Video Article

A Reverse Genetic Approach to Test Functional Redundancy During Embryogenesis

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Abstract

Gene function during embryogenesis is typically defined by loss-of-function experiments, for example by targeted mutagenesis (knockout) in the mouse. In the zebrafish model, effective reverse genetic techniques have been developed using microinjection of gene-specific antisense morpholinos. Morpholinos target an mRNA through specific base-pairing and block gene function transiently by inhibiting translation or splicing for several days during embryogenesis (knockdown). However, in vertebrates such as mouse or zebrafish, some gene functions can be obscured by these approaches due to the presence of another gene that compensates for the loss. This is especially true for gene families containing sister genes that are co-expressed in the same developing tissues. In zebrafish, functional compensation can be tested in a relatively high-throughput manner, by co-injection of morpholinos that target knockdown of both genes simultaneously. Likewise, using morpholinos, a genetic interaction between any two genes can be demonstrated by knockdown of both genes together at sub-threshold levels. For example, morpholinos can be titrated such that neither individual knockdown generates a phenotype. If, under these conditions, co-injection of both morpholinos causes a phenotype, a genetic interaction is shown. Here we demonstrate how to show functional redundancy in the context of two related GATA transcription factors. GATA factors are essential for specification of cardiac progenitors, but this is revealed only by the loss of both Gata5 and Gata6. We show how to carry out microinjection experiments, validate the morpholinos, and evaluate the compensated phenotype for cardiogenesis.

Video Link

The video component of this article can be found at http://www.jove.com/video/2020/

Protocol

Our goal here is to test the functional redundancy of two transcription factors for specification of cardiomyocyte progenitors. The factors are encoded by the two related genes *gata5* and *gata6* and we are using the zebrafish model to understand their relative functions[1]. Our strategy is to block gene function using morpholinos[2]. We will inject morpholinos for one or the other gene into fertilized eggs, and compare the embryonic phenotype with embryos derived from eggs injected simultaneously with both morpholinos. To simplify the evaluation of phenotypes, we use eggs derived from fish that carry a transgene that expresses GFP in cardiomyocytes.

Step 1. Preparing for microinjection.

A) Setting up breeding pairs of fish

The evening prior to injection, single male and single female adult fish (3-18 months of age) are placed in pairs into breeder tanks separated by a divider until the next morning. We typically set up 10-20 pairs of fish, and these are mated once a week, regardless of whether the eggs will be used, to maintain fecundity. For this experiment we are using a reporter strain, transgenic for *cmlc2:egfp*, because it provides a simple readout to identify differentiating cardiomyocytes. The next morning, once the lights are turned on, the water is changed, and dividers are pulled from tanks allowing the fish pairs to mate and produce eggs. Egg production can be monitored, and may initiate immediately, or after a period of an hour or more, depending on the fish. Typically, after approximately 20 minutes of uninterrupted mating, eggs are collected using a pipette or strainer and poured into 100 mm Petri dishes containing system water. It is important to monitor egg production in order to assure that embryos are injected between the 1-4 cell stage. By releasing several pairs at a time, it is possible to stagger the egg production and thereby maximize the amount of eggs that can be injected at an early developmental stage. A good pair of fish may generate more than 200 embryos, and it makes no practical sense to collect thousands of eggs at the same time, since a single investigator would not be able to inject them quickly enough before they develop beyond the 4-cell stage. Fish are stimulated to breed on the light cycle, so this is an experiment that requires an early start, and eggs are not usually obtained after noon.

B) Preparing needles

1. Use a Micropipette Puller and a 3.5" glass capillary tube to generate two needles using the following condition: Heat=626, Pull=60, Velocity=60, and Time=250. We use open core needles that do not contain a filament, because we front-fill them with suction.
2. The tip of each needle is then gently broken using a clean razor blade or forceps, and subsequently beveled at a 30 degrees angle for about 20 seconds using an EG-4 micro-grinder. This sharpens the tip and is important if you inject through the chorion.
3. It is important to obtain a consistent needle diameter tip size of about 15 micron (calibrated as 4 units using a gridded microscope eyepiece, to be able to calibrate a 1-2 nl injection volume).
4. Needles should be prepared in advance, stored safely in a secure location, and each is individually calibrated just prior to starting injections to assure consistent injection volume (see section D for details). If you are fortunate, you may be able to use a single needle for an entire experiment. However, a needle can easily break in the middle of an experiment, and you don't want to use that time to pull new needles.

C) Making injection plates

1. Prepare 100 ml of a 2% agarose solution in system water, by boiling in a microwave oven.
2. Cool to the touch, and pour about 12ml into the inverted lid of a 100mm Petri dish.
3. Insert 2 microscope slides, slanted at approximately 45-degree angles, into the two sides of the lid. Once the agarose has solidified, gently pull the two slides away from the lid.
4. This creates troughs where embryos will be placed and subsequently injected. Multiple plates can be prepared ahead of time. They can be stored indefinitely at 4°C after adding a layer of 70% ethanol, adding the bottom of the original plate, and sealing with parafilm. Remember to wash well before using.

D) Microinjection station and needle calibration

1. Turn on the compressed nitrogen source and PLI-100 microinjector. The injector should be adjusted for a constant flow pressure of 18 PSI.
2. Before inserting a needle, assure that the micromanipulator is in a proper position to allow a wide range of movement, and that an inserted needle will not strike the table. Insert one of the needles prepared previously (see section C) into the micromanipulator making sure there is a tight seal. Be careful not to break the needle.
3. Observe the needle tip under the microscope to make sure it is not clogged before proceeding to calibration.
4. Place a drop (2-3 µl) of injection solution or ddH_2O onto a small piece of parafilm and fill about 1.5 µl by pulling it into the needle using the fill button. Be sure that you have the needle tip fully in the solution, and observe the liquid suction through the microscope to avoid drawing in any air bubbles.
5. Place a drop of mineral oil onto the grid of a micrometer.
6. Adjust empirically the time of injection to ensure that the drop size is approximately 1.5 micron in diameter. This will deliver an injection volume of ~ 1.8 nl. The actual volume can be adjusted to your choice, but in a practical sense it is difficult to accurately calibrate volumes below 1 nl, and the embryos may not tolerate volumes above 2 nl. Regardless of the volume size you choose, keep it the same for all your injections including controls, and adjust your solution concentrations (rather than the injection volume) to titrate the amount of injected morpholino.
7. Recalibration is necessary each time a needle is replaced, since the needle tip sizes will vary.

Step 2. Validation of morpholinos targeting two distinct genes

Morpholinos are designed to target the translation initiation site or splice sites of a target mRNA thereby blocking protein synthesis or proper mRNA processing. When analyzing a gene for the first time, we use both types of morpholinos to test if they generate the same phenotype, indicating a specific knockdown. An advantage of the splice-blocker is that you can verify the knockdown of the normal transcript by RT-PCR, which is especially useful if antibodies for the target gene are not available. We will illustrate how a consistent cardiac phenotype is achieved for the *gata5* and *gata6* genes using morpholinos (*gata5* 5'UTR MO sequence: 5'-AAGATAAAGCCAGGCTCGAATACAT-3'; *gata5* Splice Site MO sequence: 5'-TCTTAAGATTTTTACCTATACTGGA-3'; *gata6* 5'UTR MO sequence: 5'-AGCTGTTATCACCCAGGTCCATCCA-3').

1. Morpholinos are obtained from Gene Tools, LLC (Philomath, OR). Gene Tools will help you choose the best sequence to target, if you ask their support staff. You only need to send them the accession number, and they will advise. However, there is no guarantee that the morpholino will work, which is why you need to be prepared to validate the activity. You can also check if the morpholino is available from OpenBiosystems. Gene Tools provides stocks of synthesized morpholinos to OpenBiosystems. While there is again no guarantee it will work, they sell smaller quantities at a lower cost, which may help defray costs for testing new targets. Be sure to BLAST your morpholino against the transcriptome; we use the BLAT feature on the UCSC zebrafish genome browser (genome.ucsc.edu). Of course, if a morpholino has been validated (rigorously) in the literature, it is recommended that you purchase that same sequence.
2. The morpholino is dissolved in sterile ddH_20 to a final concentration of 1 mM and stored at room temperature. DO NOT FREEZE the morpholino solutions, as for some reason low temperatures can sometimes cause them to aggregate and precipitate. Morpholinos are insensitive to proteases or nucleases, so as long as the water you add is sterile, they should be quite safe at room temperature.
3. Before injecting, incubate the morpholino at 65°C for 5 minutes to assure it is completely dissolved, and allow to cool to room temperature. Do NOT place the sample on ice. Keep the morpholino at room temperature until injection.
4. To titrate a morpholino, we typically prepare serial dilutions of the stock concentration in ddH_20 to yield working concentrations of 1 mM, 0.5 mM, 0.25 mM, and 0.125mM. Depending on the sequence, using 1.8 nl of injection volume, this represents approximately 20ng, 10ng, 5ng, and 2.5ng of morpholino per injection. This is just a starting point, since the tolerance and efficacy of each morpholino will vary and cannot be predicted. Most morpholinos will not be tolerated much above 20 ng, but some can be effective at doses well below 1 ng. Our approach is to define the concentration at which a phenotype "plateaus". In other words, if a defined phenotype is not seen using 2.5 ng, but is similar using any amount at or above 5 ng, we would choose 5 ng as a threshold amount. Of course, it might be useful to next titrate between 2.5 ng and 5.0 ng, to ensure that you are working reproducibly beyond the threshold, rather than right "on the border".
5. Fill the attached and calibrated needle (see above) with the lowest working concentration (most dilute) morpholino solution. Avoid over-filling the needle, as it will simply waste material. If you fill only 1 µl, there is sufficient solution for 500 injections.

6. Using a disposable pipette, transfer fertilized one-cell embryos into the troughs of the microinjection plate. Remove excess water to ensure that the embryos fall to the bottom of the injection troughs. They should not float, but rather adhere to the agarose bed.
7. Position the injection plate so that the micromanipulator can descend the needle tip through the chorion and into the yolk for injection. To position individual embryos, manipulate the injection plate such that the tip of the injection needle pushes or pulls the embryos to the proper position. Be careful not to break the needle tip or damage embryos! This takes some practice, but as a rule you use the micromanipulator simply to move the injection needle into the embryo and then after the injection, rather quickly and cleanly out. Embryos are brought into injection position by moving the plate using your free hand (left hand if you inject with your right hand). For a new learner, it is useful to practice using a solution containing 0.25% phenol red in order to visualize the injected solution. Confidence is also gained by injecting a solution containing fluorescent-tagged microspheres (Molecular Probes; F-8794), to confirm that solution was injected into the embryo, rather than the inter-chorion space. However, after a few rounds of practice, these aids are typically no longer required. It is a good idea to occasionally inject into the air, just to confirm that the needle is not clogged and that the volume being injected appears appropriate.
8. Injected embryos are transferred back to a 100mm Petri dish with system water and cultured at 28.5C. After injection of the lowest working concentration, any remaining solution can be expelled and the next highest working concentration of morpholino used to fill the needle. Repeat for each concentration of morpholino. Although it is possible to rinse out the needle using water, when testing a distinct morpholino, we prefer to change needles and recalibrate. Be sure to keep a cohort of uninjected embryos to verify whether the specific batch of embryos was healthy and normal.

Step 3. Co-injection of morpholinos at individual threshold doses

The goal of the titration carried out above was to define a threshold dose for each morpholino. In the best-case scenario, essentially 100% of the morphants will display a defined phenotype, if the targeted gene has an essential function. However, you can expect some variability due to mis-injection artifacts. With practice, this should not exceed 10%. Some batches of embryos (including the uninjected controls) may not survive well, in which case the experiment may need to be discounted. Meanwhile, there is also biological variability, since individual embryos may be more or less tolerant to the knockdown. Finally, some morpholinos may just not be efficient enough to achieve a robust (penetrant) knockdown. If a phenotype is generated in a low percentage of embryos, but is reproducible, it may be worth testing a distinct morpholino. The goal of this next experiment is to determine if an entirely new phenotype is seen when both genes are targeted simultaneously. We are not simply looking for an increase in the percentage of embryos that have a morphant phenotype, but rather for the generation of a distinct phenotype that is not seen at or above the threshold level when testing either of the individual genes.

1. Prepare a mixture of the combination of morpholinos tested previously, at the threshold concentration of each individual morpholino that generated a defined phenotype. For example, if the threshold for each gene was 5 ng, prepare a working solution that will contain 5 ng + 5 ng of each (10 ng total). Since embryos may not tolerate much more than 20 ng total morpholino, it is important, based on the previous experiments, to use the minimal amount of each that is sufficient to efficiently target each gene.
2. Controls should include sets of embryos injected with each individual morpholino alone at the same concentration that was used for the combination. Volumes of injection solution should be kept constant (e.g. 1.8 nl). A big advantage of using a reporter strain is that you can evaluate the phenotype (for example cardiomyocyte differentiation) at any time, and repeatedly. If embryos are to be evaluated for gene expression by *in situ* hybridization, stage-matched embryos and controls can be fixed at various times post-injection. For our experiment, we are going to evaluate GFP expression as a surrogate for cardiomyocyte differentiation, at approximately 24 hpf.

Step 4. Evaluation of phenotypes

1. Injected embryos should be monitored, just after the injection, and several times during the day, to remove any dead or dying embryos, since these can compromise the viability of the remaining morphants. To evaluate expression of the reporter gene, embryos are examined using a dissecting microscope outfitted with fluorescence capacity. We use a Nikon SMZ1500 microscope, with a 1X or 1.6X objective and a range of magnification from 0.75x to 11.25x. When using a fluorescent filter, be sure to open the diaphragm to the fully open setting to maximize the light intensity. Also be sure to use the correct filter, depending on the reporter gene (GFP, RFP, etc.).
2. Embryos are typically sedated using a 1/20 dilution in system water of 4mg/ml stock of tricaine methanesulfonate. The stocks are stored frozen at -20C. Alternatively, embryos can be euthanized using the 20x tricaine stock. Embryos can usually be screened even if they are still in the chorion. However, particularly if they will be photographed, it is a good idea to remove the chorions using sharp forceps. Embryos can be viewed after placing in depression slides in the tricaine solution, or to better immobilize them for photography placed into a small drop of 3% methylcellulose.
3. Observe embryo phenotypes in those injected with the combination of morpholinos compared to single morphants. Here we are looking for the pattern of GFP expression in the primordial heart tube. In this reporter strain GFP is expressed exclusively in cardiomyocytes via the *cmlc2* promoter, which allows detailed analysis of cardiac specification and heart tube morphogenesis.

Representative Results

In our experiment, both the *gata5* and *gata6* single morphants show reproducible cardiac phenotypes when injected with threshold levels, although there is considerable biological variability[3,4]. For example, most of the gata5 morphants generate a bifid heart, with GFP expression located in two regions. This occurs because the cardiac progenitors fail to migrate properly to fuse at the midline during heart tube formation. However, some of the gata5 morphants do generate a defective heart tube, and in a small number (5%) there is a significant decrease in the amount of GFP+ cells. We believe this reflects biological variability, but may also be due to the fact that morpholinos are not equivalent to a "null" mutation.

Likewise, the *gata6* morphants all show a cardiac phenotype, but there is a range of phenotypes including a small number of bifid heats, and hearts that are morphological disturbed compared to the uninjected control embryos. This variability is not unusual for a cardiac morphogenetic phenotype, since heart formation is a dynamic process and at least some of the heart phenotype may be due indirectly from embryonic

morphogenetic defects. However, importantly, the range of cardiac morphogenetic defects for both *gata5* and *gata6* morphants is consistent and reproducible, and for the most part the embryos generate substantial GFP+ cardiomyocytes.

In stark contrast to the *gata5* and *gata6* single morphants, embryos depleted of both factors show a complete loss of GFP expression, consistent with a loss of cardiomyocytes. Thus, *gata5* and *gata6* each have non-redundant functions for cardiac morphogenesis, but the two genes are functionally redundant in a requirement for generation of differentiated cardiomyocytes. Our previous studies showed also a loss of the earliest cardiomyocyte markers, including *nkx2.5*, suggesting a requirement for cardiomyocyte specification[3].

Discussion

In the experiment described here, we combined two morpholinos, each of which alone generate a distinct range of phenotypes, and found an entirely different phenotype when they were co-injected together. Of course, we needed a justification for doing this experiment in the first place. We suspected that the two genes might compensate each other for an earlier function, in this case cardiac specification. This is because the genes are highly related (and capable of binding to the same DNA sequences) and are expressed in overlapping patterns during embryogenesis[5]. Furthermore, genetic experiments in *Drosophila* indicated that GATA transcription factors should be required for cardiac specification[6].

However, there are other situations that this strategy might be used for testing functional redundancy or more generally for genetic interactions. First, knockdown of one or both genes might not generate any obvious phenotype. In this case, there is no defined threshold level for each gene, and the amount of morpholino to use will be defined empirically, limited by how well they are tolerated by the embryos. Again, the rationale for testing compensation will likely be that the genes are highly related and from confirming co-expression patterns. Second, genetic interactions can be tested for any gene pair using sub-threshold amounts of morpholinos. In this strategy, the maximal amount that does NOT generate a phenotype is used for each morpholino. If combining both morpholinos generates a phenotype, this provides strong evidence for a genetic interaction, although it may be quite indirect or even represent parallel pathways. Third, co-injection of a second morpholino may rescue the phenotype of the first, if the two genes interact in an antagonistic manner (which again may be indirect). While there is no defined limit to how many different genes can be targeted, in a practical sense it is probably 3-4 genes, depending on the amount of each morpholino that is needed for a threshold response.

While this general approach of using reverse genetics to reveal new gene functions is relatively high-throughput, there are a number of caveats to consider regarding morpholinos. Before combining morpholinos, considerable effort is needed to validate individual reagents, if this has not already been accomplished. Any single morpholino may not function, while others can generate off-targeting "artifact" phenotypes particularly associated with activation of widespread apoptosis. The reagents are not inexpensive, and if two or more morpholinos are needed to validate each gene, this investment can be significant.

Several considerations may guide the experimental strategy.

i) Is there a known mutant phenotype? If so, a single pheno-copying morpholino should be sufficient.

ii) Is there an antibody available? If so, a translation-blocker may be preferred, and if not, it will be important to document that a splice-blocker effectively targets the pre-mRNA.

iii) Can you rescue the morphant by injection of mRNA? If this works, it provides excellent evidence for morpholino specificity. However, it may often not work, due to mis-targeting of the injected mRNA. Rescue might also be tested using transposon-based transgenesis, since this may allow tighter spatial and temporal control over expression of the targeted gene. In either case, it is important to ensure that the rescue gene is not targeted by the morpholino.

Disclosures

No conflicts of interest declared.

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