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Creating avian forebrain chimeras to assess facial development

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TITLE:

Creating avian forebrain chimeras to assess facial development

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KEYWORDS:

Quail-chick chimera, duck-chick chimera, forebrain, basal forebrain transplant, Frontonasal Ectodermal Zone, FEZ

SUMMARY:

This article describes a tissue transplantation technique that was designed to test the signaling and patterning properties of basal forebrain during craniofacial development.

ABSTRACT:

The avian embryo has been used as a model system for more than a century and has led to fundamental understanding of vertebrate development. One of the strengths of this model system is that the effect of, and interaction among, tissues can be directly assessed in chimeric embryos. We have previously shown that signals from the forebrain contribute to facial morphogenesis by regulating the shape of the expression domain of Sonic hedgehog (*SHH*) in the Frontonasal Ectodermal Zone (FEZ). In this article, the method of generating the forebrain chimeras and provide illustrations of the outcomes of these experiments is described.

INTRODUCTION:

Much contemporary research in developmental biology focuses on the role of genes in shaping embryos. There are good tools to examine developmental mechanisms from a genetic perspective. However, embryos are assembled and undergo morphogenesis in response to tissue interactions. The avian system is a classic tool used to assess the variety of tissue interactions that regulate development for the following reasons: the embryology is well-understood, the embryos are easily accessible, tools for analysis of avian systems are well-developed, and the embryos are inexpensive.

The avian transplantation system has been widely employed for lineage tracing and to assess tissue interactions during development for almost a century[1-4]. This system was used to investigate a signaling center, the Frontonasal Ectodermal Zone (FEZ), that regulates morphogenesis of the upper jaw[5], and a video was published describing that technique

previously[6]. In addition to quail-chick, other species have also been used to produce chimeras for analysis of tissue interactions. For example, the mouse FEZ was transplanted from wild type[7] and mutant mice[8], and others have used a duck, quail and chick systems to assess the role of neural crest in patterning the facial skeleton[9-12].

In this work, the role of the forebrain in regulating the pattern of gene expression in the FEZ by transplanting the ventral forebrain reciprocally among quail, duck, and chick embryos was assessed, because a signal from the forebrain is required to induce Sonic hedgehog expression in the FEZ. Forebrain transplants are not unique in the field. These transplants were used to assess development of motility in quail and duck embryos[13], although in these experiments tissues that contributed to non-neural derivatives were also transplanted. In other work, auditory circuits in birds have been assessed by forebrain transplantation[14], but these transplants contained presumptive neural crest cells, which contribute to facial shape[9,10] and participate in regulating SHH expression in the FEZ[15]. Hence, a system to transplant just the ventral forebrain from one species of bird to another prior to closure of the neural tube was devised to assess the role of the brain in facial shape[16] (**Figure 1A,B**). This method was devoid of neural crest contamination of the graft. In this article, the method is illustrated and the expected results are described, and the challenges faced are discussed.

PROTOCOL:

White Pekin duck (*Anas platyrhynchos*), white Leghorn chicken (*Gallus gallus*) and Japanese quail (*Cortunix coturnix japonica*) are incubated at 37 °C in a humidified chamber until stage-matched at HH7/8[17].

1. Preparing the donor tissue

NOTE: Preparation of reagents and tools and how to open eggs for experimental manipulation has been described[6].

1.1. Prepare DMEM media with neutral red, a glass transfer pipette, and sharpened tungsten needles.

1.2. Expose embryos (as shown in[6]).

1.2.1. Using a 10 mL syringe and an 18 gauge needle, remove 0.5 mL of albumin from the pointed end of the egg shell.

1.2.2. Make a small hole on the top of the shell using the point of scissors. Place a piece of tape over the hole, and then cut a circular opening to expose the embryo.

1.3. Harvest tissue grafts from the left side of basal forebrain of stage 7/8 embryos.

1.4. Use curved sharpened tungsten needles[6] to gently incise a piece of the forebrain measuring ~0.3 mm in length by 0.2 mm in width, making sure to not include the underlying endoderm by sliding the needle beneath the forebrain so that the needle is parallel to the axis of the neural tube.

1.5. Using the glass transfer pipette, pick the graft up from the donor embryo.

1.6. Transfer the graft into DMEM containing Neutral Red (0.01% in PBS, 23 °C) for 2 minutes to stain it, and then place the stained graft into DMEM that does not contain Neutral Red until ready for engraftment.

2. Preparing the host

2.1. Incubate fertilized eggs from white Leghorn chicken (*Gallus gallus*) at 37 °C in a humidified chamber until HH7/8 [17].

2.2. Expose embryos (as shown in[6]).

2.2.1. Using a 10 mL syringe and an 18 gauge needle, remove 0.5 mL of albumin from the pointed end of the egg shell.

2.2.2. Make a small hole on the top of the shell using the point of scissors. Place a piece of tape over the hole, and then cut a circular opening to expose the embryo.

2.3. Using sharpened tungsten needles, prepare the graft site by gently cutting, then removing a 0.3 mm by 0.2 mm piece of basal forebrain from the left side to accommodate the graft as was done to isolate the donor tissue.

2.4. Take care to avoid excessive disruption of the underlying endoderm, which will be evident as yolk granules will begin to leak through any tear that is made. This takes practice and not all attempts will be successful.

2.5. After transferring to the host[6], position the graft to replace the extirpated basal forebrain of the host.

2.6. Place tape tightly over the hole and return the embryo to the 37 °C incubator for the appropriate length of time for analysis.

REPRESENTATIVE RESULTS:

Assessment of Chimerism and Transplant Contamination

In order to assess the chimeras, the extent of chimerism and contamination of the graft with other cell types should be addressed. Creating chimeras by transplanting quail tissues into chick embryos allows for this type of analysis. Using the QCPN antibody quail cells can be visualized and distinguished from the host tissues (**Figure 1 C,D**). In this case, only tissues derived from the

ventral forebrain were stained with the antibody indicating the graft was not contaminated with other cell types including neural crest**.** The extent of chimerism could be estimated from these sections using stereology to either count host and donor cells or by assessing the area occupied by the donor and host tissues.

Assessment of Morphological and Molecular Outcomes

The objective was to assess the impact of the ventral forebrain on facial morphology and *SHH* expression in the FEZ. Initially, quail tissue was transplanted into duck embryos in order to use QCPN to assess chimerism as described above. However, the faster developing quail brain led to severely deformed chimeras (**Figure 2**), which precluded this approach in the experiments. Due to this limitation transplanting duck tissues into chicken embryos was used for experimental analysis. While this did not allow assessment of chimerism, the quail chick system was used to do this and confirmed that all of the grafts were comprised only of neural tissue[16]. The resulting duck-chick chimeras had morphological changes that suggested the brain participated in regulating morphology (**Figure 3**). The duck side of the transplant appeared to develop slower and appeared more duck-like. A quantitative analysis was used to determine these chimeras were shifted toward duck morphology[16]. As controls stage matched duck and chick embryos, as well as chick-chick chimeras were used.

Whole mount *in situ* hybridization was used to assess *SHH* expression. Similar to morphology, *SHH* expression on the duck side of the chimera appeared more duck-like (**Figure 3**). A quantitative analysis[18] was also used to show this expression domain was correlated with head shape[16].

FIGURE AND TABLE LEGENDS:

Figure 1. Transplantation and Assessment of Chimerism in Experimental Embryos

(A) Dorsal view of stage 8 chicken embryo stained with neutral red showing the location of the graft and engraftment site. Dotted line is approximate level shown in B. (**B**) Cross section through a stage 8 chicken embryo after in situ hybridization to show *SHH* expression. The approximate location of the graft is shown, red dotted box. (**C**) Immunostaining to detect QCPN in quail-chick chimeras shows the graft is widespread and contributes only to the ventro-lateral neural tube (arrow) and ventral optic cup (arrowhead). (**D**) Higher magnification in C. Scale bars: A: 500 µm, B: 100 µm, C: 1 mm, D: 200 µm.

Figure 2. Assessing Morphology of Quail-Duck Chimeras.

(**A, B**) Two examples of quail-duck chimeras at stage 22. These embryos have serious malformations that preclude further analysis. Scale bar: 2 mm

Figure 3. Assessing Duck-Chick Chimeras.

(A) Normal chick and (**B**) duck embryos at stage 22 after whole mount *in situ* hybridization to visualize *SHH* expression. (**C**) A chick-chick control shows a pattern of *SHH* and morphology that resembles a normal chick embryo. (**D**) A duck-chick chimera shows an altered pattern of *SHH* expression. On the transplanted side *SHH* expression (yellow dotted line) is more rounded and resembles the duck pattern. The nasal pit is also more rounded on the transplanted side

(yellow arrow) compared to the more “slit” like appearance on the host side (red arrow). The red bar indicates the midline and the transplant is on the right side of the image. Scale bar: 1 mm

DISCUSSION:

The method described allows examination of the tissue interactions between the basal forebrain and the adjacent ectoderm. This approach differs from previous forebrain transplant methods, because the donor tissue was restricted to the ventral forebrain. This eliminates transplantation of the neural crest cells, which have been shown to participate in patterning facial morphology[9,10]. Hence, restricting the graft to the basal forebrain was essential to evaluate the experimental outcomes planned.

In the previous research that employed forebrain transplants[13,14] the presence of extra-neural tissue did not interfere with interpretation of the planned outcome measures, because the outcomes were either behavioral, or specifically tested the host and donor environment on overall brain development. This is an important consideration in designing experiments using these embryological approaches and feasibility must be balanced with rigor. For example, the requirement to exclude the presumptive neural crest cells created significant obstacles to overcome. The transplants had to be performed on early neurula stage embryos. At these stages the endoderm is immediately adjacent to the transplant site, and extreme care had to be taken to avoid damaging the endoderm, because this reduces survival. While it is not clear contamination of the graft by endoderm would impact out outcome measures, the goal was to exclusively isolate the effect of the ventral forebrain on facial development. So, for this purpose the extra rigor was warranted.

Consideration needs to be given to the rate of development of the host and donor species. While differences in the rates of these animals have been used to advantage in assessing the role of neural crest cells in patterning the developing facial skeleton[19,20], in this case, the faster developing quail neural tissue created extremely malformed embryos when transplanted into the slower developing duck. This meant chimerism and contamination had to be assessed in another set of chimeras created by grafting quail tissue into chick hosts, and while not ideal, this did provide confidence in the grafting technique.

Overall, creating chimeras to assess tissue interactions during development can be a powerful approach to help understand mechanisms of development. Care needs to be given during the planning stages to ensure the results will be as conclusive as possible, and appropriate controls determined that include normal embryos and other chimeras to account for variation due to surgery. In this case, it was very important to employ quantitative analyses, because the changes observed were subtle.

ACKNOWLEDGMENTS:

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DISCLOSURES:

All authors have nothing to disclose.

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Figure 1

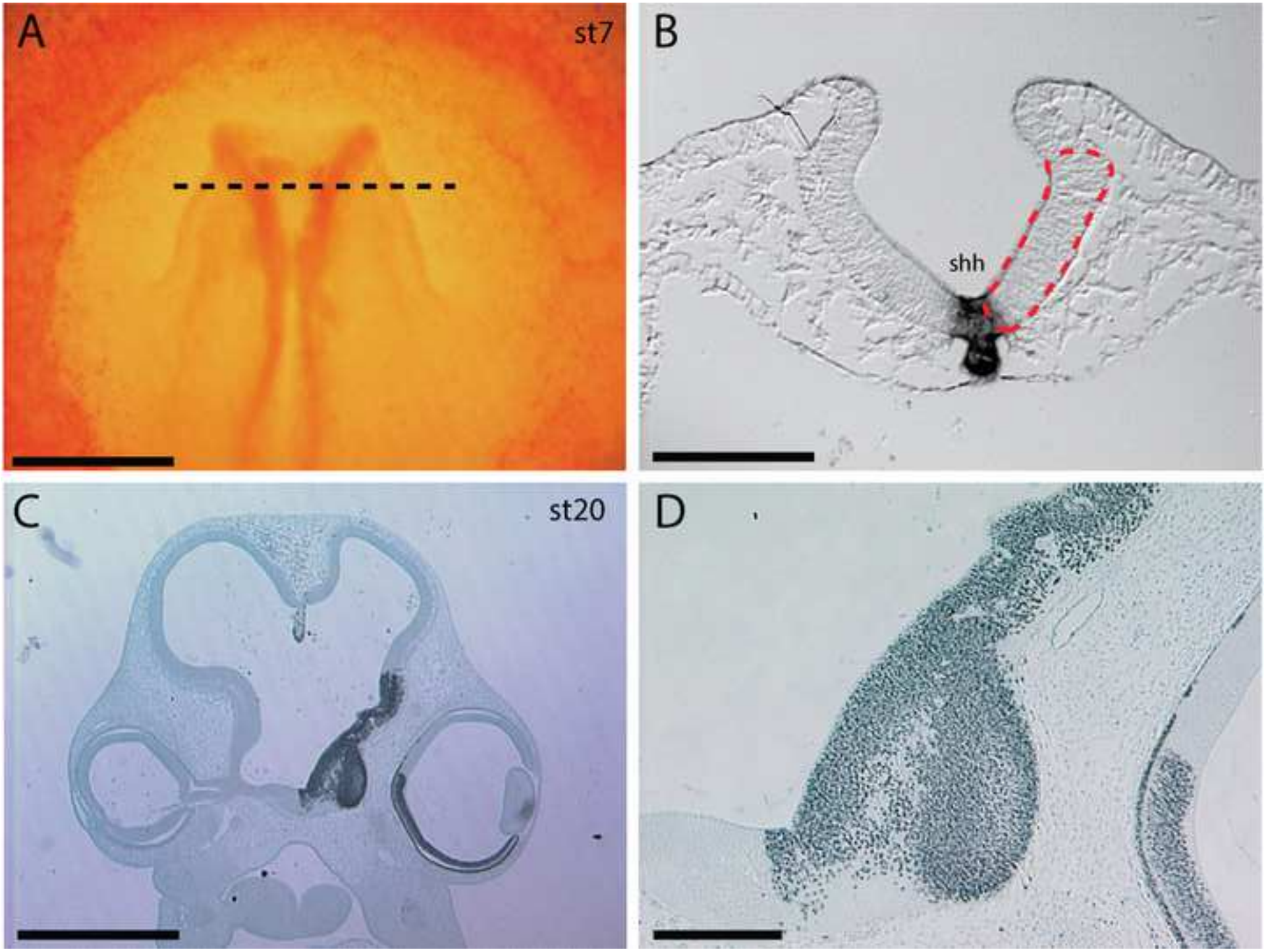


Figure 2

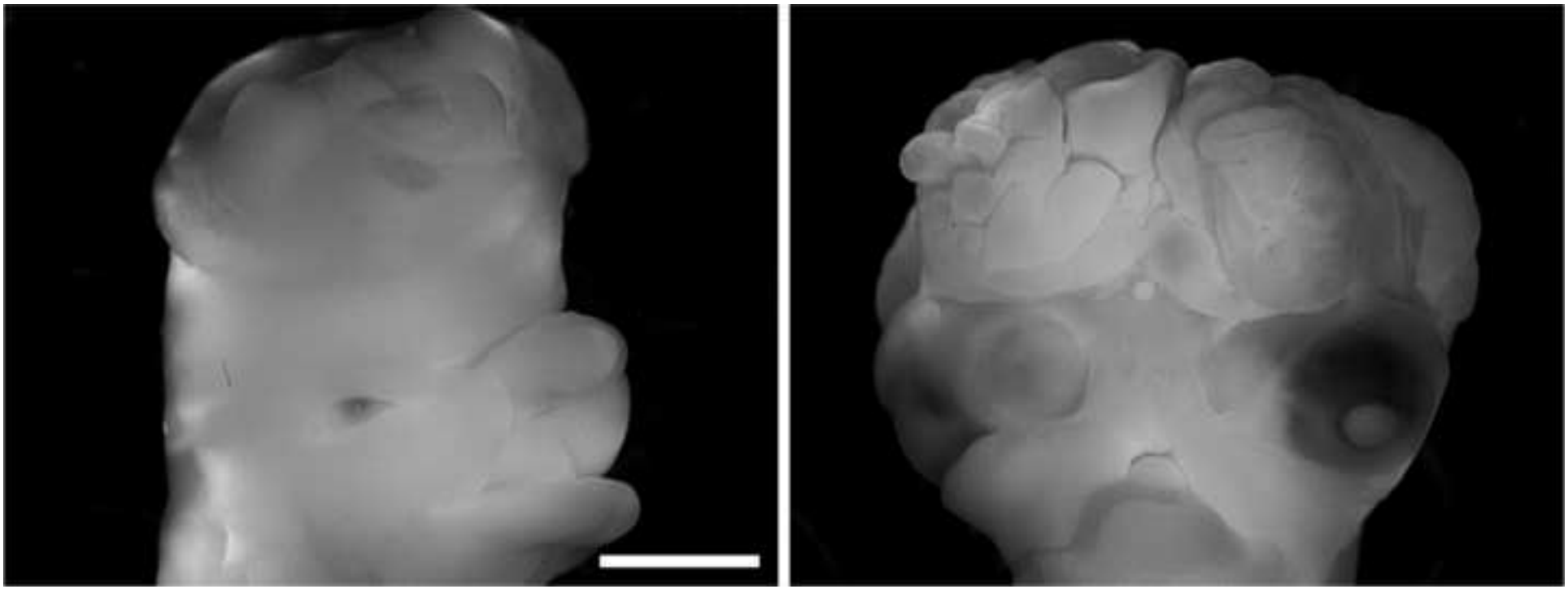
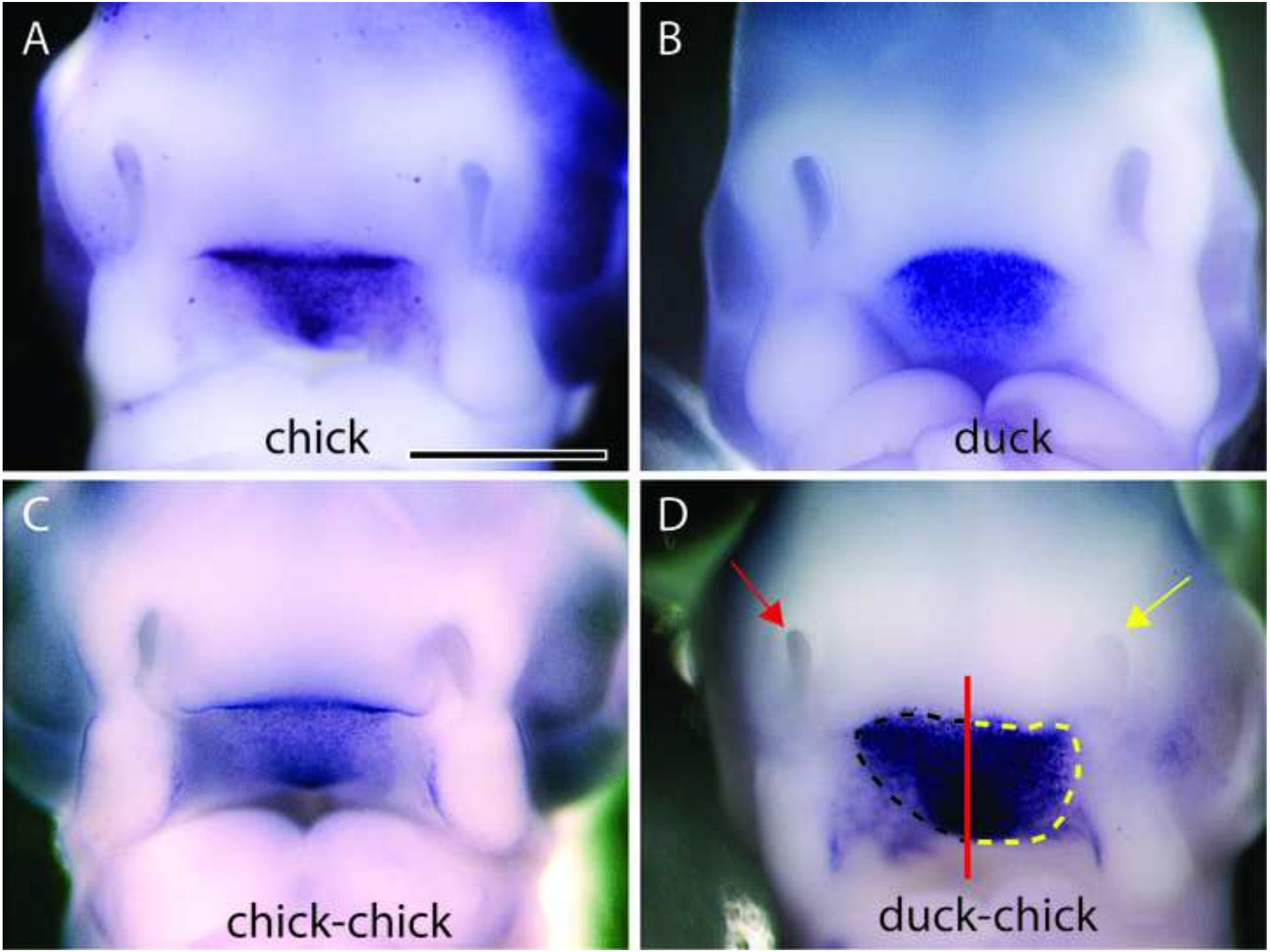


Figure 3



Table

Click here to access/download;Table of Materials;Forebrain Transplant table of materials Hu Marcucio.xls

Name of the reagent	Company	Catalog Number	Comments/Description
1x PBS	TEK	TEKZR114	
35x10 mm Petri dish	Falcon	1008	
DMEM	Thermofisher	11965084	
Needle holder	Fine Science Tools	26016-12	
Neutral Red	Sigma	553-24-2	
No. 5 Dumont forceps	Fine Science Tools	11252-20	
Pasteur Pipets	Thermofisher	13-678-6B	
QCPN antibody	Developmental Studies Hybridoma bank, Iowa University, Iowa, USA		
Scissors	Fine Science Tools	14058-11	
Tungsten Needle	Fine Science Tools	26000	

Response to Reviewers:

We would like to thank the Editor and both of the Reviewers for providing constructive feedback on our manuscript. Given that there were few comments, we have pasted the letter from JoVE with each comment. We have responded in bold and made the appropriate changes in the manuscript.

Sincerely,
Ralph Marcucio

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please define all abbreviations at first use.
2. Please revise the following lines to avoid overlap with previously published work: 75-78, **Removed** 87-93 **Removed**, 96-100 ***Removed***
3. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example: Hybridoma bank, Iowa University, Iowa, USA; ***We have moved this to the materials table***
4. Being a video based journal, JoVE authors must be very specific when it comes to the humane treatment of animals. Please include an ethics statement before all of the numbered protocol steps indicating that the protocol follows the animal care guidelines of your institution. ***Done***
5. Please revise the text, especially in the protocol, to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.). ***Done.***
6. As we are a methods journal, please add to the Discussion the following with citations: **This is discussed throughout the discussion section, and where citations are available, they are provided. This is a very unique protocol, and to my knowledge we are the only group using anything like this currently.**
a) Any modifications and troubleshooting of the technique
b) Any limitations of the technique
c) The significance with respect to existing methods
7. Please do not abbreviate journal names in the reference list. **Done.**
8. Please sort the Materials Table alphabetically by the name of the material. **Done.**

Reviewer #1:
Manuscript Summary:
The manuscript focuses around an important technique - cross-embryo transplantation of the forebrain to study how the developing brain influences the

craniofacial shaping, including the activity of FEZ - the major regulatory zone in fronto-nasal epithelium.

Major Concerns:
There are no major concerns

Minor Concerns:
The authors should mention if the transplanted forebrain contributes neural crest cells to the host, and if this can be a factor influencing facial shaping.
We have now stated that this method does not include neural crest cells in the graft.

Reviewer #2:
Manuscript Summary:
In this manuscript, entitled "Creating avian forebrain chimeras to assess facial development" Hu and Marcucio describe their technique of forebrain tissue transplantation in order to create chimeric embryos that will reveal what effect the forebrain alone has on craniofacial development. Transplantation techniques have been one of the biggest advantages of using the avian embryo as a model and the authors have extensive experience in this field. The manuscript is well written, clear, and scientifically sound. Addressing the comments below will improve the clarity and usefulness of the manuscript for a scientific audience.

Major Concerns:
1. The way the description of the PROTOCOL begins (65), having just finished reading the introduction, and now the description of all three bird species, the reader may be under the impression that these methods apply to all three bird species. Is this correct? in line 75 the authors instruct to remove 1ml of albumin from the egg. I presume this is not referring to the quail? **Since the quail is used as a donor, 0.5mls of albumin were removed. We have now stated this.** Later in the manuscript the authors state why the quail-duck is not part of this set of experiments due to disruption in development, but at this point in the manuscript, all three species are being discussed as if they will be used. If these protocols do not apply to all three species the authors should make this clear and perhaps add a separate section for the quail? If they do apply to all three, it should still probably be stated.
The method applies to all three species, the heterochrony between the duck and quail make this transplant problematic. Transplanting quail into chick and duck into chick are fine. We have now tried to make this clear in the abstract and throughout the manuscript.
2. The authors make a point that while other studies have done forebrain transplantation experiments, they often include non-neural derivatives in the transplants or neural crest cells. They instead describe a system where the forebrain

alone is transplanted. But when it comes to describing how this is done, the authors simply state "...making sure to not include the underlying endoderm" when discussing forebrain incision in lines 80-81.
Is there a specific method the authors use to make sure of this? I feel like simply telling the reader to make sure they don't contaminate the tissue isn't going to be very useful without specific information on how to do this. **We have added some detail to this section and hopefully the video will help illustrate this.**
3. Same comment as in point 2 for line 96 regarding care being taken to not cause excessive disruption of underlying endoderm when the forebrain tissue is being removed from the host animal. Is there a method the authors themselves use? is there a tissue consistency difference or texture different that the reader can look for in their own attempts? **We have added further information here and again, hope the video will be more illustrative.**
4. Lines 155-156 The authors state that in previous transplant experiments where extra-neural tissue was included in the graft, the outcome was not significant affected. If this is the case, why must the excision of the donor tissue be done so carefully? **The outcomes in the previous experiments were behavioral or examined the environment of the entire head on the brain. Our point was that the level of acceptable contamination with other tissues depends on the outcomes assessed. We have tried to make this clear.**
The authors listed the ways in which neural crest cell contamination is being avoided already, what is the harm in endoderm contamination? **It is not clear there would be harm, but for specificity of our transplants we used only the ventral neural tube. This is now stated.**

Minor Concerns:
1. Line 108 (Fig. 1) should probably specifically reference panels C and D? **Done.**