Chapter 6

Embryological and Genetic Manipulation of Chick Development

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Abstract

The ability to combine embryological manipulations with gene function analysis in an amniote embryo makes the chick a valuable system for the vertebrate developmental biologist. This chapter describes methods for those unfamiliar with the chick system wishing to initiate experiments in their lab. After outlining methods to prepare chick embryos, protocols are provided for introducing beads or cells expressing secreted factors, and for culturing tissue explants as a means of assessing development in vitro. Approaches to achieve gain of function and loss of function (morpholino oligonucleotides) in chick are outlined, and methods for introducing these reagents by electroporation are detailed.

Key words Chick, Embryo culture, Electroporation, Morpholino oligonucleotide

1 Introduction

Chick embryos have captivated developmental biologists since the days of Aristotle [1]. Large and externally developing, chick embryos are amenable to in vivo and in vitro manipulations that assay fate, inductive signaling interactions, and, more recently, gene function. Long a mainstay of limb, somite, neural crest, and spinal cord developmental studies, the ability to combine chick embryology with gain- or loss-of-function approaches has greatly increased the utility and popularity of the chick system. In addition to enabling a wider array of developmental inquiry in the chick, this new era makes possible comparative studies between the chick and other vertebrate model organisms. For example, when used in conjunction with a genetic system such as the mouse (particularly useful as both are amniotes and thus evolutionarily close), new experiments become feasible: the conservation of gene function can be evaluated, and chick embryological manipulations that are

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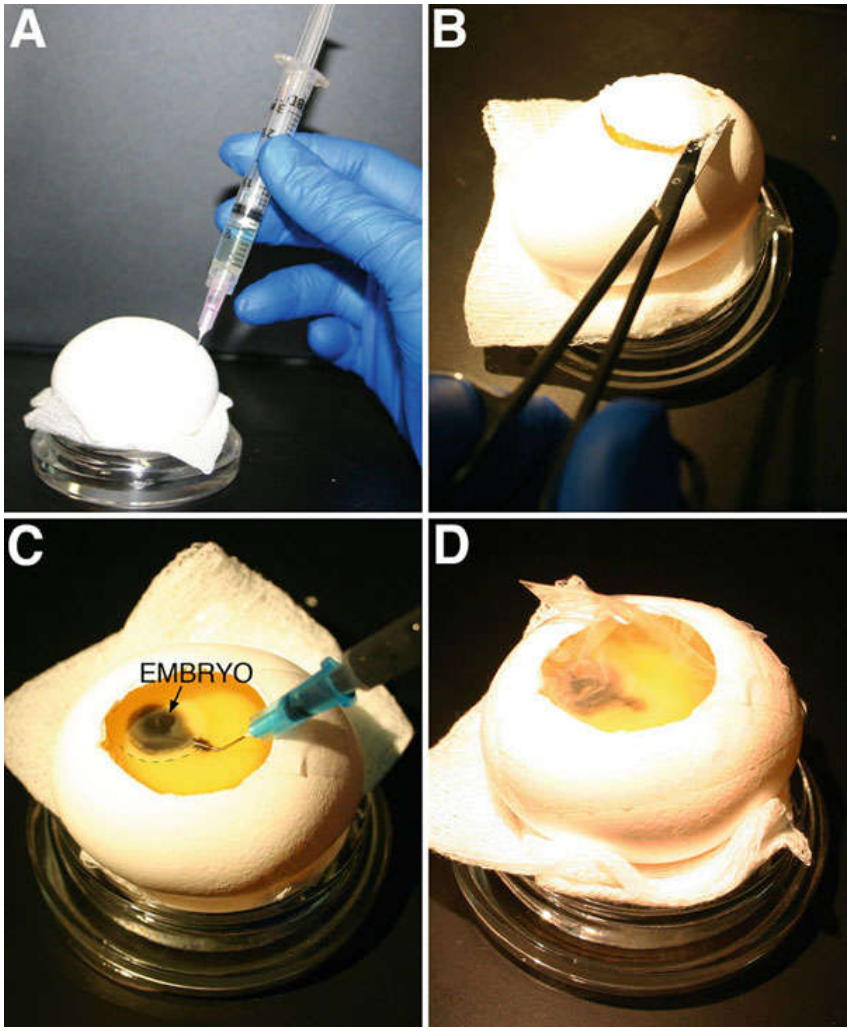

Fig. 1 Opening chicken eggs. (**a**) A 3 mL syringe outfitted with an 18 gauge needle is carefully inserted into the top side of the blunt end of the egg. The needle tip is introduced along the curvature of the shell to avoid puncturing the yolk, and 3 mL of albumin is withdrawn from the bottom of the egg. (**b**) One or two pieces of scotch tape are laid across the top of the egg, and a hole of 1.5–2 cm diameter is cut in the shell. (**c**) India ink diluted in Ringer's saline is injected under the embryo using a 1 mL syringe outfitted with a 25 gauge needle bent at a 45° angle, to enhance contrast. The needle tip is inserted just outside the perimeter of the lighter colored area opaca of the blastoderm (indicated with a dotted line along its lower edge in the photograph) and brought up under the embryo, where ink is expelled. (**d**) Following manipulation, eggs are sealed well with packing tape and placed back into 38 °C/100 °F incubator

impossible in the mouse can be used to further investigate mechanisms suggested by genetic analysis. As a result, a more complete and comprehensive view of a developmental process unfolds. This chapter serves as an introduction to chick methods. Basic protocols for culturing embryos and manipulating signaling or gene expression are described.

1.1 Culturing Chicken Embryos and Explants

A chicken embryo comes ready to use in an incubation vessel complete with its own culture medium. As a result, embryos can be manipulated in ovo (in the eggshell) by opening an access hole (Fig. 1). After procedures are completed, this hole can be sealed with packing tape to maintain a humid, sterile environment, and the embryo can be incubated to the desired stage in order to assay the consequences of any manipulations. Alternatively, if greater accessibility is needed, embryos can be cut from the yolk while attached to a filter paper frame (to maintain proper tension across the blastoderm) and cultured ex ovo (i.e., outside the eggshell, in vitro) up to 24 h on a substrate of albumin mixed with agar [2]. Working ex ovo can facilitate microsurgery, bead implantation, and electroporation of gain- and loss-of-function reagents, depending upon the stage and embryonic region being assayed. Finally, chick embryonic tissues may be dissected from the embryo and cultured in a three-dimensional collagen matrix (as in [3, 4]). The collagen supports the tissue so that it retains its normal conformation, in contrast to culturing tissue directly on a culture dish [5], which causes tissues to spread and adopt an artificial two-dimensional arrangement. With explant cultures, issues of commitment and cell-cell signaling can be addressed.

1.2 Manipulating Cell-Cell Signaling

As a model organism, chicken embryos are particularly useful to investigate the developmental consequences of secreted signals. Rather than bathing the entire embryo in a signal, cell pellets that produce the factor of interest (as in Ref. [6]) or beads soaked in purified protein (as in Ref. [7]) can be implanted to create a localized source of the signal that is gradually released and continuously replenished. The location of implanted cells or beads can be identified after a period of incubation, and neighboring cells assayed for changes in gene expression, altered morphology, or diverted migration.

1.3 Knocking Down Gene Function

Although chick is not amenable to classical genetic investigation, chick biologists can achieve transient knockdown of endogenous protein by RNA interference (RNAi; [8, 9]) or antisense morpholino oligonucleotides. Moreover, protocols using CRISPR/Cas9 to create genetic mutations recently have been developed [10–12]. This chapter covers the use of morpholinos.

Morpholino oligonucleotides (MOs) are modified nucleic acids in which the sugar backbone has been replaced with morpholine rings [13, 14]. Very stable, sequence specific, and typically eliciting minimal off-target effects in the chick, MOs can be designed to block pre-mRNA splicing or translation initiation [15, 16]. However, MOs are diluted by cell division and so are ineffective over long-term culture (i.e., greater than 24 h). MOs are produced exclusively by Gene-Tools LLC and are expensive, and multiple MOs sometimes must be tried to identify one that knocks down

protein expression efficiently. Electroporated cells are traced by including a modification on the 3′ end of the MO. This modification also provides a charge (for example, fluorescein is negative, lissamine is positive) on the uncharged MO, allowing it to be electroporated (*see* **Note 1**). While the immediate effect of a splice-blocking MO can be monitored by RT-PCR, an antibody against the target protein is the best way to document knockdown, which also depends upon protein turnover. There are comprehensive guidelines available for performing controlled morpholino experiments [14, 15].

1.4 Gain of Function

Chick biologists increase endogenous gene expression levels, or express genes at ectopic times or locations, by introducing promoter-driven DNA constructs into the embryo. A variety of vectors have been created for this purpose, such as pCIG [17], pCAβ [18], and pMES [19], all of which contain the chick beta actin promoter, an internal ribosome entry site (IRES), and a bicistronic GFP reporter. Any vector used for overexpression should include a strong promoter to drive expression (enhancers can be added to make expression tissue specific, as in [20]), a fluorescent reporter such as GFP to lineage trace cells that take up the plasmid, and an IRES or viral 2A peptide to yield separate proteins [21].

1.5 Introducing Exogenous Nucleic Acid by Electroporation

At laying, a chicken embryo has thousands of cells that are too small to be microinjected directly [22]. As such, gain- and loss-of-function reagents must be introduced into chick embryos by other means, such as retroviral transfection [23] or electroporation [16, 24]. To electroporate, a bolus of nucleic acid solution is introduced adjacent to the target structure, or inside a lumen (such as the neural tube), and electrodes are positioned on either side to create an electric field that drives the nucleic acid into the target cells. This can be done directly in ovo. However, to target very early stages when the embryo is still flat (e.g., stage 4 late gastrula), embryos must be removed from the yolk in order to create an electric field perpendicular to the blastoderm and achieve electroporation. This approach is useful when working with MOs, because perdurance of stable proteins translated prior to electroporation can mask or delay the consequences of inhibiting de novo protein synthesis with the MO. As a result, to see a phenotype it is often necessary to electroporate 10–12 h before the stage to be assayed, to allow for protein turnover and/or introduce the MO before significant protein has accumulated. For example, in order to analyze gene function at stage 10, we electroporate the cells fated to become neural crest cells at stage 4 [25].

1.6 Post-incubation

After a period of incubation to the desired stage or stage equivalent, embryos and explants can be processed in order to reveal the results

of the experimental manipulation. Embryos cultured in vivo must be removed from the yolk, and all tissues must be fixed. Then, fluorescence (MOs or GFP) can be imaged, mRNA expression visualized by in situ hybridization, or protein localization determined by immunofluorescence.

2 Materials

2.1 Culturing Chicken Embryos and Explants

1. Egg incubator (humidified) set to 100 °F/38 °C (such as G.Q.F. Manufacturing model 1202).
2. Digital outlet controller (Fisher or VWR).
3. Air incubator or tissue culture incubator set to 100 °F/38 °C.
4. Fertile chicken eggs obtained from a nearby farm, or SPAFAS-specific pathogen-free eggs from Charles River (*see* **Note 2**).
5. Ringer's saline: 123.3 mM NaCl, 1.53 mM $CaCl_2$, 4.96 mM KCl, 0.809 mM Na_2HPO_4, 0.147 mM KH_2PO_4, pH 7.4 (*see* **Note 3**).
6. 70% Ethanol prepared with water.

2.1.1 In Ovo Embryo Culture

1. 3 and 1 mL syringes.
2. 25 gauge, 5/8 in, hypodermic needles.
3. 18 gauge, 1.5 in., hypodermic needles.
4. Type A India Ink (Pelikan; note that other brands can be deleterious to embryos).
5. ¾″ wide Scotch magic tape (3 M).
6. Fine, pointed scissors with a curved blade.
7. Gauze squares.
8. Beveled-edge watch glass.
9. Parafilm squares.
10. Clear packing tape (3 M; hand-tearable or 3710 packing tape; note that some tapes are deleterious to embryos).

2.1.2 Ex Ovo Embryo Culture

1. Blunt point, straight tip, standard-pattern forceps.
2. Whatman 3 mm filter paper, cut into rectangles approximately ½″ × 5/8″ with two overlapping holes punched in the center using a standard hole punch.
3. Albumen-Agar Plates: Prepare 0.51% agar in sterile 0.12 M NaCl. Heat to boiling and equilibrate to 55 °C in a water bath. Collect an equal volume of thin albumen from unincubated chicken eggs by breaking a 1 in. slit 1/3 of the way from the blunt end of the egg (use blunt-point forceps) and tipping albumen into a 50 mL conical tube. Add 10 units/mL of

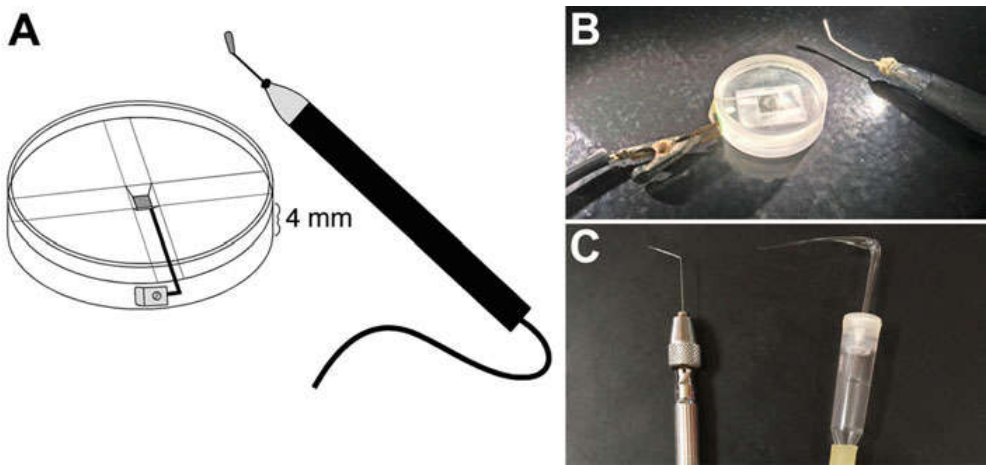

Fig. 2 Chick embryology equipment and tools. Early embryo electroporation apparatus diagram (**a**) and photograph (**b**). A platinum plate is embedded at the bottom of a 4 mm deep divet in a plastic base. Lines are etched onto the base to mark the position of the electrode plate. A wire connects the plate through the base to a flap on the side, onto which a microalligator clip attaches the bottom electrode wire. A plastic ring is attached to the top of the base, creating a dish to hold Ringer's saline. The cuvette is fixed in place under a dissecting scope using a loop of tape (visible under the dish in **b**). The top electrode is fashioned from an empty ballpoint pen casing by stringing a second wire through the casing and soldering to a 1 mm platinum wire with an end that is bent and flattened into a paddle. The wire is fixed in place with epoxy at the pen tip. Note in (**a**) that the bottom and top electrodes are not to scale. See Ref. [16] for additional details. (**c**) Useful tools. A tungsten wire sharpened to a point [26], bent at a 120° angle, and mounted in a pin holder (left) provides an efficient means to create long, straight cuts in chick embryonic tissue. Cut by pressing down through the tissue onto the bottom of the dish and sliding back and forth. A "mouth pipette" (right) is created by flame heating a glass capillary or Pasteur pipette end, which is then pulled at a 90°angle. This right-angle needle is mounted onto an aspirator assembly. The pulled needle bore can be broken off at the desired width and small pieces of tissue easily aspirated and moved without transferring much liquid

penicillin-streptomycin solution to the albumen and warm to 55 °C in a water bath. Combine the albumen and agar and swirl to mix. Pour 2 mL per 35 mm tissue culture plate. Let plates cool for 40–60 min, and then store in a sealed plastic tub at 4 °C for up to 1 week.

4. 245 mm Square bioassay dish (such as Corning 431111).

2.1.3 *Explant Culture*

1. Watchmaker's forceps #5 (Fine Science Tools).
2. Watchmaker's forceps #2 (World Precision Instruments).
3. Square glass baking dish.
4. Pin holder, to hold tungsten needles (Fine Science Tools; Fig. 2c, left).
5. Pin vise, to hold glass needles (Fine Science Tools).
6. Minutien insect pins (Fine Science Tools).
7. Sharpened tungsten wire: 1 in. lengths of 0.01″ tungsten wire sharpened to a point by dipping the wire tip into 1.0 N NaOH in an alkaline electrolysis bath [26] (Fig. 2c, left).

8. Glass capillary tubes (we prefer 0.8–1.1 mm diameter, 100 mm long), pulled into needles.
9. Needle puller (e.g., Sutter, Narashige, or Stoelting).
10. Mouth aspirator assembly (Sigma).
11. 35 mm Tissue culture plates.
12. Petri dishes.
13. Sylgard (Dow Corning) to coat 35 mm petri dishes (mix and polymerize Sylgard according to the manufacturer's instructions).
14. DMEM.
15. DMEM-F12.
16. N2 supplement (Thermo Fisher).
17. Fetal bovine serum.
18. Dispase: Prepare 0.15% Dispase II (Roche) in DMEM supplemented with 5 mM Hepes, pH 7.5. Freeze in 3 mL aliquots (*see* **Note 4**).
19. Ca^{2+}, Mg^{2+}-free Tyrode's saline (10×): 1.37 M NaCl, 0.27 M KCl, 4.2 mM NaH_2PO_4, with 1% glucose (*see* **Note 5**). Dilute 1× fresh before use and add 0.05% trypsin powder (Sigma).
20. Collagen: Mix 9 volumes rat tail collagen (BD Biosciences) with 1 volume 10× DMEM, add an aqueous solution of $NaHCO_3$ to a final concentration of 0.323%, and vortex well (*see* **Note 6**).

2.2 Manipulating Cell-Cell Signaling

1. 10 cm Tissue culture plate.
2. Cell line stably expressing a factor of interest or transfected with an expression construct (*see* **Note 7**).

2.2.1 Implanting Cell Pellets

3. PBS: 137 mM NaCl, 2.68 mM KCl, 10.1 mM Na_2HPO_4, 1.76 mM KH_2PO_4, pH 7.4 (*see* **Note 8**).
4. Trypsin-EDTA.
5. CellTracker CM-DiI (Thermo Fisher): Immediately before use, resuspend one 50 μg aliquot in 25 μL 100% ethanol, and then dilute in 500 μL of fresh, sterile 10% sucrose. Spin at full speed for 10 min in a microcentrifuge and transfer to a fresh microcentrifuge tube. Add 1 mL of Ringer's saline and mix. Make fresh before each use.
6. Bovine serum albumin, fraction V (Sigma).

2.2.2 Implanting Beads

1. Heparin acrylic beads (Sigma).
2. Affigel blue beads (Bio-Rad 153–7301 or 7302).

2.3 Knocking Down Gene Function

1. Morpholino (Gene Tools) resuspended at 1.0 mM in high-quality, nuclease-free water and stored at room temperature in the original vial (i.e., in glass and in the dark; *see* **Note 9**).

2.4 Gain of Function

1. Gene of interest cloned into a DNA expression vector, for example a construct containing the chick beta-actin promoter driving expression of the gene of interest, and including a bicistronic GFP reporter separated by the 2A peptide [21] or an internal ribosome entry site (IRES), such as in pCIG [17], pCAβ [18], or pMES [19].

2.5 Introducing Exogenous Nucleic Acid by Electroporation

1. Glass capillary tubes (we prefer 0.8–1.1 mm diameter, 100 mm long), pulled into needles.
2. Needle puller (e.g., Sutter, Narashige, or Stoelting).
3. Microloader pipette tips (Eppendorf).
4. Forced-air injection apparatus (e.g., General Valve Corporation Picospritzer or Harvard Apparatus PLI-100).
5. Electroporation chamber, custom made by local machine shop (see Fig. 2a, b or [16]).
6. Electroporation electrodes with a 4 mm gap (see [16, 24] for fabrication instructions).
7. Square pulse electroporator (see [16, 24] for recommendations).

2.6 Post-incubation

1. 4% Paraformaldehyde in PBS (or other fixative of choice); May be stored in aliquots at −20 °C.
2. 1 Dram vials, borosilicate glass with phenolic screw cap.

3 Methods

3.1 Culturing Chicken Embryos and Explants

3.1.1 In Ovo Embryo Culture

1. Use the Hamburger and Hamilton staging guide to determine the approximate time needed to incubate fertile chicken eggs to the stage of interest [25]. Set eggs horizontally (on their long sides, 3 rows will fit on a 2.5-dozen egg crate) and place into a humidified 100 °F/38 °C incubator for the necessary number of hours (*see* **Note 10**).
2. After incubation, let the eggs cool at room temperature for about 30 min (*see* **Note 11**). Swab the incubated eggs with 70% ethanol, taking care to maintain the egg in its original horizontal orientation as the embryo gradually floats to the top of the yolk. Once dry, place one or two 3 cm long pieces of Scotch tape across the top of the egg so that the shell may be cut without shattering.

3. Select an egg and place onto a bed of gauze on a watch glass (again, keep the position of the egg exactly as it was incubated). Carefully insert an 18 gauge needle mounted on a 3 mL syringe into the top side of the blunt end of the egg (Fig. 1a). Run the needle tip down along the blunt end of the egg, taking care not to puncture the yolk. Remove 3 mL albumen from the lowest part of the egg to drop the embryo away from the shell (*see* **Notes 12** and **13**).
4. Use a curved blade scissors to cut a 1.5–2 cm diameter window into the topmost surface of the egg, being careful to keep the lower scissor blade close to the inner surface of the shell so as not to disrupt the yolk (Fig. 1b).
5. Dilute 2–3 drops of India ink into 10 mL of Ringer's saline (about 5%). Fill a 1 mL syringe with diluted ink, outfit with a 25 gauge needle, and bend the needle to a 45° angle with the bevel pointed up. Tap and flush out air bubbles (*see* **Note 14**).
6. Insert the needle just outside the edge of the blastoderm (marked with a dotted line in Fig. 1c, and consisting of the lighter yellow, extraembryonic area opaca around the central embryonic area pellucida) and bring the needle tip up under the embryo. Dispense a small amount of ink to enhance embryo visibility (Fig. 1c). Gently shake the egg to disperse the ink, if necessary (*see* **Note 15**).
7. With the egg still sitting on the gauze/watch glass bed, look at the embryo under a microscope and determine the stage [25]. Use a pencil to mark the stage on the shell of good eggs, discard eggs that have not developed properly, or that are too young or too old (*see* **Note 12**). Add a few drops of Ringer's saline to keep the embryo moist and stretch a small piece of parafilm across the window to keep the embryo from drying out until ready to proceed.
8. After completing any manipulations (e.g., microsurgery or electroporation), gently add several drops of Ringer's saline to the embryo, wipe any albumen from the egg shell, and seal the window with a rectangle of clear packing tape, starting at one end and sealing around the hole (Fig. 1d). Make sure that the egg is well sealed or the embryo will dry out.
9. Incubate to the desired stage in a humidified 100 °F/38 °C incubator [25].

3.1.2 Ex Ovo Embryo Culture

1. Incubate eggs to stage 4+, usually about 25–26 h [25]. The eggs may be incubated vertically (blunt side up).
2. Wipe eggs with 70% ethanol and let cool to room temperature for 30 min (*see* **Note 11**).

3. Have ready agar-albumin plates equilibrated to room temperature ([27]; *see* **Note 16**).
4. Using a pair of blunt forceps, tap/crack/cut a 1.5–2 in. slit in the side of the egg shell about half way from the rounded end. Carefully lift off the egg shell top, and pour the yolk from the bottom shell into the palm of your gloved hand.
5. With the gloved finger of your other hand or a pair of blunt forceps, wipe and pinch off the thick albumen that adheres to the yolk and rotate the yolk until the embryo is on top (*see* **Note 17**). The embryo will appear as a light yellow circle with a clear, hourglass-shaped center. Use the edge of a pair of blunt forceps to gently wipe off the embryo to ensure that there is no thick albumen remaining attached (*see* **Note 18**).
6. Place a Whatman filter square onto the yolk so that the hole reveals the embryo. The paper will adhere to the vitelline membrane that covers the embryo. Use scissor blades to press the paper onto the yolk to ensure that it adheres well.
7. Insert a scissor blade into the yolk and cut the yolk membranes all around the paper. Grasp the embryo from one side by partially closing the scissor blades or using a #2 forceps, and gently lift one side of the embryo up and away from the yolk.
8. Place the embryo paper (dorsal) side down/yolky (ventral) side up into a dish of Ringer's saline. Gently and slowly pull the embryo back and forth through the Ringer's saline to rinse (*see* **Note 19**).
9. Place the embryo paper (dorsal) side down/yolky (ventral) side up onto an agar-albumin plate and replace the cover to maintain humidity (*see* **Note 20**).
10. Collect embryos in dishes in a humidified chamber such as a bioassay tray outfitted with moistened wipes or paper towels (*see* **Note 21**).
11. Incubate to the desired stage in a humidified 100 °F/38 °C incubator [25].

3.1.3 Explant Culture

1. Incubate several dozen eggs to the stage at which the tissue of interest is present. Use the Hamburger and Hamilton staging guide to estimate the length of incubation required [25]. The eggs may be incubated vertically (blunt side up).
2. Wipe eggs with 70% ethanol and let cool to room temperature for 30 min (*see* **Note 11**).
3. Without breaking the yolks, carefully and gently crack the eggs into a square glass baking dish a dozen at a time. The embryos will float to the top.

4. Identify the embryo. In embryos stage 12 and older, the plexus of blood vessels that surrounds the embryo marks its location. In younger embryos, find a light yellow circle with a clear, hourglass center. Using a pair of scissors, cut around the embryo to free it from the yolk (*see* **Note 22**). With the scissor blades closed, scoop under the embryo and lift, so that the embryo drapes over the closed blades (*see* **Note 23**).
5. Quickly submerge the embryo/scissor blades into a petri dish full of Ringer's saline and swish back and forth to remove the embryo. Repeat until all embryos are in the dish of Ringer's (*see* **Note 24**).
6. Lift off the vitelline membrane using a pair of forceps or a tungsten needle, and rinse the embryos using a cutoff transfer pipette. Transfer the embryos to a clean dish of Ringer's saline (*see* **Note 24**).
7. Tissues may be isolated through a variety of methods. Explants may be manually dissected from superficial structures (for example a neural fold) using glass or tungsten needles, or a pair of spring scissors.
8. To facilitate dissection of adherent tissues, minutien insect pins may be used to fasten embryos to a Sylgard-coated dish in calcium and magnesium-free Tyrode's saline +0.05% trypsin. Over time, this solution causes tissues to release from one another, and allows the tissue of interest to be more easily dissected free with pulled glass capillary needles or sharpened tungsten wire.
9. Another option is to cut blocks of tissue using a tungsten needle bent at a ~120° angle (Fig. 2c, left) by pressing the bottom edge of the wire through the tissue against the bottom of the dish and sliding back and forth (*see* **Note 25**). This tissue block can be incubated in dispase to cleave extracellular matrix proteins and gently dissociate tissues from one another (*see* **Note 26**). Dispase-treated tissue can then be "triturated" (pipetted in and out) against the side of the dish with a fire-polished Pasteur pipette to separate adhering layers.
10. Trypsin or dispase-treated tissue explants should be rinsed immediately in several changes of ice-cold media (DMEM or DMEM-F12) + 10% fetal bovine serum (*see* **Note 27**).
11. Spread 6 μL of collagen onto the bottom of a tissue culture dish (*see* **Note 28**). Cover the dish and allow the collagen to set (it will become opaque), but do not let it dry out.
12. Place the tissue/cells to be cultured on the collagen bed. Transfer as little solution as possible (*see* **Note 29**). Add 4 μL more collagen over the tissue and allow to set.

13. Cover the collagen-embedded tissue with an appropriate culture medium (we use DMEM-F12 + N2 supplement for neural crest cells) and culture at 37 °C with CO_2 for the number of hours necessary to reach the desired stage equivalent (the stage the embryo from which the explant was dissected would have been after the incubation) [25].

3.2 Manipulating Cell-Cell Signaling

3.2.1 Implanting Cell Pellets

1. Prepare a confluent 10 cm plate of a stable cell line or cells transfected with an expression construct using standard tissue culture protocols. Remember to prepare control cells as well (e.g., cells transfected with a control vector).
2. Remove the media from the cells and wash the plate with 10 mL of PBS. Aspirate well. Add 1 mL of trypsin-EDTA and rock/rotate the plate to cover the entire surface. Aspirate excess trypsin. Incubate for ~3–5 min, as needed until cells break free.
3. Collect the cells by rinsing with 10 mL of media + 20% fetal bovine serum. Transfer cells to a 50 mL conical tube. Pipette in and out to achieve a single-cell suspension, and then allow cells to recover for 30 min in the tissue culture hood, swirling occasionally to keep in suspension.
4. Bring the volume to 50 mL with PBS or Ringer's saline. Gently collect cells by centrifugation at 225 rcf for 5 min.
5. Carefully aspirate the supernatant and resuspend cells in 300 μL of DiI solution. Incubate for 15 min in the hood at room temperature.
6. Bring the volume to 50 mL with PBS or Ringer's. Gently collect cells by centrifugation at 225 rcf for 5 min. Aspirate supernatant.
7. If cells will be injected, resuspend cells in 1 mL of Ringer's saline + 0.1% BSA. Transfer to a 1.7 mL microcentrifuge tube, centrifuge at 300 × *g*, and aspirate supernatant. Resuspend pellet in an equal volume of Ringer's saline + 0.1% BSA by tapping and flicking the tube. Tube can be stored on ice. To inject, back load 2 μL of thoroughly resuspended cells into a pulled glass capillary needle using a microloader pipette tip. Air pressure inject groups of cells into a region of interest (e.g., the lumen of the neural tube, the head mesenchyme; *see* **Notes 30** and **31**).
8. If cells will be surgically grafted, resuspend cells from **step 6** in an equal volume of complete culture medium by tapping and flicking. Pipette 20–50 μL drops of cell suspension onto the underside of a tissue culture plate lid. Fill the dish itself with media or PBS (for humidity), invert the lid to cover the dish, and incubate the "hanging drop" at 37 °C in a CO_2 incubator for 24 h. The cells will form spheroids that can be grafted into

host embryos using a pair of forceps to insert them through incisions cut with sharpened tungsten wire.

9. After either **steps 7** or **8**, add several drops of Ringer's saline to the embryo, wipe any albumen from the egg shell, and seal the window with a rectangle of clear packing tape, starting at one end and sealing around the hole (Fig. 1d). Make sure that the egg is well sealed or the embryo will dry out.
10. Incubate to the desired stage in a humidified 100 °F/38 °C incubator [25].

3.2.2 Implanting Beads

1. Obtain Heparin Acrylic or Affigel Blue beads according to the application (*see* **Note 32**).
2. Wash the beads three times by rolling/moving them through a series of PBS drops on the lid of a tissue culture plate.
3. Place a drop of protein diluted in PBS + 0.1% BSA at the center of a 35 mm tissue culture dish (*see* **Note 33**). Place additional drops of PBS around the bottom of the dish for humidity.
4. Select a bead and place it on a dry area of the plate. Move the bead back and forth to draw out excess PBS. This step is important so that you do not transfer PBS along with the bead and dilute the protein.
5. Place the dried bead into the protein drop and incubate for 2 h at room temperature to 4 °C overnight.
6. Wash the bead by rolling/moving it through two or three drops of PBS.
7. Implant the bead into a region of interest in the embryo. Typically a slit must be prepared with a sharpened tungsten needle, and the bead pushed into the slit with forceps (*see* **Note 34**).
8. Add several drops of Ringer's saline to the embryo, wipe any albumen from the egg shell, and seal the window with a rectangle of clear packing tape, starting at one end and sealing around the hole (Fig. 1d). Make sure that the egg is well sealed or the embryo will dry out.
9. Incubate to the desired stage in a humidified 100 °F/38 °C incubator [25].

3.3 Knocking Down Gene Function

3.3.1 Morpholino Oligonucleotides (MOs)

1. Find your target gene of interest in the latest chick genome assembly using www.ensembl.org.
2. Decide on the type of MO to use. A translation blocking MO anneals over the translation start site to sterically block the translation initiation complex. A splice blocking MO anneals over a splice acceptor or splice donor site in a pre-mRNA to disrupt splicing and induce exon skipping, causing a frameshift

Table 1
Issues to consider when selecting the type of morpholino to use in your experiments

Translation blocking MO	Splice blocking MO
Sterically blocks the translation initiation complex to prevent translation	*Blocks a splice donor or acceptor site in the pre-mRNA to cause exon skipping, a frameshift, and premature stop*
A good choice when: – Sequence annotation includes a high-confidence translation start site without alternate/cryptic translation start sites – A specific start site/isoform is to be targeted	*Advantages:* – The efficacy of the MO can be assayed by PCR amplification of the targeted exon and the splicing outcome determined by sequencing the product – When MOs are designed against a splice junction acceptor and donor site, a "dose-synergy" phenotype generated by sub-phenotypic doses of each MO is evidence of specificity [14]
A challenging approach when: – There is no antibody available for the target protein in order to assay knockdown efficiency	*Not an option when:* – The gene contains no introns – The first coding exon is very long – Length of initial exons is divisible by three so splicing is in frame even when an exon is skipped

and a premature stop. A variety of factors will determine which MO type is best suited to an application (Table 1; see also guidelines in [14]).

3. Decide on the type of 3′ end modification to add. Unmodified morpholinos have no charge and thus will not be transfected into cells by electroporation. Addition of a 3′ modification provides a charge to allow for electroporation as well as a means to visualize targeted cells directly (fluorescein, lissamine) or indirectly (biotin). Different modifications carry different charges (fluorescein and biotin have a net negative charge, while lissamine has a net positive charge; *see* **Note 1**), which dictates the polarity of the electric field necessary for transfection.
4. www.gene-tools.com provides design recommendations and offers a free design service.

3.4 Gain of Function

1. Use standard molecular biology methods to subclone your gene of interest into an expression vector [28]. If the gene is not already cloned, we design primers based upon the genome sequence and use a high-fidelity DNA polymerase to PCR amplify the gene from chick embryo cDNA, following standard molecular biology methods [28]. It is important to limit the amount of untranslated sequence that is included with the gene of interest, as untranslated sequence can cause genes to be expressed less efficiently.

2. Prepare plasmid DNA that is endonuclease free and concentrated enough to yield 1 μg/μL to 4 μg/μL dilutions for electroporation (1 μg/μL is usually a good starting point; *see* **Note 35**).

3.5 Introducing Exogenous Nucleic Acid by Electroporation

3.5.1 In Ovo Electroporation

1. Prepare windowed eggs as in Subheading 3.1.1.
2. Use a microloader tip to back-fill a glass needle with 2–5 μL of plasmid or MO. Place the needle in the needle holder of a forced-air injection apparatus (*see* **Note 30**). Break off the very tip of the needle and expel any air remaining in the tip. Test and adjust the injection time until only a small drop is produced.
3. Introduce a bolus of plasmid or MO adjacent to the tissue to be electroporated (*see* **Notes 31**, **36**, and **37**). Best results will be obtained by injecting into a lumen (e.g., the interior of the neural tube) that will act as a reservoir to contain the injected material and limit diffusion. Keep in mind that negatively charged DNA and the negative charge of a fluorescein modification on the MO (MOs themselves are uncharged) will enter cells in the direction of the positive electrode. Positively charged lissamine-modified MO will enter cells in the direction of the negative electrode. *See* **Notes 1** and **38** and [16, 24] for additional pointers.
4. Moisten the electrodes with Ringer's saline and place them 4 mm apart on either side of the embryo, with the tissue to be electroporated located between the injected material and the relevant electrode (positive for DNA and fluorescein-conjugated MOs, negative for lissamine-conjugated MOs). The degree to which the electrode contacts the embryo surface and the angle between the electrodes will determine the extent and direction of electroporation (*see* **Note 39**).
5. For ~10-somite embryos, apply five, square-wave 20 volt, 50-ms pulses with 100-ms intervals between pulses (*see* **Note 40**).
6. Let the embryo recover for 1–2 min, then gently place a few drops of Ringer's saline onto the embryo, wipe any albumen from the egg shell, and seal the window with a rectangle of clear packing tape, starting at one end and sealing around the hole (Fig. 1d). Make sure that the egg is well sealed or the embryo will dry out.
7. Incubate to the desired stage in a humidified 100 °F/38 °C incubator [25].

3.5.2 Ex Ovo Electroporation

1. Prepare embryos on agar-albumin plates as in Subheading 3.1.2.

2. When ready to electroporate, excess yolk that is adhering to the embryo must be removed as it will impede visualization as well as electroporation. Tilt the agar-albumin plate toward you at a 45° angle and use a transfer pipette to drip Ringer's saline onto the elevated side of the plate (avoiding dripping directly onto the embryo) so that Ringer's saline washes over the embryo to moisten it. Then, either tip the dish back and forth to further clean yolk from the embryo and use the transfer pipette to aspirate the Ringer's wash from the dish or use a pair of forceps to lift the embryo from the plate and drag it gently back and forth through a dish of clean Ringer's saline.
3. Place the embryo paper (dorsal) side down/yolky (ventral) side up in an electroporation cuvette (Fig. 2a, b) containing clean Ringer's saline.
4. Using published fate maps, determine the location of the precursors of the cell type to be targeted in a stage 4+ embryo [29–32]. Use the cuvette alignment lines to orient the embryo so that this target region lies over the center of the bottom electrode (*see* **Note 41**).
5. Use a microloader tip to back-fill a glass needle with 2 μL of diluted plasmid or MO. Place the needle in the needle holder of a forced-air injection apparatus (*see* **Note 30**). Break off the very tip of the needle and expel any air remaining in the tip. Test and adjust the injection time until only a small drop about 1×–1.5× the width of the primitive streak is produced (*see* **Note 42**).
6. Gently insert the needle through the embryo from the top-facing ventral side into the subvitelline space and expel a puddle of plasmid or MO adjacent to the cells of the blastoderm to be electroporated (*see* **Note 31**). Embryos may be targeted unilaterally (useful when characterizing a knockdown phenotype, as the untargeted internal control establishes the baseline for that embryo), or on both sides (useful when collecting tissue for other manipulations) by varying the size and location of the puddle.
7. Before the injected solution has a chance to disperse, place the upper electrode into the Ringer's saline directly over (but not touching) the region of the embryo containing the injected solution (top and bottom electrodes should be ~4 mm apart). If the paddle of the top electrode is not covered with Ringer's saline, add more. Ideally, the upper electrode should be placed symmetrically so that both the targeted (injected) and untargeted control sides are exposed (*see* **Note 43**).
8. Apply five, square-wave 7 volt 50-ms pulses with 100-ms gaps between pulses (*see* **Note 40**).

9. Transfer the embryo from the electroporation cuvette back to the agar-albumin plate, paper/embryo dorsal side down.
10. Incubate to the desired stage in a humidified 100 °F/38 °C incubator [25].

3.6 Post-incubation

3.6.1 In Ovo Embryo Processing

1. When the embryo has reached the desired stage [25], cut the tape sealing the egg window and add a few drops of Ringer's saline to the embryo to moisten the surface (*see* **Note 44**).
2. Use a pair of scissors to cut around the embryo. Lift the embryo from the yolk by grasping a cut edge with a pair of forceps, or by scooping and lifting under the embryo with a pair of closed scissor blades (*see* **Note 45**).
3. Place the embryo into a petri dish with Ringer's saline. Rinse yolk from the embryo, lift off and discard the vitelline membrane, and trim extraembryonic tissues (*see* **Note 25**).
4. Fix embryos with 4% paraformaldehyde and store in 1 dram vials according to standard protocols for immunocytochemistry or in situ hybridization [33] (*see* **Notes 46** and **47**).

3.6.2 Ex Ovo Embryo Processing

1. Embryos may be fixed while still attached to the paper in order to maintain a flat conformation. To do this, remove embryos from agar-albumin plates and place them embryo side up in a dish of Ringer's (keep them sorted in separate dishes according to the construct that was electroporated). Spread them out around the bottom of the dish.
2. Alternatively, the embryo may be lifted off of the filter paper for more compact storage (e.g., in 1 dram vials). Place the embryo into a petri dish of Ringer's saline with the paper side down. Find a place where the membranes are rolling up or detaching. With a pair of forceps, gently peel the embryo away (the vitelline membrane will remain attached to the paper) and trim extraembryonic tissues (*see* **Notes 25** and **48**).
3. Fix embryos with 4% paraformaldehyde and store in 1 dram vials according to standard protocols for immunocytochemistry or in situ hybridization [33] (*see* **Notes 46**, **47**, and **49**).

3.6.3 Explant Processing

1. After incubation, tissue embedded in collagen can be fixed and processed for immunocytochemistry or in situ hybridization using standard protocols [33]. The collagen may be left attached to the culture dish for staining, or may be gently lifted off with a pair of forceps and processed as a single mass (*see* **Note 50**).

4 Notes

1. Although lissamine effectively traces the MO and allows for electroporation, because it is positively charged, it cannot be co-electroporated with negatively charged DNA in order to assay rescue of a knockdown phenotype (as a specificity control). Thus, lissamine modification of MOs is not recommended.
2. Eggs can be stored at 13–20 °C for up to 1 week before use, although fertility (i.e., number of viable embryos) will be highest when eggs are fresh and will decline over time. An old refrigerator outfitted with a WINE-STAT (Walnut Creek, CA) makes an inexpensive egg-storage cooler. Eggs may also be placed in a cool corner of the lab.
3. 1 L of Ringer's saline is prepared by dissolving 7.2 g NaCl, 0.17 g $CaCl_2$, 0.37 g KCl, 0.115 g Na_2HPO_4, and 0.02 g KH_2PO_4 in ~900 mL of water, adjusting pH to 7.4, and adding H_2O up to 1 L. Filter sterilize; do not autoclave.
4. Excess DMEM plus HEPES may be stored in 50 mL aliquots at −20 °C for later use.
5. 1 L of 10× Ca^{2+}, Mg^{2+}-free Tyrode's saline is prepared by dissolving 80 g NaCl, 2 g KCl, 0.5 g NaH_2PO_4, and 10 g glucose in H_2O up to 1 L. Filter sterilize; do not autoclave.
6. 100 μL of collagen is prepared using 90 μL of rat-tail collagen, 10 μL of 10× DMEM, and 4.5 μL of 7.5% $NaHCO_3$. The collagen mix can be stored for short periods on ice before use. The solution should be light pink and set in 15–20 min at room temperature. More (or less) $NaHCO_3$ can be added if this is not the case.
7. We have successfully implanted cell pellets of many different cell lines.
8. 1 L of PBS is prepared by dissolving 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, and 0.24 g KH_2PO_4 in ~900 mL of water, adjusting pH to 7.4, and adding H_2O up to 1 L. Autoclave. PBS can also be prepared as a 10× stock and diluted to 1× with sterile water before use.
9. Storing resuspended morpholino in plastic and at cold temperatures may result in precipitation and morpholino loss. If aliquots need to be made, they should be freeze-dried according to the protocol on the Gene Tools website (www.gene-tools.com), and individually resuspended before each use.
10. Incubators may be placed on digital outlet controller timers in order to start eggs at inconvenient times (e.g., the middle of the night).

11. Yolks are more fragile and break easily when hot.
12. Before starting, have ready a bag to discard damaged or unneeded eggs and a beaker to contain removed albumen. Egg waste should be disposed as required by your institution.
13. It is typically not necessary to apply scotch tape over the syringe needle hole as the egg usually seals itself; however, tape should be applied if the hole is extensive. Alternatively, if the syringe needle hole is far enough toward the top of the egg, it may be used as a starting point to cut the access hole in the next step (Subheading 3.1.1, **step 4**).
14. If bubbles are injected into the yolk along with diluted ink, they will eventually be resorbed and will not alter development; however, bubbles make it more difficult to visualize the embryo.
15. Over time the ink may disperse within the yolk, and the embryo may be re-inked. When possible, reinsert the needle through the same hole next to the blastoderm.
16. While collecting and working with embryos, agar-albumin plates can be stored in a bioassay tray to keep them protected and organized.
17. Make sure that the gloved finger you use to wipe the yolk is wet with albumen and not dry, or you will break the yolk.
18. If albumen remains on the yolk, the paper will not stick well, the embryo will detach, and development will be abnormal or arrested.
19. Be gentle and patient. Do not wash the embryo vigorously, and be sure that the embryo does not detach from the paper. Both over- and underwashing lead to problems with viability.
20. Embryos are stable on agar-albumin plates at room temperature for about 2–4 h. Depending on the size of your embryos/filter papers, two to three embryos may be cultured on a single agar-albumin plate.
21. After culture, bioassay dishes can be washed and reused multiple times.
22. When cutting the yolk membranes, make your first cuts along the side of the embryo that is lowest on the yolk. The contents of the yolk will spill out once it is broken; thus if you cut the highest point first, the embryo will be pulled under the yolk.
23. Embryos stage 10 and younger are small and very fragile. Paper disks (as described in Subheading 3.1.2) may be used to support young embryos during isolation from the yolk.
24. Chick embryos will stick to untreated plastic dishes and transfer pipettes. To avoid sticking, rinse unused plastic with Ringer's saline that is milky with egg yolk debris (for example, Ringer's

saline in which embryos have been rinsed, or into which a small amount of egg yolk has been added). After coating the plastic with yolky Ringer's, discard and rinse with fresh Ringer's saline, and then proceed.

25. Bent tungsten needles (Fig. 1c) are also convenient for trimming excess extraembryonic membrane after embryo fixation.
26. To isolate trunk neural tubes, we incubate stage 14–15 caudal trunk segments in dispase for 15 min on ice, and then 10 min at 37 °C, until tissues begin to fall apart. Dispase incubation conditions will need to be optimized for each tissue type.
27. Neural crest cell cultures grow best in DMEM-F12 media. However, to rinse away enzyme, either DMEM or DMEM-F12 is acceptable.
28. The collagen should be spread only a little. It should not be too thin, nor should it be significantly domed. More collagen can be used if larger explants are cultured.
29. With practice, a glass needle bent in a ~90° angle (created by holding a capillary tube or Pasteur pipette tip in a flame and pulling in a perpendicular direction) fitted onto an aspirator assembly (shown in Fig. 1c) provides a controlled means to move small tissue explants from one dish/location to another while transferring very little liquid.
30. Injections can be performed using a commercial forced-air microinjection instrument, or a homemade apparatus with a capillary microinjection holder (such as Warner MP-S10 T or similar) outfitted onto tubing attached to compressed air with tubing incorporating a "Y" connector (covering the open branch of the "Y" will force air out of the capillary tip). If the material being injected is not too dense/viscous a mouth aspirator assembly can also be used.
31. We generally hold the needle assembly in our hand to inject, but a micromanipulator can also be used to hold the needle steady.
32. Affigel blue beads work for a variety of proteins, while heparin acrylic beads work well for growth factors that bind heparin. Affigel blue beads are "stickier," while heparin acrylic beads can be easier to manipulate. Deciding which bead to use is a combination of the factor being released, preference, and empirical evidence/experience.
33. The protein concentration used depends upon the factor of interest and must be determined empirically. Generally, FGFs, Shh, and Noggin are typically used at 1 mg/mL, while BMPs can be used at lower concentrations, for example 0.1 mg/mL.

34. If your bead will not stay in place, try using a smaller bead. Heparin acrylic beads can also be chipped and a smaller fragment used instead.
35. DNA can be ethanol precipitated prior to electroporation if more concentrated samples are needed; however, be sure to include a 70% ethanol wash to remove salt as it can interfere with electroporation.
36. If the injected solution dissipates rapidly, sterile 10% sucrose can be added at 1:4 or 1:1 to make the solution more viscous and less easily displaced. Be sure to account for the extra volume when preparing the DNA or MO dilution.
37. Although the fluorescein or lissamine modifications give the MO solution color, 2% vegetable dye (FD&C Blue, Spectra Colors Corporation) can be added to the MO at 1:10–1:20 for improved visibility of the injected solution. Fast Green is not recommended because it can inhibit the uptake of the MO [34].
38. When mixed, MO and DNA each facilitates the electroporation of the other. This must be kept in mind when attempting to rescue a MO knockdown phenotype by co-electroporating a DNA expression construct, but can also be used to increase electroporation efficiency. For example, adding 0.3 μg/μL DNA (any non-biologically active DNA will do) to the MO can vastly improve its electroporation efficiency. Likewise, including low concentrations of a non-targeting MO can increase the efficiency of DNA electroporation as well.
39. The tips, bends, or arms of the electrodes may be placed on the embryo for electroporation. The angle of electroporation is changed by placing more pressure on one electrode than the other.
40. The number of pulses and voltage can be adjusted to optimize survival and targeting efficiency for your tissue and age of embryo. Generally speaking, younger embryos must be electroporated with lower voltages.
41. Ensure that the embryo is clean so that large amounts of yolk are not blocking the lower electrode or covering the embryo.
42. The drop size produced will determine how localized versus dispersed the solution that is introduced becomes, and can be varied according to preference and the requirements of the experiment. Multiple injections can also be performed to increase the size of the puddle.
43. To ensure that both sides of the embryo are treated equally, we place the upper electroporation paddle perpendicular to the primitive streak and over both sides of the embryo, even if only one side is being transfected. Thus, both sides are

electroporated, but there is a targeted and an untargeted side for comparison to control for electroporation.

44. The surface of the embryo will be very sticky without being moistened first.
45. It may be necessary to grasp one edge of the embryo with a pair of forceps while you cut so that the embryo isn't pulled under by spilling yolk.
46. Fixation can attenuate GFP fluorescence; thus embryos electroporated with GFP-expressing constructs may need to be imaged prior to fixation or after a brief initial fixation.
47. It is a good idea to image fluorescein-modified MO-electroporated embryos before subsequent staining protocols as the fluorescence quickly fades. Keep the embryos in the dark at all times to minimize this.
48. Embryos may also be removed from the filter papers in this manner after fixation and before storing in a 1 dram vial.
49. Ex ovo culture can result in nonspecific midbrain neural tube closure defects. By electroporating only one side of the embryo, phenotypes can be interpreted (by comparing to the untargeted side) as long as appropriate controls are included.
50. Occasionally the collagen gives high background staining, in which case it is necessary to dissect the pieces of tissue from the collagen and stain them individually (this should be done right before staining as small explants can be very difficult to retain and track).

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Chapter 7

Identifying Protein-DNA and Protein-Protein Interactions in Avian Embryos

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Abstract

The chick embryo is a powerful model for experimental embryology due to its accessibility, sturdiness, and ease of manipulation. Here we describe protocols for analysis of protein-DNA and protein-protein interactions in tissues and cells isolated from the developing chick. These assays are aimed at the identification of interactions between transcription factors and regulatory elements in the genome, and, in combination with functional assays, can be used for the delineation of gene regulatory circuits.

Key words Chick embryo, Protein-DNA interactions, Protein-protein interactions, Chromatin precipitation (ChIP), Co-immunoprecipitation (Co-IP)

1 Introduction

Characterizing molecular interactions is an important step in the study of the regulatory programs that orchestrate embryonic development. In particular, the identification of protein-DNA and protein-protein complexes that are present in specific spatial domains of the embryo can inform upon the mechanisms controlling patterning and morphogenesis [1]. There are, however, considerable challenges in performing such assays in embryonic tissue. Most of the established protocols aimed at testing protein-DNA and protein-protein interactions require large amounts of starting material. Here we present protocols for two gold standard biochemical assays in the chick embryo: *chromatin immunoprecipitation* (ChIP) [2, 3] for identification of binding of transcription factors to DNA targets, and *co-immunoprecipitation* (Co-IP) [4] for characterization of protein complexes. These protocols have been optimized for a relatively low input, requiring a small number of embryos (or small amounts of embryonic tissue) as starting material.

Both methods are based on the immunoprecipitation of a target protein via specific antibodies that have been complexed to

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magnetic beads. In the ChIP assay, cross-linked fragments of DNA-protein complexes are immunoprecipitated from the cell lysates using a protein-specific antibody. Associated DNA fragments can subsequently be amplified through qPCR analysis using primers for the sequence of interest. In our Co-IP protocol, we utilize ex ovo electroporation [5] to drive the expression of two proteins of interest. The bait protein is fused to an Avi tag, which can be isolated using streptavidin-coated beads upon in vivo biotinylation mediated by the BirA enzyme. Cofactors bound to the bait protein are subsequently identified by western blotting. We routinely perform these experiments with tissue explants obtained through microdissection, although cells obtained through FACS can also be used. We find that the chick embryo, which is easy to obtain and dissect, is a convenient vertebrate model organism for biochemical studies.

2 Materials

2.1 Chromatin Immunoprecipitation: ChIP-qPCR

1. Formaldehyde stock solution: 37% Formaldehyde.
2. Glycine stock solution: 1 M Glycine.
3. DTT stock solution: 1 M DTT.
4. Sucrose stock solution: 1.5 M Sucrose.
5. PMSF stock solution: 0.2 M PMSF, in EtOH (*see* **Note 1**).
6. Protease inhibitor tablet (Complete, Mini EDTA-free)—Roche.
7. Protease inhibitor stock solution: Prepare 7× stock solution by dissolving 1 tablet in 1.5 ml of ddH_2O (*see* **Note 2**).
8. Tris–HCl, pH 7.5 stock solution: 1 M Tris–HCl, pH 7.5.
9. Tris–HCl, pH 8.0 stock solution: 1 M Tris–HCl, pH 8.0.
10. Hepes-KOH stock solution: 1 M Hepes–KOH, pH 8.
11. NaCl stock solution: 5 M NaCl.
12. LiCl stock solution: 5 M LiCl.
13. $CaCl_2$ stock solution: 0.1 M $CaCl_2$.
14. 10× TE: 100 mM Tris–HCl pH 8, 10 mM EDTA.
15. SDS stock solution: 20% SDS.
16. EDTA stock solution: 0.5 M EDTA pH 7.5.
17. Triton X-100 stock solution: 10% Triton X-100.
18. NP-40 stock solution: 10% NP-40.
19. Accumax—Innovative Cell Technologies.
20. Dynabeads™ Protein G beads—Invitrogen.
21. DynaMAg™-2 magnet—Invitrogen.

22. RNase A.
23. Proteinase K.
24. Phenol:chloroform:isoamyl alcohol (25:24:1, v/v).
25. 2 mL Small glass Dounce homogenizer set (pestle B).
26. Ringer's solution (0.125 M sodium chloride, 1.5 mM calcium chloride, dehydrate, 5 mM potassium chloride, 0.8 mM sodium phosphate, dibasic).
27. PBS (137 mM NaCL, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4).
28. Blocking solution: 0.5% BSA in PBS, filtered.
29. Nuclei extraction buffer (prepared fresh): 0.5% NP-40, 0.25% Triton X-100, 10 mM Tris–HCl, pH 7.5, 3 mM CaCl2, 0.25 M sucrose, protease inhibitor tablet (1 mini tablet/10 mL of buffer), 1 mM DTT, and 0.2 mM PMSF.
30. Lysis buffer: 1% SDS, 10 mM EDTA, 50 mM Tris–HCl, pH 7.5, 1× Protease Inhibitor.
31. Dilution buffer: 0.01% SDS, 1.2 mM EDTA, 16.7 mM Tris–HCl, pH 8.0, 167 mM NaCl, 1 mM DTT, 0.4 mM PMSF, and protease inhibitors 1× (added just before use).
32. Triton dilution buffer: 0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris–HCl, pH 8.0, 167 mM NaCl. 1 mM DTT, 0.4 mM PMSF, and protease inhibitors 1× (added just before use).
33. RIPA (washing buffer): 50 mM Hepes-KOH, pH 7.6, 500 mM LiCl, 1 mM EDTA, 1% NP-40, 0.7% Na-deoxycholate.
34. TE/NaCl: 10 mM Tris–HCl, 1 mM EDTA pH 8.0, 50 mM NaCl.
35. Elution buffer: 1% SDS, 10 mM EDTA, 50 mM Tris–HCl, pH 8.0.

2.2 Co-immuno-precipitation: Co-IP

1. DTT stock solution: 1 M DTT.
2. PMSF stock solution: 0.2 M PMSF, in EtOH (*see* **Note 1**).
3. Protease inhibitor tablet (Complete, Mini EDTA-free)—Roche.
4. Protease inhibitor stock solution: Prepare 7× stock solution by dissolving 1 tablet in 1.5 ml of ddH_2O (*see* **Note 2**).
5. Ringer's solution (0.125 M sodium chloride, 1.5 mM calcium chloride, dehydrate, 5 mM potassium chloride, 0.8 mM sodium phosphate, dibasic).
6. PBS (137 mM NaCL, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4).

7. 1 mL Syringe and 27.5 gauge needles.
8. Hepes-KOH stock solution: 1 M Hepes-KOH pH 8.
9. NaCl stock solution: 5 M NaCl.
10. 1% BSA in PBS, filtered.
11. Dynabeads™ MyOne™ Streptavidin T1—Invitrogen.
12. DynaMAgTM-2 magnet—Invitrogen.
13. LDS Sample Buffer (4×)—Life Technologies.
14. Sample Reducing Agent (10×)—Life Technologies.
15. Hypotonic buffer: 10 mM Hepes pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM DTT, 0.4 mM PMSF, and protease inhibitors 1× (added just before use).
16. IP extraction buffer: 20 mM HEPES, 1.5 mM MgCl2, 0.2 mM EDTA, 20% glycerol, 420 mM KCL, 1 mM DTT, 0.4 mM PMSF, and protease inhibitors 1× (added just before use).
17. Modified RIPA buffer: 50 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40.

3 Methods

3.1 Chromatin Immunoprecipitation: ChIP-qPCR

Here we describe our approach for performing ChIP-qPCR in tissue explants dissected from chick embryos (Fig. 1). The protocol is standardized for small amounts of tissue; we found that most abbreviated protocols result in loss of sensitivity and thus are not suitable for small amounts of embryonic cells. Wild-type embryos are collected from eggs and dissected. Embryonic explants are dissociated to a single-cell suspension with Accumax, and cross-linked with formaldehyde. We use a Diagenode Bioruptor for chromatin shearing, although Covaris can be used as well. For immunoprecipitation, we found that Protein G Dynabeads give us the best results, even for rabbit antibodies. Throughout the protocol, it is essential to employ careful pipetting during the washes to minimize loss of material.

3.1.1 Embryo Dissociation and Cross-Linking

1. Dissect embryos in Ringer's solution (*see* **Note 3**).
2. Transfer dissected tissue (~50–100,000 cells) to a microcentrifuge tube.
3. Quickly wash embryos with Ringer's to remove yolk and other debris. Spin 2′ at 200 rcf to collect the tissue at the bottom of the tube.
4. Rinse quickly with 300–500 μL Accumax to remove excess salt from Ringer's.

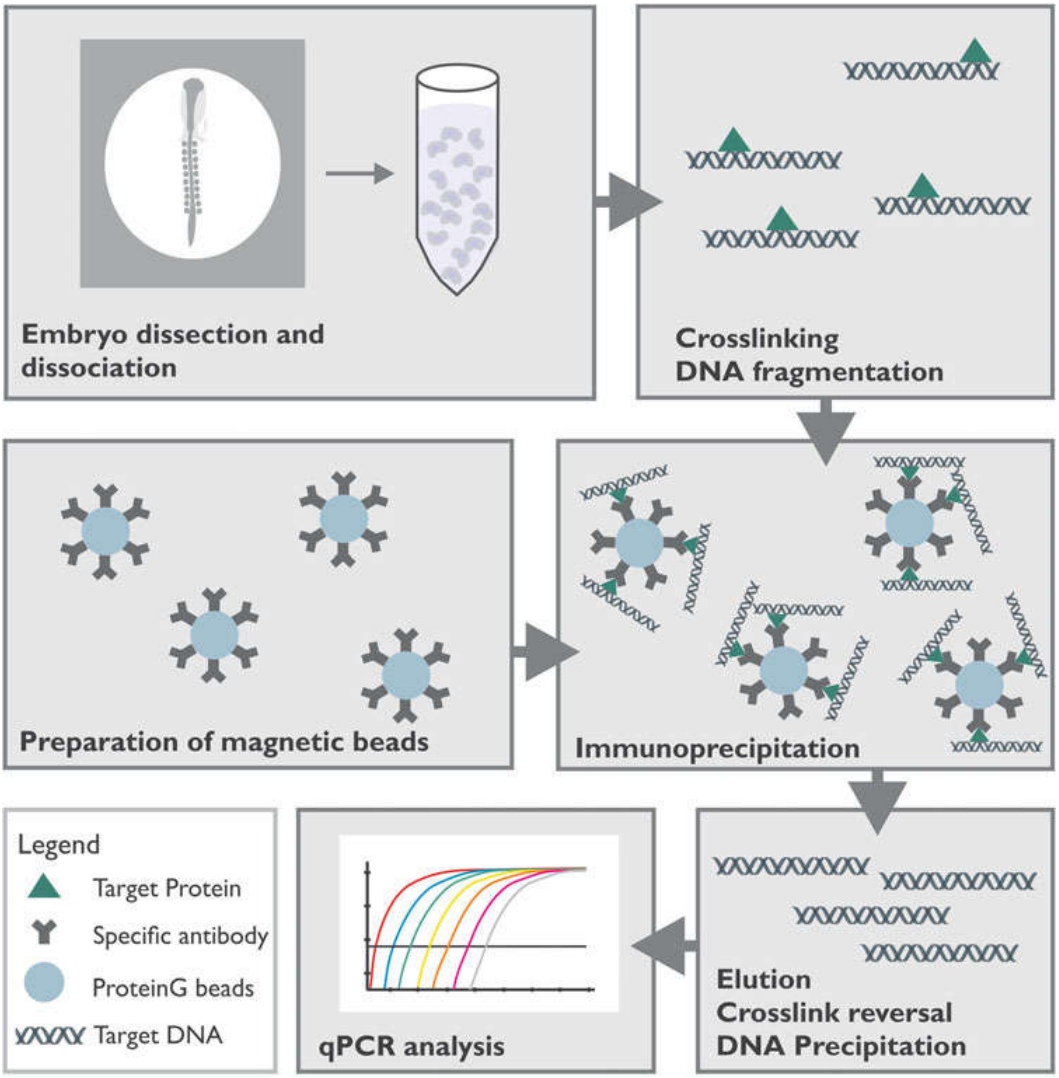

Fig. 1 Schematic overview of the ChIP-qPCR protocol

5. Add 973 μL of Accumax and incubate the tissue at RT for 20–30′, rocking at orbital shaker. During this period, gently pipette the tissue up and down to help tissue start dissociate.
6. Add 27 uL of 37% formaldehyde to each tube. Cross-link for exactly 10′ at RT, rocking.
7. Add 143 μL of cold 1 M glycine to each tube to quench the cross-linker. Make sure that the glycine is properly mixed with the cell suspension.
8. Immediately spin down the cells for 4′ at 2000 rcf, 4 °C.
9. Gently resuspend cells with ice-cold PBS supplemented with protease inhibitors (1× protease inhibitor, 10 μL 1 M DTT, and 20 μL 0.2 M PMSF). During washes, samples should be kept on ice to avoid protein degradation.
10. Spin down the cells for 4′ at 2000 rcf, 4 °C.

11. Repeat washes two more times. After the last wash, remove all of the PBS and immediately freeze the pellet with liquid nitrogen. Store cells at −80 °C.

3.1.2 Lysis and Chromatin Fragmentation

1. Resuspend pellet (fresh or frozen) in 1 mL nuclei extraction buffer and transfer the sample to a 2 ml small glass Dounce homogenizer that has been kept on ice (*see* **Note 4**).
2. Re-homogenize with 10 strokes using Pestle B. This will disrupt the cellular walls and help expulse the nuclei into the solution.
3. Transfer the sample to a 1.7 mL tube. Spin for 1′ at maximum speed at 4 °C.
4. Remove the supernatant and wash the pellet once with ice-cold PBS supplemented with protease inhibitors.
5. On ice, resuspend the pellet in 140 μL lysis buffer.
6. Pipette up and down to release the cross-linked chromatin into the solution. Allow to lay on ice for 10–20′ (this time can be extended to 1 h).
7. Add 2 volumes (280 μL) of dilution buffer.
8. Shear the chromatin to approximately 200 to 1000 bp fragments using a sonicator (for Diagenode Bioruptor, set the equipment at "High," 30" ON, 30" OFF, for 11′). The samples should be kept in ice bath throughout the process to avoid overheating and foaming.
9. Add 42 μL of 10% Triton X-100 to the sonicated chromatin. Centrifuge the sonicated solution at 20,000 rcf for 10′ at 4 °C.
10. Transfer the supernatant to a fresh tube (~400 μL) without disturbing the pellet. The supernatant contains the sheared chromatin, and the pellet can be discarded.

3.1.3 Preparation of Magnetic Beads

1. Gently homogenize the bead suspension extensively. For each IP (protein-specific antibodies and negative control), add 100 μL of magnetic beads to a microfuge tube.
2. Add 1 mL blocking solution.
3. Collect the beads using magnetic stand. Let the beads separate for ~3′ and remove supernatant.
4. Wash beads in 1 mL blocking solution two more times. Each time resuspend the beads in the blocking solution by gentle tapping on the tube and allow the beads to wash while nutating for 2–3′. When removing the liquid, leave the tubes for a minimum of 3′ on the magnetic stand, to collect all the beads.
5. Resuspend beads in 250 μL blocking solution and add 10 μL of antibody for each IP (*see* **Note 5**).
6. Incubate overnight on the rotisserie in the cold room.

7. Next day, wash beads as described above (three times in 1 mL block solution).
8. Resuspend Dynabeads in 100 μL of blocking solution.

3.1.4 Chromatin Immunoprecipitation

1. Dilute the sheared chromatin (**step 10** in Subheading 3.1.2) to 800 μL (for ChIP with two antibodies) or 1200 μL (for ChIP with three antibodies) with triton dilution buffer.
2. Save an aliquot of 20 μL of the diluted chromatin and keep at −80 °C to be used as input sample.
3. Add ~400 μL of the diluted chromatin to antibody/magnetic bead mix from **step 8** in Subheading 3.1.3.
4. Incubate O/N on rotisserie in the cold room.

3.1.5 Washing, Elution, and Cross-Link reversal

1. Prechill the magnetic stand (leave at −20 °C O/N).
2. All washing steps should be done in a 4 °C cold room. Let tubes sit for at least 3′ in magnetic stand to collect the beads. Remove supernatant.
3. Add 1 mL of ice-cold RIPA to each tube. Remove tubes from magnetic stand and tap gently to resuspend beads. Leave tubes on the nutator for 1–2′ for more efficient wash. Replace tubes in magnetic stand to collect beads. Remove and discard supernatant.
4. Repeat this wash seven more times.
5. Wash once with 1 mL of TE/NaCl.
6. To reduce the nonspecific background, after 3′ wash, change to chromatin/bead suspension to a new chilled tube. Separate the beads using the magnetic stand and remove the supernatant as previously.
7. Spin the tube at 960 rcf for 3′ at 4 °C and remove any residual TE buffer.
8. Add 220 μL of elution buffer.
9. Elute at 65 °C for 1 h in the thermomixer at 200 rcf (1400 rpm).
10. Spin down beads at 16,000 rcf for 1′ at RT.
11. Remove 200 μL of supernatant and transfer to a new screw-cap tube. Reverse cross-link of this IP DNA by incubating at 65 °C overnight.
12. Thaw 20 μL of input sample reserved after sonication (**step 2** in Subheading 3.1.4), add 180 μL of elution buffer and mix. Reverse cross-link of this input DNA by incubating at 65 °C overnight.

3.1.6 DNA Precipitation

1. Add 200 µL of TE to each tube of IP and input DNA to dilute SDS in elution buffer.
2. Add 8 µL of 10 mg/mL RNaseA (0.2 ug/ml final concentration).
3. Mix and incubate at 37 °C for 1 h.
4. Add 4 µL of 20 mg/mL proteinase K (0.2 µg/mL final concentration).
5. Mix and incubate at 55 °C for 1 h.
6. Add 400 µL phenol:chloroform:isoamyl alcohol, vortex extensively (~1′), and spin at 16,000 rcf at RT for 5′.
7. Transfer 380 µL of aqueous layer to a new centrifuge tube containing 16 µL of 5 M NaCl (200 mM final concentration) and 1.5 µL of 20 ug/µL glycogen (30 µg total).
8. Add 1140 µL EtOH. Incubate for 1 h at −80 °C.
9. Spin at 20,000 rcf for 30′ at 4 °C to pellet DNA. Wash pellets with 1 mL of 70% EtOH.
10. Dry pellets and resuspend each in 30 µL of ddH_2O.
11. Dilute input ~25 times, and run analytical qPCR reaction, using 1–2 µL of ChIP'd DNA and 1–2 µL of diluted input DNA (*see* **Note 6**).

3.2 Co-immunoprecipitation: Co-IP

This is a rapid Co-IP protocol to test if pairs of nuclear proteins can interact in chick embryo cells (Fig. 2). The proteins of interest are cloned in separate PCI expression vectors (PCI-H2B-RFP [6] or similar), with AVI or Flag tags, respectively (we usually add the tags in the C-terminus of the protein, followed by a GSG linker). The AVI tag is added to the bait protein and the Flag tag is linked to the putative cofactor. A third vector, driving expression of nuclear BirA enzyme, is co-transfected in the embryos [7] to biotinylate the AVI tag of the bait protein. Following electroporation [5] (*see* **Note 7**), embryos will be cultured until the desired stage (HH9–10), dissected, and processed for extraction of nuclear proteins. Streptavidin beads (Dynabeads™ MyOne™ Streptavidin T1) are used for the immunoprecipitation of the bait protein. We then employ western blot to test the interaction between the bait and the putative cofactor. We often use this protocol in conjunction with proximity ligation assays (PLA) [8], which can be used to confirm that the interaction takes place in vivo under endogenous conditions.

3.2.1 Embryo Extract

1. Dissect embryos using filter paper and transfer to Ringer's solution (*see* **Note 8**).
2. Cut embryos out of filter paper, remove as much of membrane as possible, and transfer to a 1.7 mL tube (*see* **Note 3**). Perform 1 extract per sample.

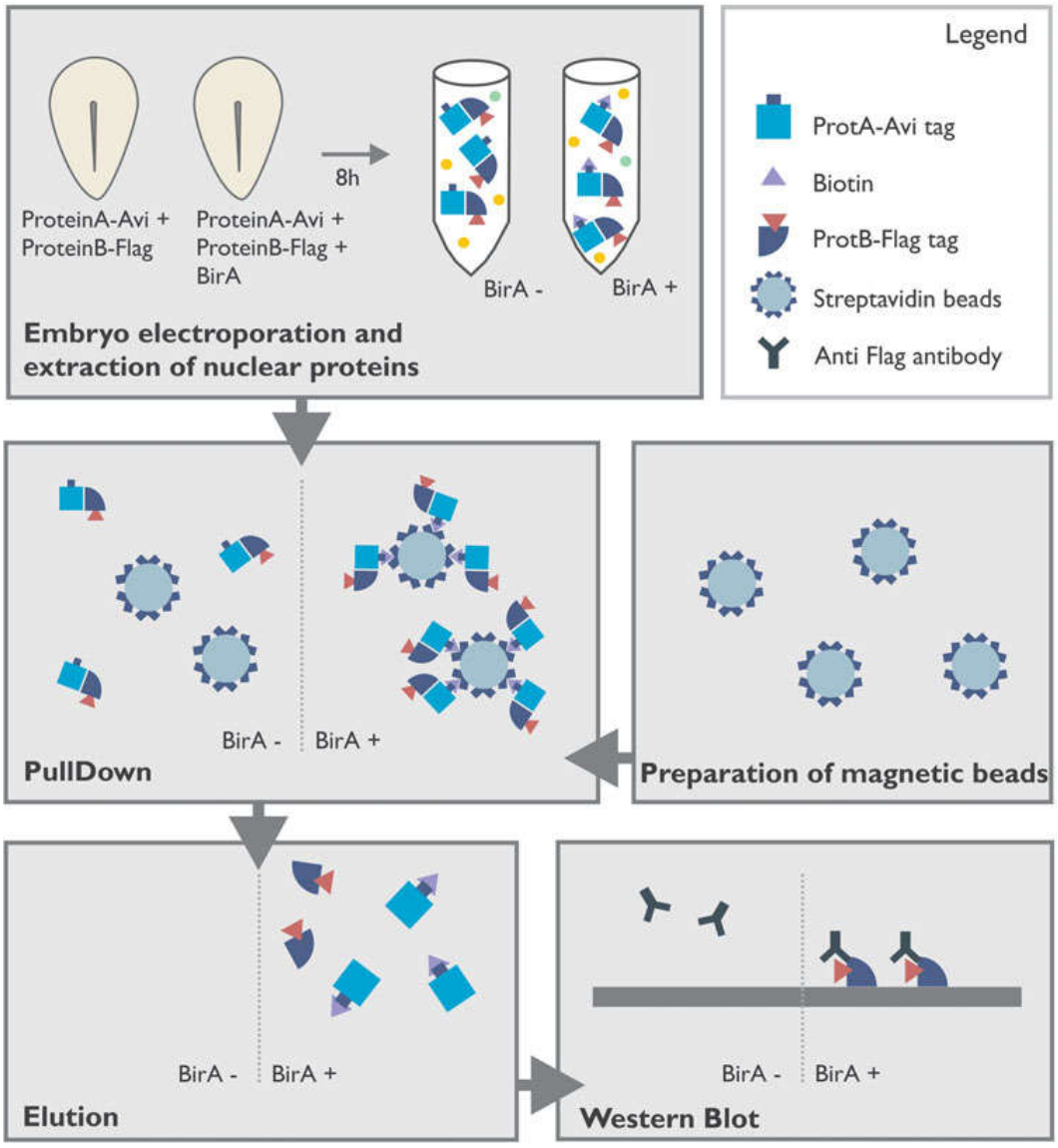

Fig. 2 Experimental design of a Co-IP experiment

3. Wash once with PBS.
4. Spin down at ~300 rcf, being careful not to lyse the embryos. Remove PBS.
5. Add 100 μL of hypotonic buffer to the embryos. Leave on ice for 15′ (*see* **Note 4**).
6. Dissociate the cells by aspirating and releasing the solution (~7 times) using a 1 mL syringe and a 27.5 gauge needle.
7. Incubate on ice for 15′.
8. Centrifuge at top speed for 2′ at 4 °C to spin out the lysate. The cytoplasmic protein is in the supernatant. If you wish to keep it, transfer the supernatant to a fresh tube and snap freeze in liquid

nitrogen. If you are only interested in the nuclear proteins, it can be discarded.

9. Resuspend the pellet in 10 μL of IP extraction buffer.
10. Rotate at 4 °C for 30′ to extract nuclear proteins.
11. Centrifuge at top speed for 2′ at 4 °C to spin out the debris.
12. Collect supernatant (nuclear extract) and transfer to a fresh tube.
13. Dilute 10 μL nuclear extract in 390 μL modified RIPA buffer for a final volume of 400 μL (*see* **Note 9**).

3.2.2 Preparation of Magnetic Beads

1. Gently resuspend the MyONE Dynabeads.
2. Transfer the 50 μL of magnetic beads to a microcentrifuge tube.
3. Using a magnetic stand to separate the bead, wash beads three times in PBS + 1% BSA FV (filtered).
4. Block beads at 4 °C in PBS + 1% BSA FV for 1 h on rotisserie.
5. Separate on a magnetic stand and remove supernatant.

3.2.3 Pull Down

1. Save an aliquot of 40 μL of the diluted nuclear extract from **step 13** in Subheading 3.2.1 and keep at −80 °C to be used as input sample.
2. Add the remaining diluted nuclear extract to the magnetic beads from **step 5** in Subheading 3.2.2.
3. Incubate for 2 h on rotisserie at 4 °C.
4. Let tubes sit for at least 3′ in magnetic stand to collect the beads. Remove and discard supernatant.
5. Add 1 mL of modified RIPA buffer to each tube. Remove tubes from magnetic stand and tap the tube gently to resuspend beads. Leave tubes on the nutator for 1–2′ for more efficient wash. Replace tubes in magnetic stand to collect beads. Remove supernatant.
6. Repeat this wash four more times.
7. After the last wash, change to chromatin/bead suspension to a new chilled microcentrifuge tube. This step largely reduces the nonspecific background.
8. Separate the beads using the magnetic stand and remove the supernatant as previously.
9. Resuspend each sample with:

   Sample reducing agent (10×)—5 μL.

   LDS sample buffer (4×)—12.5 μL.

   Modified RIPA buffer—32.5 μL.

10. Heat samples at 80 °C, for 15′ at 1400 rpm.
11. Separate the beads using the magnetic stand. Transfer the supernatant to a fresh tube. Store at −80°C.
12. To each aliquot of input (**step 1** in Subheading 3.2.3), add:

    Sample reducing agent (10×)—5 μL.

    LDS sample buffer (4×)—12.5 μL.
13. Heat samples at 80 °C, for 15′ at 1400 rpm. Store at −80 °C.
14. Run samples in a protein gel and perform western blot using an anti-Flag antibody. Use an anti-Flag antibody conjugated with HRP to speed up the analysis and minimize background.

4 Notes

1. PMSF stock solution is dissolved in ethanol as the half-life in water is 30′ in water.
2. The protease inhibitor stock solution is stable for 12 weeks at −15 to −25 °C.
3. For all steps in the procedure use low-binding safe-seal tubes. The use of low-retention pipette tips is preferred for accurate solution transfers. Also, the use of P1000 REACH tips helps to not disturb the beads during washes.
4. All steps prior to DNA elution should be done on ice to avoid protein degradation.
5. Use a ChIP-grade rabbit or mouse protein-specific (transcription factor) antibody. Also, use a ChIP-grade rabbit or mouse IGG as negative control.
6. Samples should be kept at −80 °C and analyzed within 2 weeks as the low-DNA-content samples degrade quickly. Enrichment of target region should be presented as percentage of input or as fold over the IGG DNA pull down.
7. For ex ovo electroporation [5], we recommend using the following concentrations of the PCI-H2B-RFP vectors: (a) BirA positive sample: Avi-tag plasmid (500 ng/μL) + Flag-tag plasmid (500 ng/μL) + BirA plasmid (500 ng/μL); (b) BirA negative sample: Avi-tag plasmid (500 ng/μL) + Flag-tag plasmid (500 ng/μL) + empty PCI-H2B-RFP (500 ng/μL).
8. Each aliquot of nuclear extract is prepared from at least 5–10 whole embryos (HH9–10) overexpressing the proteins of interest fused to Avi or Flag tags.
9. We generally use 10–20 μg of nuclear extract per IP.

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Chapter 8

Experimental Manipulation of Ploidy in Zebrafish Embryos and Its Application in Genetic Screens

Triveni Menon and Sreelaja Nair

Abstract

Metazoan animals are typically diploid, possessing two sets of a chromosome in the somatic cells of an organism. In naturally diploid species, alteration from the endogenous diploid state is usually embryonic lethal. However, the ability to experimentally manipulate ploidy of animal embryos has fundamental as well as applied biology advantages. In this chapter we describe experimental procedures to convert normally diploid zebrafish embryos into haploid or tetraploid states. We also describe methodologies to verify the ploidy of embryos and the utility of ploidy manipulation in expediting the isolation of mutations using both forward and reverse genetic strategies in zebrafish.

Key words Zebrafish, Mutagenesis, Ploidy, Haploid, Tetraploid, Gynogenesis, Heat shock, Genetic screen

1 Introduction

The original version of Hugo De Vries' mutation theory of evolution was based on his observation that species of the evening primrose *Oenothera lamarckiana* (*O. lamarckiana*) often displayed dramatic changes in physical form. De Vries ascribed these changes to mutations in *O. lamarckiana* genes [1]. However, it is now known that what De Vries interpreted as mutations was in fact the duplication of all 14 chromosomes of *O. lamarckiana* resulting in a new polyploid species, *O. gigas* with 28 chromosomes [1]. In the century that followed De Vries' original observation, the remarkable ability of plants to tolerate polyploidy has been a horticultural and agricultural boon to mankind, leading to production of seedless fruits, larger size produce, and drought-resistant crops. In stark contrast, the diploid state of chromosomes is an obligate requirement for embryonic survival in metazoans. The fundamental biological principles underlying this phenomenon remain unknown and are not pertinent for this chapter. However, the ability to manipulate the endogenous ploidy of embryos can be an

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Chapter 19

Germ Cell Transplantation in Avian Species

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Abstract

Germ cell transplantation technology has played a critical role in germline modification and preservation of genetic resources. Several germ cell transplantation systems have been developed, including sperm, oocyte, or germline stem cell transplantation systems in mammals. Meanwhile, in avian species, this has mostly relied on primordial germ cell (PGC) transplantation for efficient germline transmission. In this chapter, we describe how to isolate PGCs from avian embryos and produce germline chimeras through transplantation of donor PGCs to recipient embryos.

Key words Aves, Germline chimera, Primordial germ cells, Transplantation

1 Introduction

Avian species are important not only as food resources but also for use in multidisciplinary studies across many fields including developmental biology, immunology, toxicology, physiology, and behavioral science [1, 2]. Therefore, the preservation of avian species is important for future generations [3]. However, unlike other model animals, it is difficult to perform technical procedures such as pro-nucleus injection into the oocyte, somatic cell nuclear transfer, embryonic stem cell (ESC)-mediated germline transmission, transgenesis, and genome editing in avian species due to their oviparous development and the physiological features of the ovum [4]. To overcome this limitation, much effort has focused on the development of a unique germ cell transplantation system via primordial germ cells (PGCs), which are progenitor cells of gametes containing genetic information that can be delivered to the next generation. Avian PGCs reportedly originate from maternally inherited germplasm factors, such as the chicken vasa homolog (*CVH*) and the chicken deleted in azoospermia-like (*DAZL*) gene, which indicates that avian germ cell specification follows the germplasm model [5, 6]. In the Eyal-Giladi and Kochav (EGK) stage X embryo, PGCs migrate toward the germinal crescent region until

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Hamburger and Hamilton (HH) stage 4 [7]. Subsequently, PGCs enter the vascular system via the anterior vitelline vein during HH stages 10–12, and they ultimately start to settle in the genital ridge at around HH stages 15–17 [8, 9]. Because avian PGCs have a unique migratory pathway during embryonic development, it is possible to isolate them from donor embryos and transplant them into recipient embryos [10–12]. For this reason, the PGC-mediated germ cell transplantation has been considered the most efficient germline transmission system in avian species, and it has successfully produced germline chimera and germline-transmitted progenies as well as transgenic and genome-edited progenies with much higher efficiencies than other germline-competent cells, such as ESCs and spermatogonial stem cells (SSCs) [13–16]. In this chapter, we introduce the methods to isolate vascular circulating PGCs using cell surface-specific antibody-mediated cell sorting and size-dependent cell isolation system, and describe the PGC-mediated transplantation system in detail.

2 Materials

2.1 Isolation and Purification of PGCs

2.1.1 Blood Isolation from Dorsal Aorta of HH Stage 13–16 Embryos

1. Korean Oge (KO) and white Leghorn (WL) chicken (*Gallus gallus domesticus*), Japanese quail (*Coturnix japonica*), mallard duck (*Anas platyrhynchos*), and Muscovy duck (*Cairina moschata*) at HH stage 13–16 [17] (*see* **Note 1**).
2. Microelectrode pipette puller.
3. Microgrinder.
4. 25 μm diameter glass micropipette.
5. Mouth-controlled pipette.
6. Sanitary cotton.
7. 70% Ethanol in distilled water.
8. 3.8% Sodium citrate buffer: 3.8% (w/v) Sodium citrate in distilled water, pH 7.2.
9. Sharpened microdissection forceps.
10. 1.5 mL Microtube.

2.1.2 PGC Purification Via Size-Dependent Isolation (SDI) System

1. 6.5 mm Transwell insert with 8 μm microporous membranes (Corning, CLS3422).
2. 1× Phosphate-buffered saline (1× PBS): 137 mM sodium chloride, 2.7 mM potassium chloride, 10 mM sodium phosphate dibasic, and 1.8 mM monobasic potassium phosphate in distilled water, pH 7.2.
3. 3.8% Sodium citrate buffer.

4. Laboratory wipes (Kimberly-Clark Corp.).
5. Chicken DAZL (cDAZL) polyclonal antibody for purified PGCs (*see* **Note 2**).

2.1.3 PGC Purification Via Magnetic Activated Cell Sorting (MACS)

1. Magnetic activated cell sorting (MACS) separator (Miltenyi Biotec).
2. MACS column (MS columns, Miltenyi Biotec).
3. MACS buffer: 0.5% Bovine serum albumin (BSA), 2 mM ethylenediaminetetraacetic acid (EDTA) in 1× PBS, pH 7.2.
4. Chicken PGC-specific surface antibody: Stage-specific embryonic antigen-1 (SSEA-1) antibody (Santa Cruz Biotechnology, Inc., SC-21702).
5. Anti-Mouse IgM MicroBeads (Miltenyi Biotec).
6. 1× PBS.

2.1.4 Immunocytochemistry of Purified PGC

1. Fixation solution: 4% (w/v) paraformaldehyde (Sigma-Aldrich) in 1× PBS.
2. Permeabilization solution: 0.1% Triton X-100 (Sigma-Aldrich) in 1× PBS.
3. Blocking solution: 10% (v/v) normal goat serum, 1% (w/v) BSA in 1× PBS.
4. Primary antibodies: SSEA-1 (Santa Cruz Biotechnology, Inc.).
5. Secondary antibodies: Goat anti-mouse IgM-phycoerythrin (PE) or mouse anti-rabbit IgG-fluorescein isothiocyanate (FITC).
6. ProLong Gold antifade reagent (with DAPI, or 4′,6-diamidino-2-phenylindole; Invitrogen).

2.2 PGC Transplantation

2.2.1 Fluorescent Labeling of Donor PGCs and Microinjection of PGCs into Recipient Embryos

1. Purified PGCs.
2. Fluorescent labeling dye: PKH26 red fluorescent cell linker kit (Sigma-Aldrich).
3. PKH26 staining stop solution: 10% (v/v) fetal bovine serum (FBS) in Dulbecco's modified Eagle medium (DMEM).
4. Hanks' balanced salt solution (HBSS).
5. Microelectrode pipette puller.
6. Microgrinder.
7. 25 μm diameter glass micropipette.
8. Mouth-controlled pipette.
9. Sharpened forceps for egg shell opening.
10. Parafilm.
11. HH stage 13–16 recipient chicken embryo.

2.2.2 Migration Assay of PGC

1. PKH26-labeled donor PGC-transplanted 6-day-old embryonic gonads (at HH stages 26–28).
2. Sterilized dissection petri dish.
3. Dissection pin.
4. Fine tweezers.
5. 1× PBS.
6. Inverted fluorescence microscope.

2.3 Test Cross and Identification of Germline Chimera

1. Sexually maturated male recipient (putative male germline chimera).
2. 1 mL syringe without needle (for artificial insemination).
3. 1.5 mL Microfuge tubes.
4. 1× PBS.
5. DNeasy Blood and Tissue Kit (QIAGEN).
6. Breed-specific primers for determination of KO chicken donor and WL chicken recipients [18]:

   KO-specific primers:

   – F: 5′-AGCAGCGGCGATGAGCAGCA-3′.
   – R: 5′-CTGCCTCAACGTCTCGTTGGC-3′.

   WL-specific primers:

   – F: 5′-AGCAGCGGCGATGAGCGGTG-3′.
   – R: 5′-CTGCCTCAACGTCTCGTTGGC-3′.
7. PCR kit (Takara, Ex Taq) or equivalent PCR kit.
8. PCR machine.

3 Methods

Here, we explain how to isolate avian PGCs from embryonic blood vessels using magnetic activated cell sorting (MACS) and size-dependent isolation (SDI), and describe production of germline chimeras in details (Fig. 1).

3.1 Isolation and Purification of PGCs

3.1.1 Isolation of Whole-Blood Cells from Avian Embryos

1. Incubate fresh eggs (EGK stage X) from various avian species such as white Leghorn chicken, Japanese quail, mallard duck, and Muscovy duck at 37 °C until the desired stage (HH stages 13–16) is reached. Incubate the fertilized mallard and Muscovy duck eggs until their morphology is similar to that of chicken or quail at the various stages (Fig. 2a).
2. After incubation, place the eggs horizontally on an egg plate and wipe the eggshells gently using sanitary cotton with 70% ethanol.

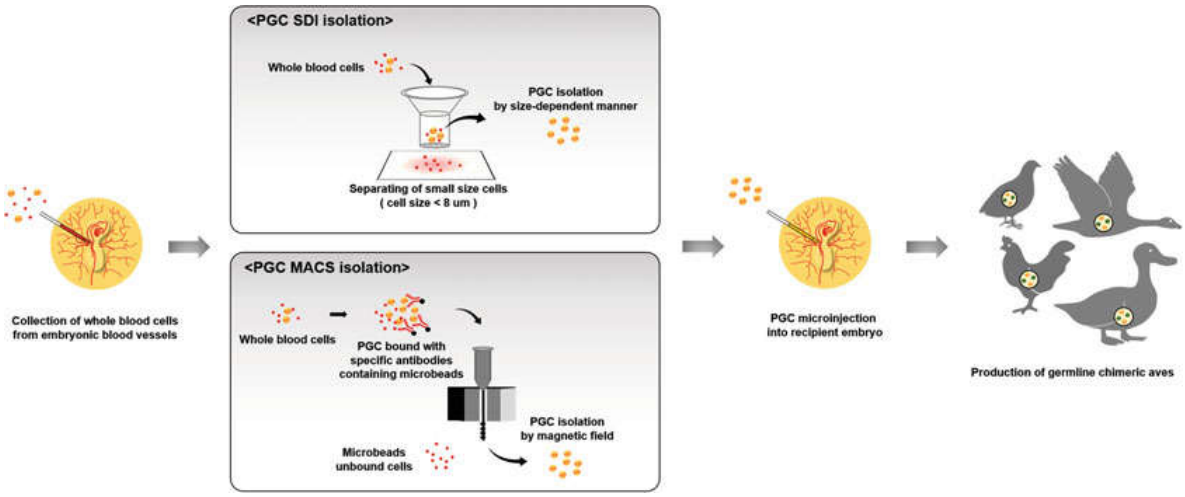

Fig. 1 Schematic representation of PGC isolation from avian embryonic blood vessels and subsequent transplantation. PGCs are isolated from avian embryonic blood vessels by size-dependent isolation (SDI) and magnetic activated cell sorting (MACS) system. Purified PGCs are injected into Hamburger and Hamilton (HH) stage 13–16 recipient embryos. Subsequently, the recipient embryos are hatched and raised until sexual maturation

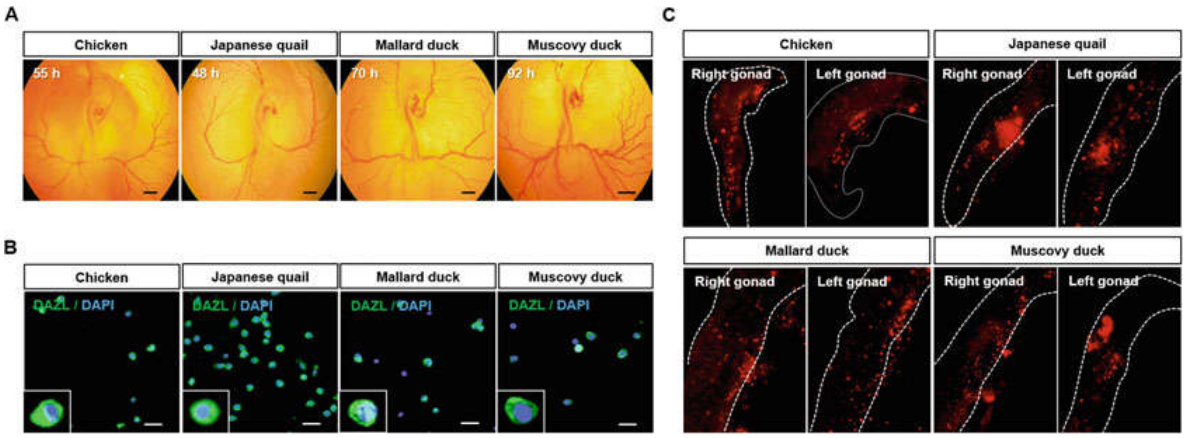

Fig. 2 Separation of avian PGCs by SDI system. (**a**) HH stage 13–16 embryos of the various avian species. Fertilized eggs of white Leghorn chickens, Japanese quail, mallard ducks, and Muscovy ducks are incubated for 55, 48, 70, and 92 h, respectively. Scale bar, 1 mm. (**b**) Immunocytochemical analysis of SDI-purified cells against DAZL in the various avian species. Scale bar, 50 μm. (**c**) Migration of SDI-purified cells in recipient chicken embryos. Approximately 500 cells isolated from the four avian species are labeled with PKH26 fluorescent dye, injected into the dorsal aorta of HH stage 13–16 chicken embryos, and incubated until HH stage 27 (reproduced from ref. [17] with permission from *Molecular Reproduction and Development*)

3. Using sharpened forceps, cautiously crack the eggshell (<1 cm diameter for the chicken and ducks, and <0.5 cm diameter for the quail) to isolate whole-blood cells.
4. Collect approximately 2–5 μL of embryonic blood from the dorsal aorta of each embryo using a thinly ground glass micropipette and mouth pipette under a microscope.

5. Transfer whole-blood cells to 1.5 mL microtubes with 100 μL of 3.8% sodium citrate buffer to prevent blood coagulation, and then centrifuge the whole blood cells at 200 × *g* for 5 min for further isolation of PGCs.

3.1.2 PGC Isolation Via SDI System

1. For PGC isolation via SDI system (Fig. 1), rinse a 6.5 mm Transwell insert with 8 μm microporous membranes with 500 μL of 1× PBS.
2. Resuspend the isolated embryonic blood cells in 500 μL of 3.8% sodium citrate buffer, pipette onto the Transwell insert, allow to pass through, and wash twice with 1× PBS using laboratory wipes.
3. Resuspend the cells on the microporous membranes in 500 μL of 1× PBS (*see* **Note 3**).
4. Centrifuge the suspended cells at 200 × *g* for 5 min and then resuspend the pellets in 1× PBS.
5. Confirm the identity of these cells by immunocytochemistry using cDAZL polyclonal antibody (Fig. 2b).

3.1.3 PGC Isolation Via MACS System

1. For PGC isolation via MACS system (Fig. 1), resuspend the cell pellets and label with anti-SSEA-1 antibody for chicken at 1:200 titers in 1 mL PBS; then incubate the cells for 15 min at room temperature (RT).
2. Wash the cells to remove unbound primary antibody by adding 5 mL MACS buffer per 10^7 total cells and centrifuge at 200 × *g* for 5 min.
3. Aspirate supernatant completely and resuspend cell pellet in 80 μL MACS buffer per 10^7 total cells.
4. Add 20 μL rat anti-mouse IgM MicroBeads per 10^7 cells. For higher cell numbers, scale up the buffer volume accordingly.
5. Incubate the cells for 20 min at 2–8 °C.
6. Wash the cells by adding 2 mL MACS buffer per 10^7 cells and centrifuge at 200 × *g* for 5 min.
7. Aspirate supernatant completely and resuspend up to 10^7 cells in 500 μL MACS buffer.
8. Place MACS column in the magnetic field of a suitable MACS separator.
9. Prepare column by rinsing with 500 μL MACS buffer.
10. Apply cell suspension to the column. Wash the column with 500 μL of MACS buffer three times and collect unlabeled cells that pass through. New buffer should be added only when the column reservoir is empty.
11. Remove the column from the separator and place it on a suitable collection tube.

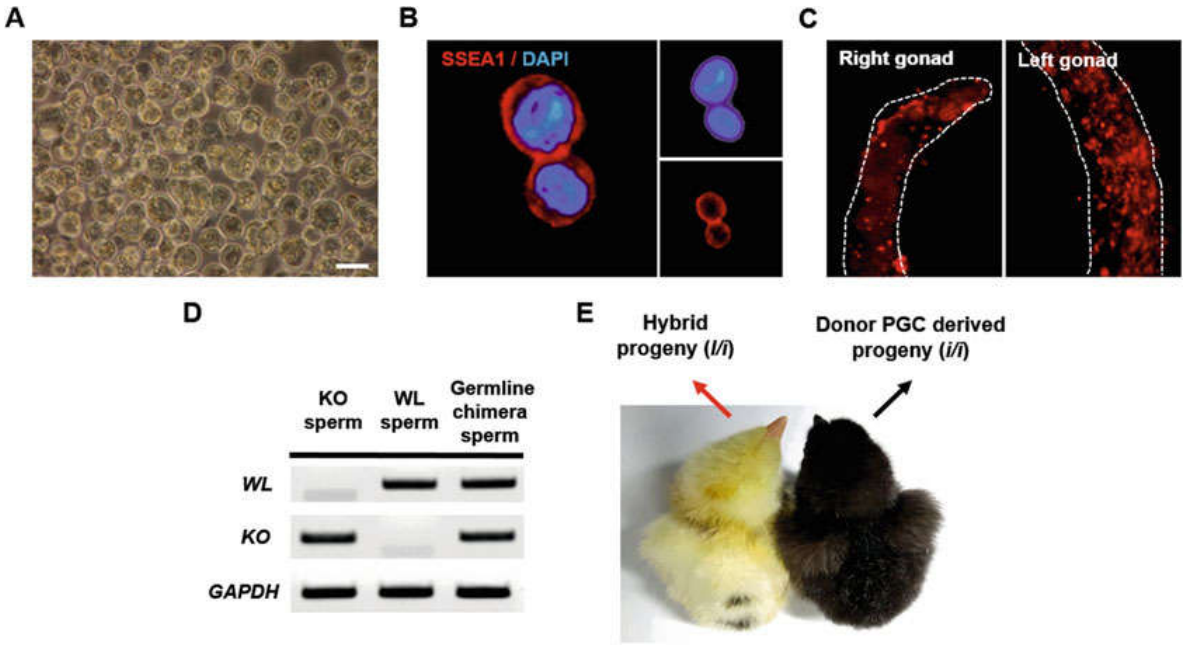

Fig. 3 Separation of avian PGCs by MACS and generation of donor PGC-derived progeny via PGC transplantation. (**a**) PGCs are isolated by MACS from HH stage 13–16 Korean Oge (KO) embryonic blood. Scale bar, 20 μm. (**b**) Immunocytochemical analysis of MACS-purified cells against SSEA1 in KO chicken embryonic blood is performed. (**c**) Approximately 500 cells isolated from KO chicken blood vessels are labeled with PKH26 fluorescent dye, injected into the dorsal aorta of HH stage 13–16 WL chicken embryos, and incubated until HH stage 27. (**d**) Genomic DNA PCR for breed determination of germline chimeric rooster. Genomic DNA is isolated from germline chimera sperm and analyzed by PCR using WL and KO chicken-specific primers. (**e**) Donor KO (*i/i*) PGCs are injected into the dorsal aorta of WL (*I/I*) recipient embryos, and after sexual maturation progeny is derived from the donor KO PGCs (black arrow, *i/i*). Hybrid progeny (red arrow, *I/i*) derived from the endogenous WL PGCs exhibits slightly yellow feathers

12. Add 1 mL MACS buffer to the column and immediately flush out the magnetically labeled cells by firmly pushing the plunger into the column.
13. Centrifuge the separated cells at 200 × *g* for 5 min and resuspend the cell pellets in 1× PBS.
14. Confirm the isolated cells by immunocytochemistry using anti-SSEA-1 antibody (Fig. 3b).

3.1.4 Immunocytochemistry of purified PGC

1. Centrifuge PGCs isolated by SDI or MACS system at 200 × *g* for 5 min and resuspend approximately 1 × 10^4 PGCs in 100 μL of 1× PBS on glass slides and dry slowly in a 37 °C slide warmer.
2. Fix the cells using 500 μL of 4% paraformaldehyde in 1× PBS at RT for 10 min. Wash the slides in 1× PBS three times.
3. Incubate with blocking solution for 10 min at RT.
4. Add anti-SSEA-1 antibody or anti-cDAZL antibody at 1:200 titers in 1 mL blocking solution and incubate the cells for 1 h at

RT under humidity using an airtight container. After 1 h, wash the slides in 1× PBS three times.

5. Add secondary antibody (e.g., goat anti-mouse IgM-PE for anti-SSEA1 antibody and mouse and anti-rabbit IgG-FITC for anti-cDAZL antibody at 1:500 dilution in 1 mL blocking solution) and incubate for 1 h at RT. After 1 h, wash the slides in 1× PBS three times.
6. Mount the slides using ProLong Gold antifade reagent (with DAPI) and analyze under a fluorescence microscope (Figs. 2b and 3b).

3.2 PGC Transplantation

3.2.1 PGC Microinjection into Recipient Embryos

1. Prepare approximately 1 × 10^4 isolated PGCs in 1× PBS and centrifuge at 200 × *g* for 5 min.
2. Mix the cell pellet with 2 μL PKH26 and 500 μL diluent buffer and incubate for 5 min at RT without light exposure.
3. After 5-min incubation, stop the staining by adding 1 mL of 10% FBS in DMEM and centrifuge the stained cells at 200 × *g* for 5 min.
4. Resuspend the cell pellet with 20 μL HBSS to prepare a cell density of approximately 500 cells in 1 μL medium.
5. Incubate recipient eggs with the pointed ends down until they reach HH stages 13–16.
6. Make a small window (approximately <1 cm diameter) in the pointed end of each recipient egg.
7. Remove 1–2 μL whole-blood cells from recipient blood vessel using mouth pipette (*see* **Note 4**).
8. Inject approximately 1 μL of PKH26-labeled 500 PGCs into the upper portion of the dorsal aorta of the recipient embryo.
9. Seal the window twice with parafilm, and continue incubating the eggs.

3.2.2 Migration Assay of PGCs

1. Incubate recipient eggs until HH stages 28–30 at 37 °C in air with 60–70% relative humidity.
2. Dissect embryonic gonads from recipient embryos at embryonic day 6.
3. Retrieve gonads from the recipient embryos and count the number of fluorescent PGCs in each gonad under a fluorescence microscope (Figs. 2c and 3c).

3.3 Identification and Testcross of Germline Chimera

1. Collect semen from sexual maturated WL recipients (putative germline chimera; if the donor KO PGCs were transplanted to WL recipient).
2. Isolate genomic DNA from collected semen using a DNA Purification Kit.

3. Perform breed-specific PCR using allele-specific primers under the following thermocycling conditions: 10 min at 94 °C followed by 35 cycles of 30 s at 94 °C, 30 s at 69 °C, 30 s at 72 °C, and finally 10 min at 72 °C (Fig. 3d).
4. For artificial insemination, introduce 50 μL semen from germline chimeric WL roosters to egg-laying KO hens.
5. Collect eggs from recipient hens at 1 day after artificial insemination and incubate the eggs with the pointed ends down until hatching at 37 °C in air with 60–70% relative humidity.
6. Donor-derived progenies can be distinguished by feather color (Fig. 3e) (*see* **Note 5**).

4 Notes

1. Incubate fertilized chicken, Japanese quail, mallard duck, and Muscovy duck eggs to HH stages 13–16 [17]. Incubate the chicken eggs for 55 h and the quail eggs for about 48 h. The duck eggs should then be incubated until their morphology is similar to that of the chicken and quail at the various stages (about 70 h for mallards and 92 h for Muscovy ducks).
2. Raise polyclonal antibodies against N-terminal peptides of cDAZL (aa 2–17, SANAEAQCGSISEDNTH) in a rabbit, followed by purification of antisera [6].
3. Add PBS to transwells and gently pipette the cells at an angle of 45° along the wall to keep the microporous membrane from the transwell and to isolate the PGCs with minimal damage.
4. Since the recipient embryos (HH 13–16) contain about 200 endogenous PGCs in whole blood [19], removing 1–2 μL whole-blood cells would be helpful for efficient germline chimera production.
5. Because of differences in their pigmentation (e.g., WL have a dominant pigmentation inhibitor gene (*I/I*) whereas KO have a recessive pigmentation inhibitor gene (*i/i*)), the progenies of germline chimera are distinguishable. Donor-derived progenies exhibit all black feathers (*i/i*), whereas hybrid progenies (*I/i*) exhibit slightly yellow feathers with small black spots.

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