Video Article

Cell Labeling and Injection in Developing Embryonic Mouse Hearts

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Abstract

Testing the fate of embryonic or pluripotent stem cell-derivatives in *in vitro* protocols has led to controversial outcomes that do not necessarily reflect their *in vivo* potential. Preferably, these cells should be placed in a proper embryonic environment in order to acquire their definite phenotype. Furthermore, cell lineage tracing studies in the mouse after labeling cells with dyes or retroviral vectors has remained mostly limited to early stage mouse embryos with still poorly developed organs. To overcome these limitations, we designed standard and ultrasound-mediated microinjection protocols to inject various agents in targeted regions of the heart in mouse embryos at E9.5 and later stages of development. Embryonic explant or embryos are then cultured or left to further develop *in utero*. These agents include fluorescent dyes, virus, shRNAs, or stem cell-derived progenitor cells. Our approaches allow for preservation of the function of the organ while monitoring migration and fate of labeled and/or injected cells. These technologies can be extended to other organs and will be very helpful to address key biological questions in biology of development.

Video Link

The video component of this article can be found at http://www.jove.com/video/51356/

Introduction

More than a decade ago, human embryonic stem cells (HuESCs) have been derived from human blastocysts[1]. Since then, these cells have become the subject of an important field of research which addresses unmet questions in human developmental biology. HuESCs have furthermore provided hopes in regenerative medicine. In recent years, human induced pluripotent stem cells (iPSCs) have been generated from patient-specific somatic cells, providing models of genetic disease[2]. Many *in vitro* protocols for differentiation of embryonic or induced pluripotent stem cells towards various cell lineages, including heart lineages[3], have been reported. The differentiated cells are often phenotyped by analysis of RNA and protein expression, immunostaining, and/or *in vitro* functional tests. However, pluripotent stem cell derivatives have to be placed in a proper embryonic environment in order to test whether they fully acquire the cell fate of their embryonic counterpart and whether they recapitulate the genuine *in vivo* function in response to regional cues. While tissue engineering is promising, it does not yet provide all the known and unknown cues of the proper *in vivo* developing embryonic tissue[4,5].

Cell labeling with dyes or retroviral vectors in embryos, including mouse embryos, have brought important information as to the embryonic origin of cell lineages during cardiac development[6]. For example, dye injection into the pericardial space of mouse embryos *ex vivo*, followed by *in vitro* culture of isolated hearts, was used to label epicardial cells and their descendants[7]. However, dye and retroviral cell labeling have been mostly applied to early mouse embryos with still poorly developed organs, or chicken embryos, which are more easily accessible[8]. An exception is the brain, which is easier to target in embryos[9,10]. Such an approach has not yet been applied to the beating embryonic mouse heart.

To complement direct labeling with dyes or virus and to perform lineage tracing in more advanced stage mouse embryos and adult mice, the cell labeling approach has been combined with analysis of transgenic mice using the Cre/Lox technology. The Cre/Lox approach[11] however features some limitations due to the spatiotemporal specificity of the genomic regulatory regions used to drive expression of the recombinase, and the efficiency of the Cre/Lox recombination[12]. Furthermore, this approach does not fully address the specific questions of cell migration-driven acquisition of cell fate as it can only label a precursor after activation of the regulatory region used to drive Cre expression. It also cannot apply to human embryos for obvious ethical issues.

Given these limitations, we designed a series of new protocols to inject a variety of cell labeling agents such as fluorescent dyes, virus, gene expression modulators such as shRNAs and DNA-based cell labeling vectors, or cells in the mouse embryo at E9.5 and later stages of development in targeted regions of the heart.

The DNA/cell injections use a stereomicroscope and a simple microinjection device combined with *ex vivo* embryo culture up to 48 hr, or isolated heart or embryonic explant culture for 48-72 hr. We also report an ultrasound-mediated microinjection protocol in mouse embryonic hearts *in utero*. This technique allows monitoring the development of embryos[13] and allows for long-term follow-up of the injectates and/or labeled cells.

We found that these approaches preserve the function of the organ and provide a more representative environment than *in vitro* testing of stem cell potential. It also provides the opportunity to follow migration of labeled and/or injected cells to monitor their fate. Ultimately, this should yield a better understanding of regional tissue patterning and key biological processes.

Protocol

1. Preparation

Animal procedures

Obtain approval from an animal ethical committee and follow institutional guidelines for work with virus, HuESC and/or iPSC (when applicable), as well as mouse handling, obtaining mouse embryos, and performing mouse surgery. For timed matings, the day of the plug is considered embryonic day (E)0.5 / 0.5 days post-coitum.

1. Microinjection needles:

   For *ex utero* microinjection, pull glass pipettes (1.2 mm external diameter borosilicate capillary tubes) using a pipette puller/beveller to get a 1 or 10 µm internal tip diameter and a sharp and conic tip shape to inject DNA or cells, respectively. For ultrasound-mediated injections, use customized sterile needles, which have an outer/inner diameter (OD/ID) of 1.14 mm/0.53 mm, with a 50/35 µm OD/ID tip.
2. Silicon-filled Petri dishes:

   Prepare silicon as described in the manual. Fill a clean glass 60 mm diameter Petri dish with an elastomer layer of ~1 cm. Avoid air bubbles.
3. Instruments:

   Keep all surgical instruments sterile prior to and during the procedure.
4. DNA preparation:

   Add 3 µg DNA and 12 µl Opti-MEM in one tube. Add 3 µl Lipofectamine 2000 and 12 µl Opti-MEM in another tube. Incubate for 5 min at RT and mix both solutions. Use immediately.
5. Cell preparation:

   Prepare a suspension of 10^6 cells/ml DMEM (Dulbecco's Eagle Medium, high glucose and 50% rat serum). 50 µl of final cell suspension is enough for 8-10 embryos.
6. Dye preparation:

   Use the green fluorescent CDCFDA-SE at 25 mg/ml DMSO. Prepare 20 µl aliquots and store at -20 °C. Dilute 1:100/1:200 in sterile saline or PBS before use.
7. Virus preparation:

   Use a commercial 3rd generation PGK-GFP-expressing lentivirus with a titer of ~10^9-10^{10} transducing units/ml DMEM. For the preparation of lentivirus, see Tiscornia *et al.*[14] Wear personal protective equipment and safely use and dispose of virus.

2. Collection of E9.5 and E10.5 Embryos for *Ex vivo* Injection

1. Euthanize a pregnant mouse at embryonic day 9.5 or 10.5.
2. Sterilize the chest and abdomen of the mouse with 70% ethanol.
3. Make a ventral midline incision to open the abdomen and retrieve the uterine horn at RT in PBS and transfer it after two PBS washes to a silicone-filled Petri dish with M16 medium.
4. Pin the uterine horn with insect pins to the silicone layer so that the vascularized side of the uterus is at the bottom.
5. Cut the surface of the uterus with fine forceps, and a cap of the decidua at 1 mm below the surface.
6. Gently retrieve the embryo in the yolk sac by spreading out the walls of the decidua.
7. Store the embryos at 37 °C in M16 medium before cell injection. For injection, each embryo is transferred to the silicone dish filled with M16 medium pre-warmed at 37 °C.

3. DNA or Cell Injection Under a Stereomicroscope

1. Fill the glass pipette from the back with a Hamilton syringe and shake the pipette to remove bubbles at the tip.
2. Set the pipette on the microinjection holder; set the holding pressure to 50 (DNA) or 30 (cells) hPa, and the injection pressure to 150 (DNA) to 300 (cells) hPa. Set the injection time to 0.2 sec (DNA) or 0.5 sec (cells). These settings might slightly vary according to the type of microinjector and pipette.
3. Pin the embryo to the silicone bottom through the yolk sac without touching the embryo proper. Watch the region of the heart and open the sac just above this region.
4. Add 4 pins around the embryo to prevent any motion during cell injection.

5. Using the micromanipulator (set to manual), slowly position the pipette tip at an angle of 45° above the pericardium, just above the region of the heart that is to be injected **(Figure 1**).
6. Move the pipette into the region of interest and trigger the injection. Take out the pipette as quickly as possible. Make sure the heart is properly beating after DNA/cell injection.

4. Embryo, Isolated Heart, and Explant Culture

1. Culture the E9.5 embryos in 25% M2 medium and 75% rat serum, supplemented with penicillin/streptomycin, pre-warmed at 37 °C and oxygenated with 40% O_2.
2. Add 2 ml culture medium in a 25 ml glass tube, gently add the embryo, and oxygenate with 40% O_2 before screwing the cap onto the tube. Incubate up to 36 hr at 37 °C while rotating or shaking (30 rotations/min).
3. At the desired time point (*e.g.* 24 hr), dissect the hearts carefully with fine forceps, without touching the hearts, by first decapitating the embryos; the heart is easy to excise by pulling it out using the distal outflow tract.
4. Culture the isolated heart for another 36-48 hr in DMEM with 50% FCS on a Matrigel-coated insert set in a 12-well plate. See Dyer & Patterson (2013) for an improved protocol of heart culture[15].
5. Make any explant of the region of interest. As an example, dissect the atrio-ventricular canal (AVC) and culture it for 48 hr on collagen type 1 gel[16].

5. Ultrasound-guided Injection in the Heart *In utero*

1. Setup of ultrasound machine and microinjector:
    1. Use the high-resolution ultrasound system with a 40 MHz transducer and microinjection setup on a rail.
    2. Set the injection volume on the microinjector at 69 nl per injection. See the manufacturer's microinjection manual for programming the microinjector.

2. Preparation of the microinjection setup and injection:
    1. Place a glass micropipette in the microinjector. To ensure efficient injection, first coat the inner side of the pipette with mineral oil by aspirating up to the maximum volume. Then eject the oil again, leaving 1-2 mm of oil inside the needle.
    2. Aspirate the injectate until maximum volume is reached. Be careful not to touch any surfaces with the needle tip as this will blunt the tip and make injection more difficult.
    3. To stabilize the uterine horn containing the embryos during injection, use a modified Petri dish with a hole in the middle (2.5 cm diameter), covered by a thin silicone membrane with a small slit cut in the center, and position it above the incision site. Stabilize the Petri dish on the mouse handling table by positioning 3-4 clumps of clay under the edges of the dish.

3. Injection procedure:
    1. Shave the abdomen of a pregnant mouse (at the desired embryonic stage) prior to anesthesia to remove hair. Treat the abdomen with a chemical hair removal agent to remove residual hair and sterilize with 70% ethanol.
    2. Anesthetize a pregnant mouse with oxygen/isoflurane via a face mask. Use 3-5% isoflurane in 100% oxygen for initial anesthesia, followed by 1-1.5% during the rest of the procedure. Ensure that the mouse is adequately anesthetized by gently pinching its paws and/or tail.
    3. Place the mouse supine on the mouse handling table (set to heat at 37 °C) and cover the eyes with lubricant to prevent dehydration of the cornea.
    4. Apply electrode gel on the electrodes and tape the paws to the electrodes to monitor heart rate, respiration rate, and ECG. Position a rectal thermometer to monitor body temperature.
    5. Verify first that the mouse is pregnant by visualizing and counting the embryos by ultrasound. Apply ultrasound gel on the abdomen and position the scan head above the bladder. For consistency, visualize the bladder first, and count embryos in the left and right uterine horns, starting from the position of the bladder. Ensure that embryonic hearts are beating normally.
    6. If the mouse is pregnant, position the Petri dish above the future incision site with clay. Press the dish slightly onto the abdomen so that the dish is in direct contact.
    7. Remove the dish and sterilize the abdomen again. Make a 1.5-2 cm ventral midline incision, approximately 0.75-1 cm above the vagina, to open the abdomen and peritoneum. Avoid injury to internal organs.
    8. Exteriorize the left or right uterine horn with forceps, gently pull it through the silicon membrane of the dish, and stabilize the dish on the clay again. Prevent extensive pulling or manipulation, since this may cause bleeding of the uterine vessels and premature death of the female and/or embryos.
    9. Count the embryos again to ensure adequate record keeping of which embryos were injected.
    10. Apply ultrasound gel on the uterine horns to image the heart and to prevent dehydration.
    11. Image the first embryo to be injected. Check normal beating of the embryonic heart and keep records of the cranial-caudal and left-right orientation of the embryo. If necessary, reposition the uterine horn slightly to ensure proper orientation. If this proves difficult, move on to the next embryo.
    12. Puncture the uterus carefully with the microinjection needle to position the needle in a 45° angle above the embryo, slightly outside of the pericardium (**Figure 3**). For labeling of epicardial cells, image the heart in a four-chamber view and position the needle so that it points towards the atrio-ventricular groove (**Figure 3**). If the angle needs to be adjusted, pull out the needle, and reposition. Do not adjust the angle with the needle inside the uterus, since this might break the needle. Avoid puncturing other tissues, such as limbs or placenta.
    13. Move the needle onto the pericardial wall and puncture it with a gentle, but swift motion. In general, there will be some resistance, but moving the needle too fast may cause the needle tip to puncture the heart itself. The tip should end up in the pericardial space of the atrio-ventricular groove. In the case of pericardial bleeding, blood cells will be visible as a white snow-like pattern. If this is the case, stop injecting the embryo and move on to another embryo.

14. Inject the injectate. Inject maximally 700 nl into the pericardial space to prevent adverse effect on hearts development. Monitor the proper injection by slight, temporal swelling of the pericardial sac.
15. Following injection, pull the needle out and confirm that injectate can still be ejected to ensure that the needle was not clogged by tissue fragments.
16. Reposition the handling table to image and inject the next embryo. Keep the number of injected embryos to maximally 3-4 (in one uterine horn) to prevent extended anesthesia, to allow for control embryos in the opposed uterine horn, and to ensure that the procedure did not disturb embryonic development.
17. After injecting the desired number of embryos, remove excess ultrasound gel and gently place the uterine horn back into the abdomen in its proper position.
18. Close the maternal abdomen by using one layer of suture for the peritoneum and abdominal muscles, and a second layer of suture for the skin.
19. Inject the mouse with the first dose of pain medication. Use one injection of 0.05-0.1 mg/kg Buprenorphine 15 min prior to ending anesthesia and three additional injections with 12 hr intervals.
20. Discontinue anesthesia and monitor the mouse for adequate recovery from the procedure. House the pregnant mouse in an individual cage for the desired duration of follow-up.

Representative Results

Using the injection protocols described above, cells can be labeled and/or injected into the embryonic mouse heart. As proof of concept, several examples are shown in which the injection protocol and the *ex vivo* AVC explant, isolated heart, or whole embryo culture were combined (**Figure 1**).

Figure 1 shows the preparation of the embryo before cell injection. The E9.5 embryo is removed from its decidua while maintaining integrity of the yolk sac (**Figure 1A**). The yolk sac is opened just above the heart (**Figure 1B**). The pipette is approached (**Figure 1C**) and the cells injected. The heart retains its shape (**Figure 1D**) and remains beating.

To address a more specific biological question in developmental biology such as the epithelial to mesenchyme transition (EMT), an E9.5 AVC explant was injected with a shRNA that down-regulates a protein required for endothelial-mesenchymal transition of endocardial cells (**Figure 2A**). The control embryo was injected with an empty backbone vector. Likewise, HuES cell–derived endothelial pre-valvular cells expressing GFP under the control of the *Sox9* promoter were injected in the AVC at E10.5, followed by 48 hr explanted heart culture. These cells acquire markers of EMT such as periostin similar to the endogenous endocardial cells (**Figures 2B** and **2C**).

These *ex vivo* approaches are relatively easy, but limited to short-term follow-up (maximally 48-72 hr). The ultrasound-guided injection procedure provides a technically more challenging approach, but with the option of long-term, even post-natal, *in utero* follow-up. The *in utero* injection of dye or viral vectors to label cells in specific cardiac regions is shown in **Figures 3A-3C**. With this method, specific labeling of epicardial cells can be achieved with fluorescent dyes (**Figures 3D** and **3E**) or virus (**Figure 3F**), and the method can be expanded upon with cells or alternative injectates.

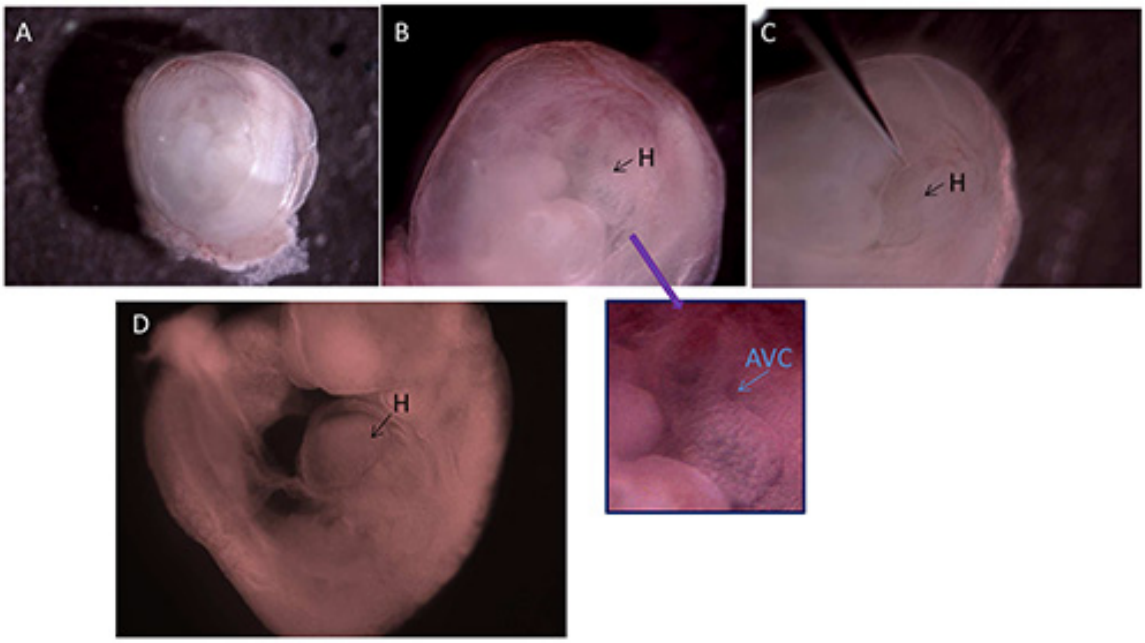

Figure 1. Cell injection in the heart. A: E9.5 embryo in the yolk sac. **B**: visualization of the heart after opening the yolk sac just above the heart. The inset below shows a magnification of the heart and points the AV canal. **C**: approach of the pipette towards the AVC. **D**: injected embryo after 48 hr culture (H = heart).

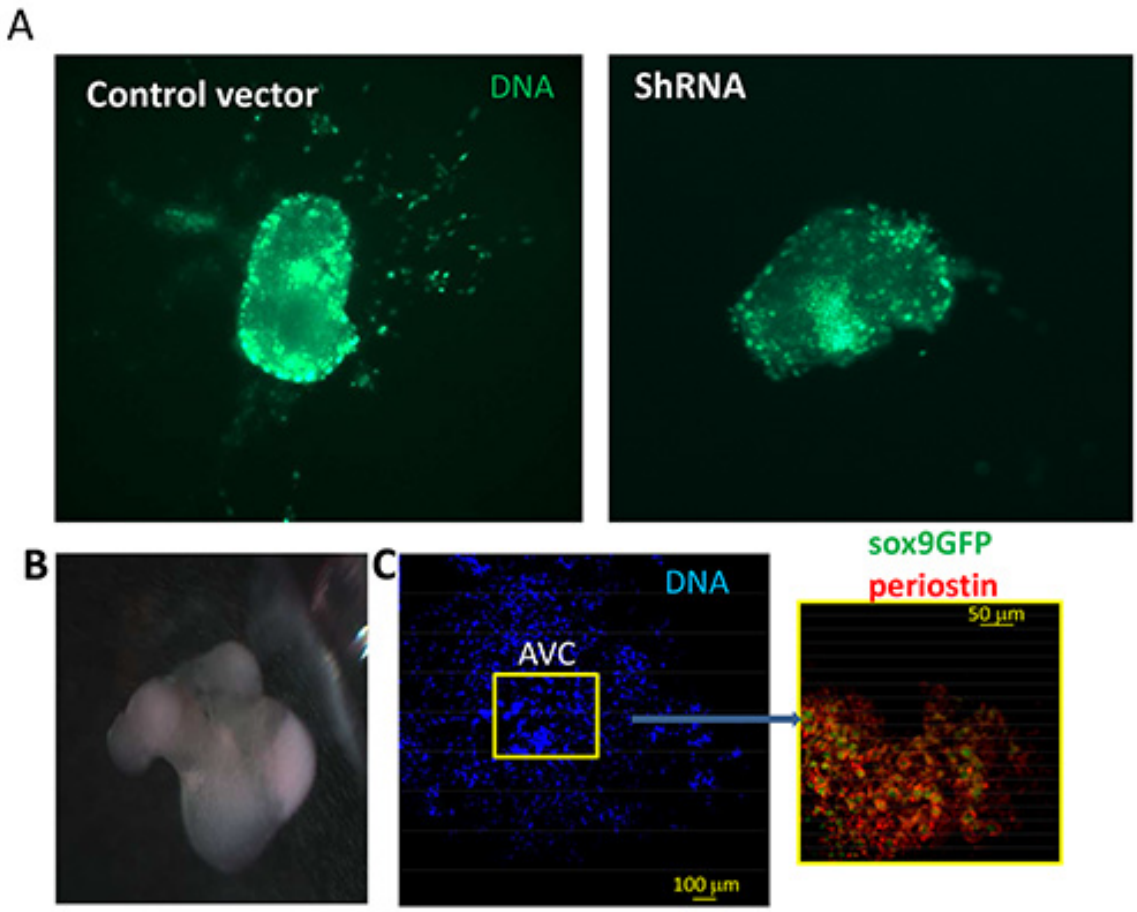

Figure 2. Injection of a shRNA in the AVC of an E9.5 embryo that prevents *ex vivo* EMT of endocardial cells in a cardiac explant. A: control backbone vector or a shRNA was injected together with lipotectamine in the AVC of an E9.5 mouse embryo; 3 hr later, the AVC was dissected out and grown on a collagen gel in order to trigger EMT of endocardial cells. The shRNA downregulated a protein that is required for EMT. **B-C**: HuES cell-derived valvular cells engineered to express GFP under the control of the Sox9 promoter were injected in the AVC of E10.5 embryos. The heart was cultured for 2 days (**B**), and subsequently fixed and stained with an anti-periostin antibody (**C**). The inset shows a magnification of the AVC region.

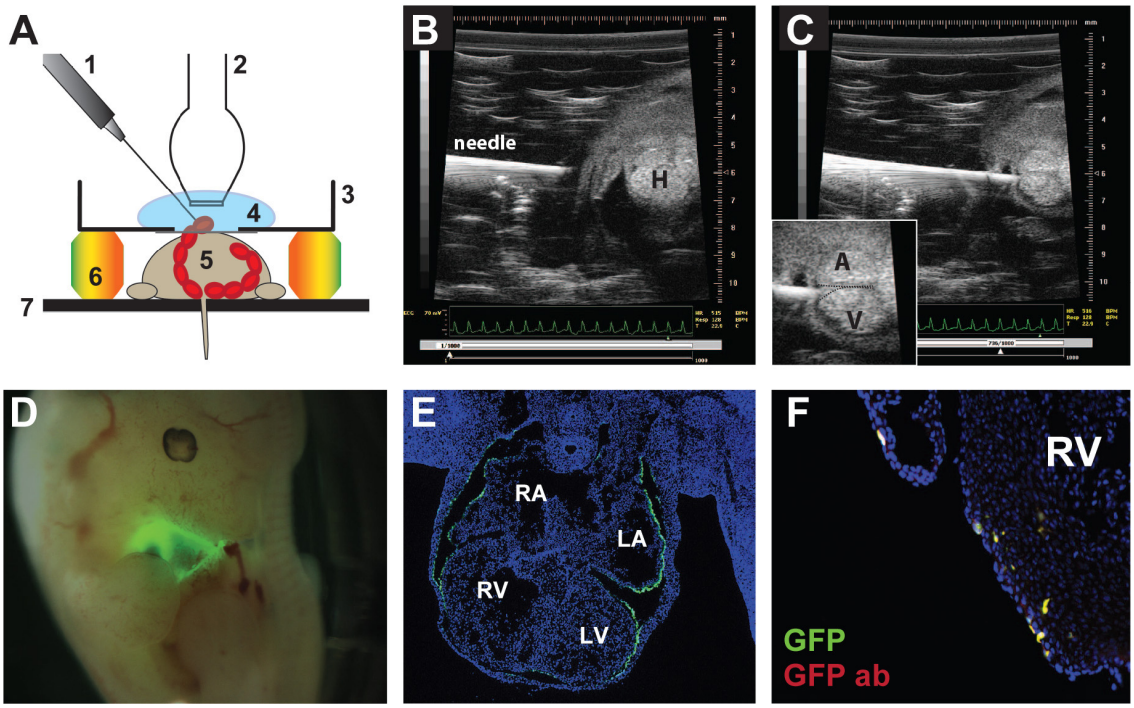

Figure 3. *In utero* injection. A: scheme depicting the experimental setup. The pregnant mouse is positioned supine and a Petri dish with silicone membrane is stabilized above the incision site. 1 = microinjector, 2 = transducer, 3 = Petri dish with silicone membrane, 4 = ultrasound gel, 5 = mouse with uterine horns (red), 6 = Play-Doh clay, 7 = mouse handling table. **B**: still frame of an ultrasound movie (M mode) showing the position of the needle and embryonic heart prior to injection. The female ECG is shown at the bottom (green), and heart and respiration rate in the lower right (yellow). H = heart. **C**: ultrasound image showing the position of the needle and embryonic heart during injection. Inset: the tip has passed the pericardium, but does not touch the myocardium, so that the injectate can be injected into the pericardial space of the atrio-ventricular groove. Black dotted lines mark the borders of the atrium (A) and ventricle (V). **D**: whole mount image of an E11.5 mouse embryo showing strong fluorescence of the dye in the pericardial space. **E**: representative section of an E11.5 embryonic mouse heart showing pericardial and epicardial cell labeling with a fluorescent dye (CDCFDA-SE, green) 4 hr after injection. Nuclei are labeled with DAPI (blue). LA = left atrium, LV = left ventricle, RA = right atrium, RV = right ventricle. **F**: representative section of an E14.5 embryonic mouse heart showing mosaic epicardial cell labeling with GFP-expressing lentivirus 3 days after injection. To confirm GFP specificity, the GFP protein was also stained with a GFP antibody (red). Nuclei are labeled with DAPI (blue).

Discussion

The intra-cardiac *ex vivo* injection protocols described above are designed to preserve myocardial function for at least 48 hr in mid-stage (E9.5-E11.5) mouse embryos. These injection approaches allow for spatially targeted injection of DNA or cells. The few examples shown in **Figures 1-3** provide proof of concept for delineating *ex vivo* and *in vivo* molecular mechanisms of developmental processes that take place in restricted cardiac regions, such as EMT of endocardial or epicardial cells. Most importantly, these protocols provide an opportunity to follow migration and fate of injected cells such as human embryonic cells or huESC derivatives in a proper embryonic environment, a so far unmet challenge. *In vivo* labeling (like the epicardial cells in **Figure 3**) with saturating or limiting dilutions of dye or virus allows for lineage tracing of cell (sub)populations on a short- as well as long-term basis, without the need for Cre/lox technology (although combining these techniques may be useful as well).

Several critical steps while applying these protocols were observed. First, the embryos are very sensitive to light. It is therefore recommended to practice the injection protocol under a stereomicroscope so that the procedure can be carried out with high reproducibility and efficiency within 15 min per embryo. In the same line, the entire ultrasound-mediated injection procedure should be performed within 30 min to preserve a good viability of embryos and to not compromise their *in utero* development. Manipulation of the uterus should be kept to a minimum. Additionally, it should be noted that surgery on pregnant mice tends to result in premature delivery (1-2 days earlier than normal). However, neonates should be generally healthy and develop normally. Further, the penetration of the pipette through the uterine wall, the yolk sac and the embryo to reach the pericardium and finally the myocardium is a delicate procedure (step 5.3.13). This step should be done carefully and gently. For optimization, it is recommended to perform several short-term dye injections to determine the reproducibility and exclude injection in non-targeted areas. Finally, we have not found developmental cardiac defects related to the injection procedures for the examples given above. However, a more detailed, stage-by-stage, morphological and functional analysis should be performed to completely ascertain normal development and function during the stages of interest.

The limitations of these technologies are several. (i) The injection under the stereomicroscope is limited by the light and temperature sensitivity of the mouse embryos. This can be overcome by the practice of the microinjection procedure in order to be as fast as possible, regularly replacing or warming the medium, and working in a dark room. (ii) The *ex vivo* culture of embryos or explants is limited in time, in contrast to the *in utero* ultrasound-guided injection, which allows for long-term follow-up. Vital dyes tend to label cells almost immediately and remain visible up to three to four days post-injection. However, the intensity of the dye will be diluted by successive cell divisions. Labeling cells with lentivirus may

overcome this problem. However, uptake of the virus takes several hours and expression is optimal after 24-48 hr, which prevents short-term analysis. (iii) The ultrasound-mediated injection is limited by the cost of the equipment. The resolution of the 40 MHz transducer is sufficient for mid- and late-stage embryos, but more limiting for stages prior to E11.5.

In summary, we propose that the cardiac injection protocols described above should be used in mid-stage mouse embryos. The *ex vivo* technique is compatible with the culture of embryos, isolated hearts, or explants. The *in vivo* procedure allows for long-term follow-up, including post-natal development. These techniques will aid significantly in testing the differentiation potential of human stem cell derivatives in a proper environment, as well as in addressing specific questions of developmental biology such as cell migration and regional cues, which are key events in the road toward shaping a functional heart. Additionally, our methods and those of others[10] may in the future be used for investigation of other organs than the heart, dependent on available *ex vivo* culture protocols and/or developmental stage *in vivo*.

Disclosures

The authors have nothing to disclose.

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