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Combining in ovo electroporation and heterospecific grafting to unravel the role of Six1-Six2-Six4 genes in cephalic neural crest for head development

--Manuscript Draft--

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Abstract:	Embryonic development is a highly complex process, which depends on multistep interplays between cell lineages over a prolonged period of time. Therefore, investigation in this field requires a powerful and refined experimental strategy, which allows cells under scrutiny to be unequivocally labeled and recognized at any state of differentiation, and allows gene manipulation to be restricted to the same cell population, selectively. The experimental combination of xenografts between quail and chick embryos associated with in ovo electroporation perfectly fulfills this requirement and offers a unique strategy to unveil epistatic relationships within gene regulatory networks operating in embryonic cell populations, and to unmask functional redundancy through multiple silencing and rescue. Here, we describe how we used these techniques to uncover the complementary roles exerted by Six genes in the cephalic neural crest for vertebrate encephalogenesis. Functional assays based on stage- and tissue-specific RNAi strategy reveal that the selective silencing of each gene triggers partial and localized defects of facial skeletal development. Similarly, the development of cephalic vesicles are differently affected depending on the Six gene knocked-down, varying from the agenesis of choroid plexuses, to a severe alobar holoprosencephaly. Hence, the triple silencing results in anencephaly and prevents development of facial structures. This indicates that the three Six genes cooperate in the control of head skeletogenesis and brain morphogenesis.
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Gif-sur-Yvette, July, 10th, 2016

Jaydev Upponi
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Dear Editor,

We are sending you herewith the revised version of our manuscript entitled "Combining in ovo electroporation and heterospecific grafting to unravel the role of Six1, Six2, Six4 genes in cephalic neural crest for head development".

The present article describes how we combine xenograft between avian embryos and in ovo electroporation for multiple gene silencing and rescue, to uncover the complementary roles exerted by Six genes in the cephalic neural crest cells for vertebrate encephalogenesis.
Here is the 'point by point' list of changes addressed in the present manuscript.

Hoping the article in the present format will suitable for publication in JoVE.

Sincerely yours,

Dr. Sophie Creuzet
Principal investigator at CNRS
Group leader Neural Crest: Development & Evolution

Editorial comments:
* NOTE: Please download this version of the Microsoft word document (File name: 54180_R3_050316) for any subsequent changes. Please keep in mind that some editorial changes have been made prior to peer review pro
*Please keep the editorial comments from your previous revisions in mind as you revise your manuscript to address peer review comments. For instance, if formatting or other changes were made, commercial language was removed, etc., please maintain these overall manuscript changes.
*Formatting:
-Please include a space between 1.2.1 and 1.2.2.
-Please include spaces between all numbers and units.
-Please use the correct formatting for the materials table, and please include company and catalog number for all items.

*Grammar:
-Please copyedit the manuscript for numerous grammatical errors. Such editing should be performed by a native English speaker and is required prior to acceptance.
-Line 56 - "combine xenograft between"
-Line 72 - "on stage- and tissue-specific RNAi strategy"
-Line 74 - "knocked-down"
-Avoid the term "perfectly" to describe these techniques, as this is not an objective assessment.
-1.3.1 - "made up with glass"
-1.3.2 - "microscapel"
-2.1.4 - "so that as the blastoderm"

-2.2 note, 2.2.1 - "Sequences" aren't dispersed or transferred, but nucleic acid molecules or nucleic acids are.
-2.2.5 - "blow" - do you mean "inject"? If mouth pipetting is used, this should be specified.
-2.2.11 note - What does "it" refer to?
-2.3.5 - "the heterospecific transfected"
-Line 343 - "well tractable"
-Line 349 - "negative charged"
-Line 350 - "towards cathode"
-The discussion is difficult to understand due to numerous errors.

*Additional detail is required:
-Is a stereomicroscope used for any of these procedures? Please specify if so.
-1.3.1 - Please clarify "under the limit of capillarity." How is the tip stretched?
-2.1.3 - How is a hole made in the egg?
-2.1.5 - Where is the opening made? If this is the opening referred to in 2.1.4, this detail should be found in 2.1.4.
-2.3.4 - Please clarify "Cut out transversely".
-2.3.6 - What is "a flexible plastic pipe"? Are inhaling and blowing done by mouth?

*Results: Please include scale bars. There are none visible. Panels M, O & Q should have their own scale bars. What staining is shown in M, O, Q?

*Discussion: Please discuss any modifications/troubleshooting that can be performed as well as the future applications of the protocol.
*If your figures and tables are original and not published previously, please ignore this comment. For figures and tables that have been published before, please include phrases such as "Re-print with permission from (reference#)" or "Modified from.." etc. And please send a copy of the re-print permission for JoVE's record keeping purposes.

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*IMP: Please copyedit the entire manuscript for any grammatical errors you may find. The text should be in American-English only. This editing should be performed by a native English speaker (or professional copyediting services) and is essential for clarity of the protocol and the manuscript. Please thoroughly review the language and grammar prior to resubmission. Your JoVE editor will not copy-edit your manuscript and any errors in your submitted revision may be present in the published version.

*NOTE: Please include a line-by-line response letter to the editorial and reviewer comments along with the resubmission.

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
In this paper, authors combine hetero-specific tissue grafting and electroporation in chicken embryo to evaluate the roles of six genes in head development. The idea is well conceived and the technique will be very useful in a variety of experiments using chicken embryo as a model. A clearly and vividly illustrated video procedure will be useful to many who use chicken embryo as an experimental model, and encourage young students to get interested into developmental biology.

Major Concerns:
N/A

Minor Concerns:
N/A

Additional Comments to Authors:
N/A

	Reviewer #2: Manuscript Summary: The manuscript well explain the need of cell labeling, and up- and down regulation of the gene of interest, and show the xenografts transplantation between quail and chick embryos, and up- and down-regulation of Six genes in the cephalic neural crest cells. By this method, the authors could show hierarchy among Hoxa2, Noggin and Six genes by single and total silencing Six1, Six2 and Six4 genes, and Noggin genes by their dsRNA. The method is very useful for the researchers who use chick embryos. Major Concerns: N/A Minor Concerns: N/A Additional Comments to Authors: N/A Reviewer #3: Manuscript Summary: The protocol is nicely described and may have some importance for other labs (although electroporation has been described in JOVE several times previously, but not combined with grafting). Major Concerns: -The section of REPRESENTATIVE RESULTS should be modified, as the 2 first paragraphs are not relevant to the described protocol and representative results, and only the last paragraph discusses the representative results. The first paragraphs are more relevant to the Introduction. -The first parts of the current Introduction are too general and long and and may be edited to become more focused to the relevant published method. -The Discussion should discuss the prones and cones of the current method as compared to bilateral electroporation of NCCs in an embryo without grafting the tissue to another, as in such a case the manipulated NCCs will also invade a non-electroporated head tissue. Also, in my view a unilateral electropration will be more informative with the grafting method since the non-manipulated side of the grafted tissue can serve as an internal control for grafting effects versus electroporation effects. This should be also discussed. Minor Concerns: 1 details regarding the preparation or purchase of the triplet electrode system is needed. Additional Comments to Authors: N/A
Additional Information:	
Question	**Response**
If this article needs to be "in-press" by a certain date to satisfy grant requirements, please indicate the date below and explain in your cover letter.	

TITLE:
Combining *in ovo* electroporation and heterospecific grafting to unravel the role of *Six1-Six2-Six4* genes in cephalic neural crest for head development

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KEYWORDS:
Neural crest; electroporation; xenotransplantation; avian embryos; RNAi; triple silencing; craniofacial development; brain patterning

SHORT ABSTRACT:
To uncover the complementary roles played by three members of the *Six* gene family in cephalic neural crest cells during vertebrate encephalogenesis, xenograft between avian embryos and *in ovo* electroporation for multiple gene silencing and rescue were combined.

LONG ABSTRACT:
Embryonic development depends on multiple interactions among cell lineages over a prolonged period of time. Therefore, investigation in this field requires a powerful and refined experimental strategy, which allows 1) cells under scrutiny to be unequivocally labeled and recognized at any state of differentiation and 2) gene manipulation to be selectively restricted to the same cell population. The experimental combination of xenografts between quail and chick embryos associated with *in ovo* electroporation perfectly fulfills these requirements and offers a unique strategy to unveil epistatic relationships within gene regulatory networks operating in embryonic cell populations, and to unmask functional redundancy through multiple silencing and rescue. We herein report the used of this strategy to elucidate the complementary roles played by three members of the *Six* gene family in cephalic neural crest (NC) cells during vertebrate encephalogenesis. Functional assays based on stage- and tissue-specific RNAi reveal that the selective silencing of each gene triggers partial and localized defects of facial skeletal development. Similarly, the development of cephalic vesicles are differently affected, depending on the *Six* gene knockdown, varying from the agenesis of choroid plexuses to severe alobar holoprosencephaly. Hence, the triple silencing results in anencephaly and prevents development of facial structures. This indicates that the three S*ix* genes cooperate in the control of head skeletogenesis and brain morphogenesis.

INTRODUCTION:
A recurrent problem in developmental studies involves accurately characterizing a discrete cell population and its interactions with the neighboring tissues. Identifying the fate of cells in complex morphogenetic processes requires a reliable and refined technique for long-term cell lineage tracing. “Mosaicism”, when selectively introduced into a developing organism, can be used for the systematic exploration of cell fate. In pioneering experiments performed in lower vertebrates, labeling a subset of embryonic cells was essentially based on combination two genetically, or morphologically, different cell types in a same individual in order to characterize cell lineages. These attempts exploited intrinsic cell markers, cell size, vital dye, or radioactive, labeling. However, such marking technique is limited by instability and imprecision; therefore, these methods failed to appropriately track the dispersal of embryonic cells and to determine their long-term fate.

In higher vertebrates, avian is a popular system for descriptive morphogenesis by its accessibility throughout ontogenesis. As a model, the chick embryo was introduced to biological investigations by Aristotle, who posited the principles for systematic and comprehensive studies of living beings. Over centuries, avian development has inspired morphologists and embryologists with paradigms and major conceptual frameworks related to the notion of germ layers [1, 2, 3], which, later on, paved the way for comparative embryology [4] . In the second half of the 19th century, the German anatomist Wilhelm His first described the neural crest (NC) in chick embryo as a transient structure that delaminates from the neural

tube (NT) and subsequently coalesces to form the dorsal root ganglia [5]. Aside from its participation to the formation of the peripheral nervous system, the NC turned out to be essential for many aspects of embryonic development and for the evolution of the vertebrate phylum based on the phylogenetic implications of its derivatives [6]. The breakthrough came in the late 1960s when Nicole Le Douarin devised the quail-chick chimera system [7]. Based on species-specific heritable cellular features, this technique relies on the differential staining of the nucleus of quail and chick cells, marking it possible to discriminate the respective contribution of heterospecific primordia to the ontogenesis of chimeric individuals. As a consequence of marker stability and cellular resolution, this technique enables to trace the fate of embryonic cells over a prolonged period of time, extending from morphogenesis to organogenesis, irrespective of their state of differentiation [8]. This method turned out to be a formidable approach to track the fate of discrete migratory cell population and, along their path, perceive their interactions with the environment, finally elucidating the hierarchical interactions that control tissue induction and specification [9].

In addition to perfectly fulfilling the requirement for descriptive investigations, the avian embryo is also a resourceful support for experimentation. Fate-mapping studies gained from quail-chick chimeras have served as standards for all ensuing molecular investigations of regulatory networks governing embryogenesis. The strategy has allowed researchers to study the "right" genes inside the "right" cells that are signaling to each other in appropriately defined time windows. More recently, we devised a combination of cell tracking and gene expression manipulations that significantly increases the possibilities for developmental studies [10, 11, 12, 13, 14]. This strategy allows us to change the expression of candidate genes, as well as the cell type under scrutiny and to transplant it into a "wild-type environment". Thus, it is now possible to determine the fate of such genetically manipulated cells and their morphogenetic impacts on neighboring unmanipulated tissues. The experimental combination of xenografts between quail and chick embryos associated with *in ovo* electroporation, as herein reported, has resulted in unravelling the role of three *Six* genes as molecular determinants of craniofacial and brain morphogenesis.

PROTOCOL:

NOTE: In this paper, the experiments are performed on chick and quail embryos long before the development of nerve and connectivity.

1. 1) Avian models and respective timetables

1. 1. 1) Select fast-growing strains for chick and quail in order to synchronize the early stages of development between donor and recipient embryos.

NOTE: JA657 strain chicks and Japanese gray-pigmented quails are particularly resistant and show a high rate of hatching, thus meeting this requirement.

1. 1. 2) Precisely stage chick and quail embryos according to developmental tables [15, 16, 17].

NOTE: Chick and quail belong to the same taxonomic group and share a similar, but not identical, timetable early in development. During the early phases of development, specifically at neurula stage, embryos subjected to microsurgery are exactly staged by counting the number of somite pairs flanking the neural tube. Their developmental stage is then expressed as somite-stage (ss).

1. 2) Incubation

1. 2. 1) Initiate embryonic development by incubating fertilized eggs in brooders.

1. 2. 2) Precisely control the onset of incubation using a programmer timer, especially for the early phases of development. Regulate both temperature (38 ± 0.5 °C) and humidity (45 %) throughout embryonic development up to embryonic day (E) 17. From this stage onwards, set the humidity around 75 % in order to facilitate hatching.

1. 3) Microsurgery Instruments

1. 3. 1) Prepare micro scalpels made of glass from Pasteur pipettes. Stretch the pipette over the flame to shrink the pipette tip drawn out by hand until the disruption of capillary. Carry out this his step rapidly to ensure that the tip is short (< 1 cm).

NOTE: Since glass does not retain embryonic material, the tip of the micro-scalpel produces very neat incisions and remains sharp over many operations. This type of micro scalpel is particularly suitable for ablations or transplantations involving very tiny NC cell territories [10, 11, 13].

1. 3. 2) Alternatively, use a metal micro scalpel devised by honing steel needle on an Arkansas stone, or by sharpening tungsten wire by electrolysis in a 0.5M KOH solution. NOTE: These micro scalpels are more convenient for the excision of the neural tube or brain vesicles because they are extremely thin and robust [18].

2. 1) Egg preparation

2. 1. 1) Before the operation, incubate eggs in a brooder and maintain them in a horizontal position by a series of wired coils.

2. 1. 2) Use hollowed out slats to stably hold eggs, during and after operation, for reincubation of manipulated embryos until the stage required for analysis.

2. 1. 3) Discard a small quantity of albumen beforehand. To accomplish this, pierce the shell with the needle tip and use a syringe to pump out 1 to 2 ml of albumen at the pointed pole of the egg. Seal with wax or small piece of adhisive tape. Alternatively, perforate the air chamber with micro-tip scissors and turn the egg upside down so that the blastoderm lies away from the shell.

2. 1. 4) Make an opening in the shell using curved scissors without ripping embryos. This eggshell opening at the side has a diameter of approximately 1.5 cm.

2. 2) Bilateral electroporation for gene silencing and rescue in facial neural crest (FNC)

NOTE: Use *in ovo* electroporation to bilaterally transfer exogenous nucleic acid sequences into living FNC cells. The triplex of electrodes generates a triangular electric field yielding the bilateral dispersion of the nucleic acid sequences [10, 13].

NOTE : The manipulation of embryos must be performed with the aid of a stereomicroscope with variable magnification capacity of at least 10 to 40x.

2. 2. 1) Synthesize and purify nucleic acid sequences designed to either up- or downregulate gene activity [19].

2. 2. 2) Use constructs at the range of concentration to achieve gain-of-function [12, 13]. For either plasmid or retroviral constructs set the concentration between 1-4 µg/µl in Phosphate-buffered saline (PBS). Use double-stranded RNA (dsRNA) at a working concentration

varying between 200 ng/µl and 500 ng/µl in PBS to trigger gene silencing and loss-of-function [12, 13, 19].

2. 2. 3) Prepare an approximate volume of 0.1 µl of the working solution per embryo for the experiment.

2. 2. 4) Contrast the nucleic acid solution with 0.001 % Fast Green FCF (in PBS) (Figure 1A-B) in order to visualize the tip of the micropipette and precisely control the injection.

2. 2. 5) Carefully blow the nucleic acid solution in the lumen of the neural tube using a glass drawn-out pipette.

2. 2. 6) Use a triplex electrode to achieve a bilateral and simultaneous transfection of the neural fold (NF). Set the two positive electrodes apart from each other with a gap of 5 mm on the vitelline membrane. Place the negative electrode in front of the anterior neural ridge, at a distance of 6 mm from the two positive electrodes [10, 13] (Figure 1C).

2. 2. 7) Set the electrodes in contact to the surface of the extra-embryonic ectoderm using a 3D-micromanipulator with respect to the targeted tissue.

2. 2. 8) Ensure a homogeneous dispersion of electrical pulses by damping the tip of the triple electrodes and embryonic tissues with PBS supplemented with antibiotics.

2. 2. 9) Connect the triple electrode system to a square pulse-delivering generator (electroporator).

2. 2. 10) Preset the electroporator with the following parameters: iterative square pulses of 25V of intensity with a respective duration of 50ms and a resting interval of 500 ms.

2. 2. 11) Trigger a transient permeabilization of the cell membrane by an electrical impulse.
NOTE: First devised *in vitro*, electroporation was successfully adapted to *in vivo* transfection in the mid-1990s by turning a unique pulse of high voltage —with an exponential decay — into a series of square low voltage iterative pulses [20, 21].

2. 3) Grafting procedure
NOTE: Perform FNC cell transplantation before the onset of their migration.

2. 3. 1) Contrast the embryonic structures from the yolk by injecting a solution of Indian ink diluted 1:20 in PBS in an approximative volume of 0.2ml, supplemented with antibiotics (penicillin-streptomycin diluted 1:100 in PBS), under the blastoderm in the donor embryo (Figure 1D-E).

2. 3. 2) Incise the vitelline membrane with a micro scalpel in order to access the embryonic region elected for microsurgery (Figure 1F).

2. 3. 3) Perform a longitudinal slit through the ectoderm along the chosen level of the NF with a micro scalpel (Figure 1G), on both sides (Figure 1H).

2. 3. 4) Make a small transversal cut in the dorsal edges of neural tube to delineate the antero-posterior limits of the excised fragment with the micro scapel (Figure 1I), to isolate the NC domain subjected to transplantation from the neural primordium (Figure 1J).

2. 3. 5) Excise the endogenous NC territory in the chick recipient embryo with a micro scalpel, according to the procedure described above (from step 2.3.1 to 2.3.4), in order to remove the endogenous FNC and clear the site of transplantation prior to the implantation of the heterospecific transfected.

2. 3. 6) Transfer the excised NF fragment from the host to the recipient embryo through a glass micropipette with a flexible plastic pipe put on the larger tip of the micropipette to precisely control FNC fragment intake and release by inhaling and blowing, respectively.

2. 3. 7) Carefully lay the exogenous NC on the embryo with the micropipette. Then, gently manipulate it with a micro scalpel to implant in the elected site.

2. 3. 8) Seal the shell with a piece of adhesive tape and reincubate the recipient embryo at 38°C in the same position (Figure 1K).

2. 3. 9) Harvest and fix embryos at E5 and E8 to analyze the consequence of gene manipulation on brain development, and on craniofacial morphogenesis [13], respectively.

REPRESENTATIVE RESULTS:

The combination of *in ovo* bilateral electroporation and heterspecific grafting between quail and chick embryos has been previously used to explore the role of *Hox* genes expression in FNC cells [10]. When the expression of *Hoxa2* is forced by electroporation of retroviral constructs in the FNC cells before the onset of migration, the *Hoxa2*-transfected FNC cells then migrate and differentiate within a *Hox*-negative environment. In these experiments, *Hoxa2* expression abolished the capacity of the FNC to form the facial skeleton.

Similar in principle, experiments using *Hoxa3* and *Hoxb4* as transgenes partly prevent lower and upper face development, respectively, but severely impact the development of facial skeleton. In addition to the inhibition of the skeletogenesis, cell lineage in chimeras revealed that the ectopic expression of *Hoxa3* also selectively hampered FNC cell differentiation into pericytes. As a result, embryos died prematurely from haemorrhages due to the absence of musculo-connective walls in vessel tunicae. In contrast, neither neural nor pigment NC derivatives seemed to be affected by the translocation of *Hox* expression at their level [10]. In addition, aside from its structural role in craniofacial formation, our investigations have shown that the FNC also plays a potent morphogenetic "paracrine" role in the development of brain and sense organs. Parts of the brain that mostly depend on the regulatory activity of the FNC are those that have evolved more recently, *i.e.*, the telencephalon and the dorsal part of di- and mesencephalon [12].

More recently, we have investigated the molecular determinants of FNC-derived skeletogenesis and the mechanisms whereby FNC conveys its trophic effect on the developing brain. By associating *in ovo* electroporation and heterospecific grafting of FNC, we have shown that translocation of *Hoxa2* in FNC interferes with the activity *Six1*, *Six2* and *Six4* expressed in FNC cells [13]. We have shown that translocation of *Hoxa2* in FNC results in the global reduction of *Six1*, *Six2,* and *Six4.* These three *Six* family member genes are specifically expressed in FNC cells at neurula stage and in early emigrating NC cells,

according to a similar pattern of activation. However, when performed in FNC cells selectively, the respective silencing of each of these genes produces different defects. Despite sharing up to 75% identity, the sequences which encoding *Six1*, *Six2,* and *Six4* used as template for dsRNA synthesis generate different phenotypes. For skeletal development, dsRNA targeting *Six2* and *Six4* causes very severe defects, including diverse and complementary truncation of maxillo-mandibular skeleton, while *Six1* induces a global reduction in size without affecting the skeletal patterning. Similarly, silencing of *Six* genes selectively generates diverse brain defects, and dsRNA targeting *Six1* causes defects in brain morphology strikingly distinct from those induced by dsRNA against either *Six2* or *Six4* (Fig. 1L-S), ranging from plexus agenesis to mild or severe holoprosencephaly. Ultimately, the triple silencing of *Six1-2-4* causes extended anencephaly.

Therefore, these experiments shed light on the requirement of these genes for craniofacial and cerebral morphogenesis. We performed additional experiments to rescue the triple silencing of *Six1-Six2-Six4*, in order to determine if the activity of *Noggin* is downstream of *Six* genes activity. According to the same paradigm, the triple silencing was performed in combination with the upregulation of *Noggin*. We then observed the complete restoration of the skeletal nasofrontal and maxillo-mandibular structures. Taken together, these results showed that the control of head morphogenesis, through the cooperation of *Six* genes, is mediated by *Noggin* activity [13].

FIGURE LEGEND:

Figure 1: ***In ovo* electroporation and surgical procedure.** (A-B) Injection of the plasmid, contrasted with Fast Green solution, in the neural groove. (C) Setting of the triple electrode system at the surface of quail embryo. (D,E) India ink is injected under the blastoderm. (F-J) Surgical procedure consists of (F) a partial removal of the vitelline membrane and (G) microdissection of the NF on the right-hand side, then (H) on the left-hand side. The bilateral stands of NF are (I) separated from the more caudal level and (J) isolated from the rest of the embryo before transplantation into the recipient embryo. (K) Two hours after grafting the transfected NF, FNC cells start to exhibit GFP activity and migrate from the site of implantation. (L-S) Brain phenotypes in (L-M) control, (N-O) *Six1*-, (O-Q) *Six4*-silenced embryos. (R-S) Triple silencing of *Six1/Six2/Six4* causes anencephaly. (K-S), as modified from [13]. Cav, cavum pellucidum; Lat v, lateral ventricle; Pal, pallium; Se, septum pellucidum; Tha, thalamus; 3rd v, third ventricle. Scale bars in A and L-Q represent 200 µm and 1 mm, respectively.

DISCUSSION:

By associating two embryological strategies, gene manipulation by *in ovo* electroporation plus heterospecific combinations by quail-chick transplantations, we have elucidated the role of some molecular determinants operating in FNC cells, which govern vertebrate encephalogenesis.

The combinatorial expression of *Hox* genes is an evolutionarily ancient program underlying body axis patterning in amniotes and bilateria. However, in the head, the NC, a vertebrate innovation that contributes to evolutionarily novel skeletal and neural features, develops as a structure free of *Hox*-gene expression [22, 10, 23]. The emergence of FNC-derived structures has coincided with evolution of the most rostral parts of the brain, and we have previously shown that the FNC exerts morphogenetic cell-autonomous control of the growth and patterning of the telencephalon and the dorsal part of the di- and mesencephalon, as well as and the sense organs [11, 12].

To explore these mechanisms in depth, the conventional transgenic models are unsuitable [24]. Chick embryo is a very traceable model when combining both embryological and gene manipulations. The avian embryo with a range of developmental stages allows transfection to be performed in stage-, space-, and tissue-specific manner. Electroporation allows us combine molecular approaches with classical embryonic tissue manipulations and use embryonic chimeras for functional molecular studies.

When placed in a polarized electrical field, nucleic acids, globally negative charged molecules, are displaced towards cathode. The transfer of foreign nucleic acid into cells is controlled by electric impulse, which directs the nucleic acid towards cathode through cells situated in between (Fig. 1B-C). In this context, the exogenous nucleic acid sequences enter the targeted cells and become trapped in the cytoplasm as the cell membrane recovers its integrity. This results in an instantaneous and highly efficient penetration of the vector into the cells.

At neurula stage, the FNC forms a NF bulge that accompanies the rise of the lateral aspects of the neural plate, while the edges of the neuroepithelium come together and form the neural tube. Because of epithelio-mesenchymal transition (EMT), FNC cells can only be microsurgically manipulated before they delaminate, become mesenchymal and migrate. The ideal timing for heterospecific exchanges is therefore synchronized with the neurulation, and the neural crest (NC) level subjected to replacement must be the last formed. NC formation and delamination are first initiated at cephalic level at 5-6 ss [25] and then progressively reach the more caudal levels up to E5 and E4.5 for chick and quail embryos, respectively [26].

For electroporation, a problem arises from FNC cells that emanate from the strands of NF that merge along the dorsal midline before migrating bilaterally. As a consequence, the "control" and "experimental" sides receive a substantial contribution of transfected and untransfected cells, respectively, a phenomenon that could strongly bias the interpretation of resulting phenotypes. To circumvent this limitation, a bilateral transfection of both neural folds should be carried out simultaneously once by adding a third electrode: one anode, rostral, and two cathodes, lateral. Using such triplex electrode remarkably increases the efficacy and reliability of CNC cell transfection. Moreover, this increase in efficiency can be accompanied by electroporation of NF adjacent tissues. Combining heterospecific grafting with electroporation, however, guarantees tissue specificity of transgenesis.

If necessary, some modifications of this protocol can be made. Difficulty in positioning electrodes, or conducting electroporation *in ovo*, can be overcome by removing the embryo and performing electroporation in petri dishes. If the operator has little experience with *in vivo* electroporation, it is recommended that preliminary tests be carried out with dextran-FITC (or equivalent). Thus, the researcher will be able to immediately determine if the site being electroporated is correct.

Most commonly, this kind of experiment can result in injury to the embryo as a result of poor positioning of electrodes. As long as tissue to be transplanted is not compromised, this should pose no problem, but caution should always be taken. Moreover, in some cases, fungal contamination can occur; therefore, it is recommended that antifungals, such as Amphotericin B (Gibco, cat. no. 15.290.018), are used in PBS.

By using this approach, we have previously shown that translocation of *Hoxa2* in FNC results in the global inhibition of *Six1*, *Six2* and *Six4* expressed in FNC. Although these genes exhibit similar pattern of activity in premigratory and migrating FNC cells, the outcome of their selective silencing is variable and produces partial and complementary truncation of facial and brain development. These experiments shed light on the requirement of these genes for craniofacial and cerebral morphogenesis. We show that a "*Six* code" is central to the spatial layout of CNC-derived skeletogenesis and is required to achieve proper brain development. Therefore, while the "*Hox* code" rules the patterning of skeletal structures in the pharynx and the trunk, craniofacial skeletogenesis, which normally develops as a *Hox*-free domain, follows a "*Six* code" for its growth and patterning.

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DISCLOSURES:
Authors declare no competing financial interests.

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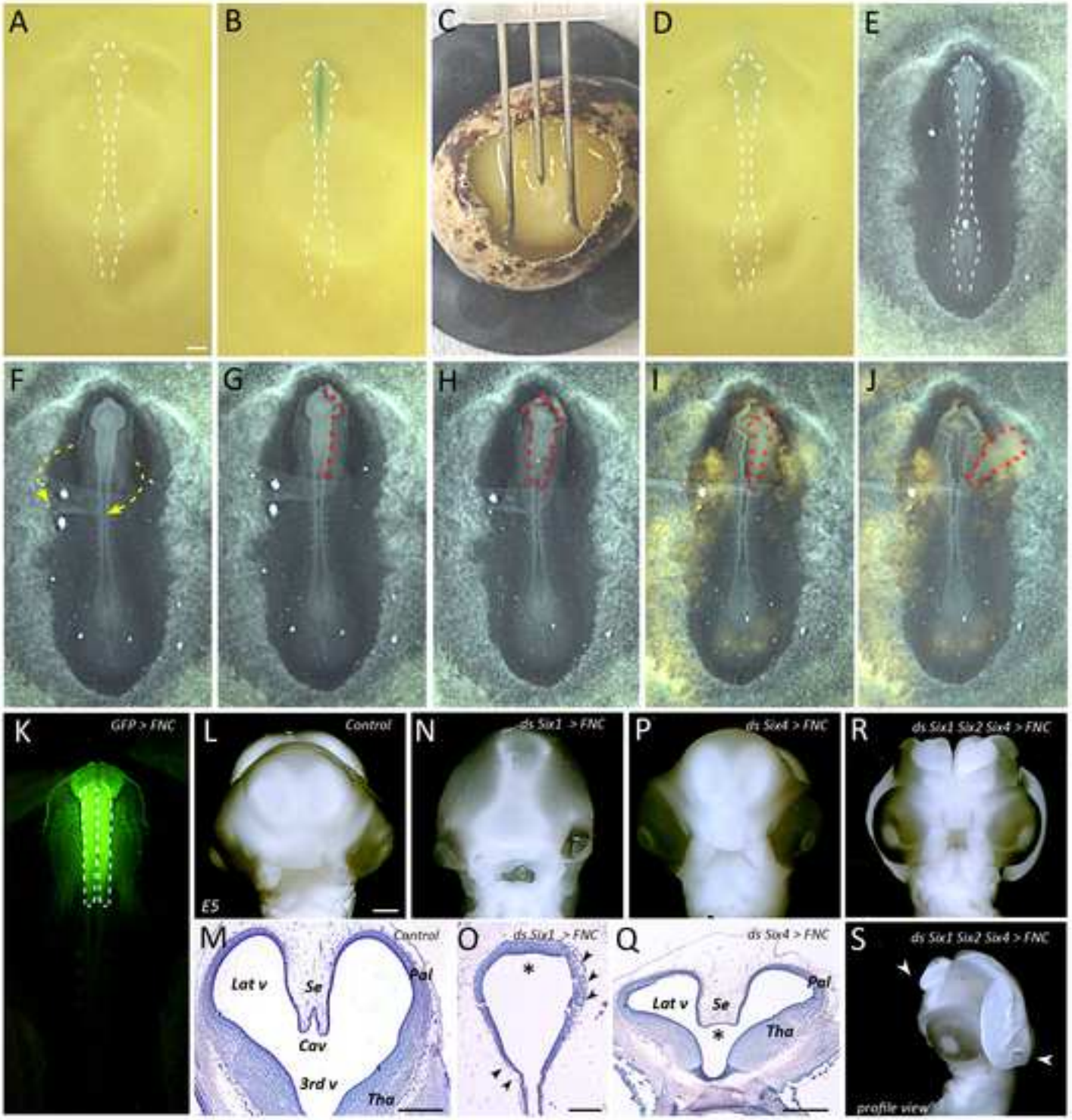

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dsRNA or plasmids previously purified	
Penicillin-streptomycin solution	Invitrogen
Fast green	Sigma-Aldrich
Indian ink	B&D
Transparent Scotch tape	3M
Stereomicroscope with swan-neck fiber optic illumination system	Leica
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4" curved scissor	Fine Science Tools
Glass pasteur pipettes	Fisher Scientific
Electroporation system	Harvard Apparatus
Egg incubator	Autoelex Co.,
Tungsten needles	Fine Science Tools
Arkansas stone	Sharpening Supplies
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Syringe and needle combination	BD
Triple electrode	Bex Co

Catalog Number	Comments/Description
10010031	
15140148	10,000 U/mL
F7258	
261194	
MZ 7.5	or equivalent
SZ	or equivalent
14.061-10	or equivalent
13.678-20D	
45-0052	BTX-ECM 830
JURAGON	or equivalent
10.130-10	
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Dear Editor,

We are sending you herewith the revised version of our manuscript entitled "Combining in ovo electroporation and heterospecific grafting to unravel the role of *Six1, Six2, Six4* genes in cephalic neural crest for head development".

The present article describes how we combine xenograft between avian embryos and *in ovo* electroporation for multiple gene silencing and rescue, to uncover the complementary roles exerted by *Six* genes in the cephalic neural crest cells for vertebrate encephalogenesis.
Here is the 'point by point' list of changes addressed in the present manuscript.

Hoping the article in the present format will suitable for publication in JoVE.

Sincerely yours,

Dr. Sophie Creuzet
Principal investigator at CNRS
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-Line 74 – "knocked-down"
-Avoid the term "perfectly" to describe these techniques, as this is not an objective assessment.
-1.3.1 – "made up with glass"
-1.3.2 – "microscapel"
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-2.2 note, 2.2.1 – "Sequences" aren't dispersed or transferred, but nucleic acid molecules or nucleic acids are.
-2.2.5 – "blow" – do you mean "inject"? If mouth pipetting is used, this should be specified.
-2.2.11 note – What does "it" refer to?
-2.3.5 – "the heterospecific transfected"
-Line 343 – "well tractable"
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-Line 350 – "towards cathode"
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Reviewers' comments:

Reviewer #1:
Manuscript Summary:
In this paper, authors combine hetero-specific tissue grafting and electroporation in chicken embryo to evaluate the roles of six genes in head development. The idea is well conceived and the technique will be very useful in a variety of experiments using chicken embryo as a model. A clearly and vividly illustrated video procedure will be useful to many who use chicken embryo as an experimental model, and encourage young students to get interested into developmental biology.

Major Concerns:
N/A

Minor Concerns:
N/A

Additional Comments to Authors:
N/A

Commented [r15]: Ok!

Reviewer #2:
Manuscript Summary:
The manuscript well explain the need of cell labeling, and up- and down regulation of the gene of interest, and show the xenografts transplantation between quail and chick embryos, and up- and down-regulation of Six genes in the cephalic neural crest cells. By this method, the authors could show hierarchy among Hoxa2, Noggin and Six genes by single and total silencing Six1, Six2 and Six4 genes, and Noggin genes by their dsRNA. The method is very useful for the researchers who use chick embryos.

Major Concerns:
N/A

Minor Concerns:
N/A

Additional Comments to Authors:
N/A

Commented [r16]: Ok!

Reviewer #3:
Manuscript Summary:
The protocol is nicely described and may have some importance for other labs (although electroporation has been described in JOVE several times previously, but not combined with grafting).

Major Concerns:
-The section of REPRESENTATIVE RESULTS should be modified, as the 2 first paragraphs are not relevant to the described protocol and representative results, and only the last paragraph discusses the representative results. The first paragraphs are more relevant to the Introduction.
-The first parts of the current Introduction are too general and long and and may be edited to become more focused to the relevant published method.
-The Discussion should discuss the prones and cones of the current method as compared to bilateral electroporation of NCCs in an embryo without grafting the tissue to another, as in such a case the manipulated NCCs will also invade a non-electroporated head tissue. Also, in my view a unilateral electropration will be more informative with the grafting method since the non-manipulated side of the grafted tissue can serve as an internal control for grafting effects versus electroporation effects. This should

Commented [r17]: Disagree, these paragraphs just present the most relevant results that the group obtained with this technique

Commented [r18]: I find it important to keep the text as it is. It is our writing style. We believe it is important to contextualize historically and experimentally the methodology presented.

be also discussed.

Minor Concerns:
1 details regarding the preparation or purchase of the triplet electrode system is needed.

Additional Comments to Authors:
N/A

Commented [r19]: Heeded suggestion
SOPHIE - I wish you confer to see if was not decontextualized

Commented [r20]: **SOPHIE, It is marked in red in the text to be filled**

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http://www.inaf.cnrs-gif.fr/ned/equipe09/accueil_09.html

Gif-sur-Yvette, July, 10th, 2016

Jaydev Upponi
Science Editor
JoVE
One Alewife Center, Suite 200
Cambridge, MA 02140

Dear Editor,

We are sending you herewith the revised version of our manuscript entitled "Combining in ovo electroporation and heterospecific grafting to unravel the role of *Six1, Six2, Six4* genes in cephalic neural crest for head development".

The present article describes how we combine xenograft between avian embryos and *in ovo* electroporation for multiple gene silencing and rescue, to uncover the complementary roles exerted by *Six* genes in the cephalic neural crest cells for vertebrate encephalogenesis.
Here is the 'point by point' list of changes addressed in the present manuscript.

Hoping the article in the present format will suitable for publication in JoVE.

Sincerely yours,

Dr. Sophie Creuzet
Principal investigator at CNRS
Group leader *Neural Crest: Development & Evolution*

Editorial comments:

• ***NOTE: Please download this version of the Microsoft word document (File name: 54180_R3_050316) for any subsequent changes. Please keep in mind that some editorial changes have been made prior to peer review pro***

•Please keep the editorial comments from your previous revisions in mind as you revise your manuscript to address peer review comments. For instance, if formatting or other changes were made, commercial language was removed, etc., please maintain these overall manuscript changes.

•Formatting:
-Please include a space between 1.2.1 and 1.2.2.
-Please include spaces between all numbers and units.
-Please use the correct formatting for the materials table, and please include company and catalog number for all items.

•Grammar:
-Please copyedit the manuscript for numerous grammatical errors. Such editing should be performed by a native English speaker and is required prior to acceptance.
-Line 56 – "combine xenograft between"
-Line 72 – "on stage- and tissue-specific RNAi strategy"
-Line 74 – "knocked-down"
-Avoid the term "perfectly" to describe these techniques, as this is not an objective assessment.
-1.3.1 – "made up with glass"
-1.3.2 – "microscapel"
-2.1.4 – "so that as the blastoderm"
-2.2 note, 2.2.1 – "Sequences" aren't dispersed or transferred, but nucleic acid molecules or nucleic acids are.
-2.2.5 – "blow" – do you mean "inject"? If mouth pipetting is used, this should be specified.
-2.2.11 note – What does "it" refer to?
-2.3.5 – "the heterospecific transfected"
-Line 343 – "well tractable"
-Line 349 – "negative charged"
-Line 350 – "towards cathode"
-The discussion is difficult to understand due to numerous errors.

•Additional detail is required:
-Is a stereomicroscope used for any of these procedures? Please specify if so.
-1.3.1 – Please clarify "under the limit of capillarity." How is the tip stretched?
-2.1.3 – How is a hole made in the egg?
-2.1.5 – Where is the opening made? If this is the opening referred to in 2.1.4, this detail should be found in 2.1.4.
-2.3.4 – Please clarify "Cut out transversely".
-2.3.6 – What is "a flexible plastic pipe"? Are inhaling and blowing done by mouth?

•Results: Please include scale bars. There are none visible. Panels M, O & Q should have their own scale bars. What staining is shown in M, O, Q?

•Discussion: Please discuss any modifications/troubleshooting that can be performed as well as the future applications of the protocol.

•If your figures and tables are original and not published previously, please ignore this comment. For figures and tables that have been published before, please include phrases such as "Re-print with permission from (reference#)" or "Modified from.." etc. And please send a copy of the re-print permission for JoVE's record keeping purposes.

•JoVE reference format requires that DOIs are included, when available, for all references listed in the article. This is helpful for readers to locate the included references and obtain more information. Please note that often DOIs are not listed with PubMed abstracts and as such, may not be properly included when citing directly from PubMed. In these cases, please manually include DOIs in reference information.

•IMP: Please copyedit the entire manuscript for any grammatical errors you may find. The text should be in American-English only. This editing should be performed by a native English speaker (or professional copyediting services) and is essential for clarity of the protocol and the manuscript. Please thoroughly review the language and grammar prior to resubmission. Your JoVE editor will not copy-edit your manuscript and any errors in your submitted revision may be present in the published version.

rcgarcez 10/7/16 23:49
Commentaire: inclued

rcgarcez 10/7/16 23:49
Commentaire: inclued

rcgarcez 10/7/16 23:49
Commentaire: new section added

rcgarcez 10/7/16 23:49
Commentaire: All text was submitted to a professional reviewer of American English

rcgarcez 10/7/16 23:49
Commentaire: add information (213-214)

rcgarcez 10/7/16 23:49
Commentaire: text changed to become more obvious

rcgarcez 10/7/16 23:49
Commentaire: added

rcgarcez 10/7/16 23:49
Commentaire: text changed to become more obvious

rcgarcez 10/7/16 23:49
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rcgarcez 10/7/16 23:49
Commentaire: yes, the inhaling and blowing done by mouth

rcgarcez 10/7/16 23:49
Commentaire: **SOPHIE**

rcgarcez 10/7/16 23:49
Commentaire: 2 new paragraphs have been added?

rcgarcez 10/7/16 23:49
Commentaire: **SOPHIE, are all originals?**

rcgarcez 10/7/16 23:49
Commentaire: Some references are very old and do not have DOI.

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•NOTE: Please include a line-by-line response letter to the editorial and reviewer comments along with the resubmission.

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rcgarcez 12/7/16 16:49
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