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TITLE:
Echocardiography-guided Injection for Targeted and Reliable Intramyocardial Stem Cell Delivery in a Rat Model of Myocardial Infarction

AUTHORS AND AFFILIATIONS:
Ellen Heeren[1], Vincent Vandenboer[1], Lotte Vastmans[1], Dorien Deluyker[1] , Marc Hendrikx[1], Virginie Bito[1]

[1]UHasselt, Cardio & Organ Systems (COST), Biomedical Research Institute, Agoralaan, 3590 Diepenbeek, Belgium

EMAIL ADDRESSES OF CO-AUTHORS:
Vincent Vandenboer Vincent.vandenboer@student.uhasselt.be
Lotte Vastmans Lotte.Vastmans@uhasselt.be
Dorien Deluyker Dorien.deluyker@uhasselt.be
Marc Hendrikx Marc.hendrikx@uhasselt.be

CORRESPONDING AUTHORS:
Virginie Bito Virginie.bito@uhasselt.be
Ellen Heeren Ellen.heeren@uhasselt.be

SUMMARY:
This article details an optimized protocol for echocardiography-guided intramyocardial injections in rat models of myocardial infarction using a 29 G x 88 mm needle. This technique ensures robust, precise, and reproducible delivery of therapeutic agents directly into the peri-infarct zone.

ABSTRACT:
Echocardiography-guided intramyocardial injection (EGI) is a minimally invasive technique for delivering stem cell therapies in preclinical myocardial infarction (MI) models. Compared to traditional open-chest approaches, EGI offers improved clinical translatability, reduced invasiveness, and minimized physiological impact on the animal. While EGI is well established in murine models, its application in rats remains limited due to anatomical and technical challenges. In particular, thinning of the left ventricular anterior wall (LVAW) in infarcted and peri-infarct regions complicates safe and accurate myocardial delivery, as wall thickness can fall below the needle bevel size of commonly used 27 G or 28 G needles, increasing the risk of ventricular perforation or failed delivery. To address this limitation, we optimized a protocol for EGI in rat MI models using 29 G Spinocan needles. The smaller-diameter, longer needle enables precise targeting of thin myocardial tissue, minimizing damage and enhancing injection accuracy, independent of LVAW thickness. This technique is compatible with standard transthoracic echocardiography platforms and eliminates the need for thoracotomy, allowing longitudinal studies in the same animal. Our refined method enables robust, reproducible delivery of therapeutic agents into viable myocardium adjacent to the infarct zone, where regenerative therapies are most effective. By improving safety and targeting precision, this

approach increases the translational relevance of preclinical cardiac research and supports the development of standardized protocols across laboratories.

INTRODUCTION:

Cardiovascular diseases remain the leading cause of morbidity and mortality worldwide, with myocardial infarction (MI) being a significant contributor to both acute and chronic cardiac conditions[1]. Despite advances in pharmacological and interventional management, the regenerative capacity of the adult human heart is limited, often resulting in adverse remodeling and progression to heart failure[2,3]. Consequently, stem cell-based therapies have gained attention as potential strategies to repair damaged myocardium, preserve cardiac function, and improve clinical outcomes[4].

Robust preclinical models are essential for evaluating the safety, efficacy, and delivery strategies of these therapies. Among small animal models, rats offer several advantages, including manageable heart size, well-characterized infarction techniques, and translationally relevant cardiac remodeling responses[5]. Conventionally, intramyocardial administration of stem cells in rat MI models is achieved via thoracotomy, allowing direct visualization of the injection site[6]. However, this approach is invasive, introduces significant procedural risks, and impedes repeated interventions in longitudinal studies. Moreover, it lacks alignment with clinical delivery modalities, such as catheter-based or percutaneous injection[7].

Echocardiography-guided intramyocardial injection (EGI) is a minimally invasive alternative that enables targeted delivery of therapeutic agents under real-time imaging guidance. While well established in murine models, the use of EGI in rat models has been limited. EGI is more extensively developed in mice than rats primarily because most cardiovascular research has historically focused on mice as the dominant preclinical model[8]. Additionally, the smaller size and heart anatomy of mice allowed for the optimization of ultrasound-guided procedures[9]. In contrast, rats have a thicker chest wall and greater respiratory motion, complicating stable, high-resolution imaging during the procedures[10].

Advances in high-frequency ultrasound technology have made EGI in rats feasible, enhancing the clinical relevance of their use. Yet, the thinning of the left ventricular anterior wall (LVAW) in infarcted regions remains a major challenge for EGI in rodents. In rats, wall thickness often decreases to less than 1 mm, whereas standard 27 G or 28 G needles have bevel lengths of 1.25–1.5 mm, increasing the risk of ventricular perforation or poorly directed cell delivery. To address this limitation, we refined the EGI technique in rats by utilizing 29 G x 88 mm Spinocan needles. These needles feature a bevel length of 1 mm, allowing precise, atraumatic delivery of cells into the thinned myocardium of the infarct and peri-infarct regions. The procedure is performed using high-resolution transthoracic echocardiography, enabling visualization of both anatomical landmarks and needle trajectory in real time.

This refined EGI protocol provides a minimally invasive, reproducible method for targeted delivery of therapeutic agents into the peri-infarct myocardium in rat models of both acute and chronic MI, independent of LVAW thickness. It facilitates injection into viable border

zones—critical sites for achieving therapeutic benefit—while significantly reducing surgical burden and recovery time compared to open-chest approaches. Moreover, it supports longitudinal studies involving repeated injections and follow-up imaging, thereby reducing animal numbers in accordance with the 3Rs (Replacement, Reduction, Refinement) principle[11]. The protocol is adaptable for the delivery of pharmacological agents, gene therapies, biomaterials, and various stem cell types. By standardizing EGI in rat models and aligning with clinical delivery modalities, this approach enhances reproducibility across laboratories and strengthens the translational relevance of preclinical cardiac research.

PROTOCOL:

All animal experiments were approved by the Local Ethical Committee of UHasselt (Ethical Commission for Animal Experimentation, UHasselt, Diepenbeek, Belgium, ID202308 and ID202497) and conducted in accordance with EU Directive 2010/63/EU.

1. Equipment setup

1.1. Turn on the ultrasound imaging platform, the integrated warmed platform, the physiology monitoring unit, and the gel warmer.

NOTE: To enable clear and sufficiently detailed visualization of the left ventricular anterior wall during the EGI procedure, a high-frequency ultrasound system equipped with a 15 MHz linear array transducer providing approximately 75 µm axial resolution is recommended.

1.2. Turn on the heating pad to maintain the body temperature of the rat during chest hair removal.

1.3. Select the appropriate transducer and initialize the 3D motor to ensure full range of motion of the transducer.

NOTE: In addition to using the micromanipulator of the animal's platform railing system, the 3D motor allows for highly precise transducer adjustments, which can facilitate the alignment with the injection needle when needed. If necessary, the procedure can also be performed without the 3D motor.

1.4. Ensure proper system alignment before imaging. Set the micromanipulator of screws controlling the animal platform to a neutral position (i.e., centered within the full range of motion). Center the animal platform itself in the middle of the railing system. Position the transducer mounting system so that the transducer is aligned directly above the center of the animal platform.

2. Animal preparation and anesthesia

2.1. Weigh the rat and calculate the required volume of buprenorphine (0.04 mg/kg) to administer for pain relief.

2.2. Place the rat in an induction chamber connected to an anesthesia machine. Induce anesthesia with 2.5% isoflurane supplemented with oxygen at a flow rate of 2 L/min.

2.3. Once anesthesia is induced, transfer the rat onto a heating pad to maintain the body temperature at 37 0.5 °C. Maintain adequate anesthesia by positioning the rat's snout into a nose cone connected to the anesthesia system, delivering a constant flow of 1–3% isoflurane supplemented with 1–1.5 L/min oxygen, adjusted as needed to ensure adequate sedation.

2.4. Shave the rat's chest and remove residual hair with depilatory cream. After hair removal, disinfect the surgical site by alternating an appropriate surgical scrub agent (e.g., povidone-iodine) with 70% ethanol, repeated 3x. Apply ophthalmic gel to both eyes to prevent dryness.

2.5. Administer the precalculated dose of buprenorphine intramuscularly into the hind leg to minimize the discomfort related to the EGI.

2.6. Transfer the rat from the heating pad to the animal platform of the ultrasound imaging system. Ensure consistent anesthesia delivery through the platform's integrated nose cone, maintaining a steady flow of 1–3% isoflurane in 1–1.5 L/min oxygen. Secure the rat's paws onto the platform electrodes, applying a small amount of electrode gel to ensure optimal signal quality. Insert the rectal temperature probe for continuous body temperature monitoring.

NOTE: Continuous monitoring of physiological parameters is essential throughout imaging and injection procedures. Heart rate (HR), respiratory rate (RR), and body temperature of the rat must remain within acceptable ranges to avoid anesthesia-related complications. As a general guide, HR should be in the range of 250–400 bpm, whereas RR should be at least 30 breaths per minute. If HR or RR drop below these thresholds, immediately decrease the isoflurane concentration (e.g., from 2–3% down to 1–2%) and verify that the animal remains properly positioned and does not become hypothermic. Adjustments should be made gradually, with close monitoring until parameters return to target levels.

2.7. At this stage, acquire any desired baseline or pre-injection images. In general, parasternal long axis (PSLAX) and short axis (SAX) images in both B-mode and M-mode, along with four-chamber images, are recommended for baseline functional and anatomical assessment.

NOTE: General imaging can be performed with the system set up as shown in **Figure 1.** For the injection procedure, the animal platform will need to be repositioned and the transducer adjusted accordingly. While PSLAX B-mode imaging is used for guidance during injection, these images may not be optimal for post-hoc functional analyses due to the necessity of precise needle alignment.

3. Echocardiography-guided intramyocardial injection procedure

NOTE: From this point onward, two operators are required to successfully perform the injection. Operator A is responsible for manipulating the animal platform and monitoring the live echocardiography image, while Operator B should position themselves behind the injection mount to accurately align the needle and transducer.

3.1. Prepare the injection system by attaching a 22 G guide needle to a 1 mL syringe. Place the syringe with the guide needle onto the injection mount and secure the injection clamp.

NOTE: The 29 G x 88 mm needle is too flexible to puncture skin and muscle layers directly; the guide needle is necessary to facilitate the injection.

3.2. Align the transducer with the injection mount to ensure the needle can be visualized. Adjust the transducer position using the transducer mount and holding clamp, and, if needed, rotate the injection mount along its rail to achieve appropriate alignment.

3.3. Without moving the rat, rotate the animal platform so that the notch of the transducer points to the rat's right shoulder. This setup allows the acquisition of a clear PSLAX B-mode image of the left ventricle. Verify that the guide needle remains within the transducer's field of motion. If misaligned, adjust the transducer position via the 3D motor system rather than altering the injection mount.

3.4. Once correct needle-transducer alignment is achieved, fine-tune the imaging by using the micromanipulator screws of the animal platform rail. Maintain the transducer position to preserve alignment with the needle. Additional rotation or translation of the animal platform might be necessary to optimize image quality.

3.5. Visualize the infarcted area. Evaluate the infarct extent, regional wall motion, and wall thinning, using both PSLAX B-mode and M-mode imaging to assess the feasibility of injection site. Apply a 16-segment wall motion scoring approach to identify akinetic or severely hypokinetic regions, which represent the infarct core.

3.6. Identify the peri-infarct zone and select the target injection site. Choose a hypokinetic area adjacent to the infarct core with an end-diastolic wall thickness greater than 1 mm, which allows complete insertion of the 29 G needle bevel at a shallow angle.

3.7. Using the rail system, advance the injection mount towards the animal platform. Fine-align the needle with the exact middle of the transducer using the micromanipulator screws on the injection mount.

NOTE: Precise alignment is critical. If the needle is not centered to the transducer, it will not be visible during the injection.

3.8. Advance the guide needle using the **inject** micromanipulator screw on the injection mount until the skin is punctured. Confirm that the needle tip is visible in the ultrasound image. If the needle is not visible, retract the needle carefully using the 'inject' micromanipulator screw and repeat step 3.7 to realign.

3.9. Activate **needle guide** feature in the ultrasound software to confirm the needle trajectory. Advance the guide needle towards the pre-determined target region, positioning the needle bevel approximately 1–2 mm from the left ventricular anterior wall (LVAW) (**Figure 2A)**.

NOTE: Avoid puncturing the myocardium with the guide needle to prevent excessive bleeding or cardiac injury.

3.10. Once the needle is positioned, have Operator A firmly stabilize the guide needle at its base ensuring constant visualization of the needle on the ultrasound screen. Simultaneously, ask Operator B to loosen the clamp securing the syringe, remove the syringe, and prepare the injectate syringe. Instruct Operator A to keep the guide needle stationary.

3.11. Attach the 29 G x 88 mm needle on the syringe containing the injectate (Operator B) and secure the syringe onto the injection mount. Manually guide the 29 G x 88 mm needle through the stationary guide needle, making minor adjustments with the micromanipulator screws in the x-axis plane (left or right of the probe) if necessary. Advance manually until the needle bevel becomes visible in the thoracic cavity on ultrasound.

NOTE: Due to the flexibility of the 29 G needle, it may bend during insertion into the thoracic cavity. To minimize this, Operator A can stabilize the external portion of the needle shaft while operator B advances the needle into the thoracic cavity.

3.12. Once the 29 G x 88 mm needle passes through the guide needle and becomes visible on ultrasound, advance it into the myocardium of the LVAW using the micromanipulator screws for added precision and safety. Confirm that the needle bevel is located entirely within the myocardium **(Figure 2B)**.

NOTE: Due to the relatively long bevel of the 29 G needle compared to the myocardial wall thickness, it is critical to ensure that the entire bevel is embedded within the myocardium. Use the 'Freeze image' function to verify placement if needed. Although ultrasound depth calibration may aid in precision, in this protocol, we verified depth by selecting injection sites with an end-diastolic wall thickness >1 mm and visually confirming the needle tip approaching—but not breaching—the endocardial border (**Figure 2B**).

3.13. Inject the injectate slowly into the myocardium (Operator B). Successful intramyocardial injection is confirmed visually by a bright/dense echogenic spot at the injection site, which should move synchronously with the LVAW **(Figure 2C)**. To minimize backflow of the injectate, wait for approximately 10 s after completing the injection before retracting the needle.

3.14. First, remove the syringe with the needles from the injection mount to minimize the risk of needle stick injury. Next, carefully remove the rat from the animal platform and allow it to recover on a heating pad. Monitor vital parameters continuously until full recovery from anesthesia.

REPRESENTATIVE RESULTS:

Validation of injection accuracy by *in*- and *ex vivo* bioluminescence imaging (BLI)

To evaluate the success and efficiency of the EGI, stem cells were first transduced to express firefly luciferase (Fluc) via a lentiviral vector, enabling *in vivo* tracking by bioluminescence imaging (BLI). BLI confirmed precise intramyocardial delivery in 86% of cases (n = 14). **Figure 3A** illustrates partial leakage resulting from an excessively rapid needle withdrawal, evidenced by diffuse luminescence in the adjacent thoracic cavity. In contrast, following a fully successful intramyocardial injection, a strong, localized Fluc signal was detected at the left ventricular mid-apex level (**Figure 3B**). To further confirm myocardial delivery, whole-animal imaging of the successful injection was immediately followed by *ex vivo* imaging of the excised heart, sectioned into three transverse levels: apex, mid-apex boundary, and mid ventricle. *Ex-vivo* imaging revealed the highest bioluminescent signal in the mid-apex section, corresponding to the targeted injection site (**Figure 3C**). Lower-intensity signals in the apex and mid sections indicate minor dispersal of injected cells throughout the ventricular tissue.

Spatial distribution of the injectate in tissue sections

To confirm targeted delivery into the peri-infarct zone, a Texas Red–dextran tracer embedded in a hydrogel matrix was injected via EGI. **Figure 4A** shows a gross image of the isolated heart slice post-mortem, with a purple hydrogel adjacent to the infarct site. After cryosectioning and DAPI nuclear counterstaining, fluorescence imaging (**Figure 4B**) revealed the red tracer signal confined to the peri-infarct region, validating accurate injectate delivery. While echocardiographic identification of the peri-infarct zone is primarily based on the LVAW thickness, postmortem Sirius Red staining provides a more precise assessment of infarct location and extent. **Figure 4C** shows infarct extent in red, confirming that the tracer was injected into a region with visibly greater wall thickness than the infarct core, yet still within an area of notable fibrosis.

Safety assessment using color Doppler imaging

Color Doppler imaging was used throughout the EGI procedure to detect any accidental bleeding. **Figure 5A** shows the 29 G needle positioned within the myocardium, with no evidence of any blood leakage or abnormal flow. During the injection phase (**Figure 5B)**, the LVAW remained intact with no extraventricular blood flow. **Figure 5C**, acquired after needle withdrawal, demonstrates the retained injectate in the myocardium and the absence of any hemorrhage. Importantly, procedural mortality associated with EGI was 0%, underscoring the safety and reliability of the technique when properly executed. These findings collectively confirm that EGI maintains cardiac integrity without inducing hemorrhagic complications.

FIGURE AND TABLE LEGENDS:

Figure 1. Setup of the ultrasound imaging station and animal placement. (A) Overview of the ultrasound imaging platform and rail system (1. injection micromanipulator screws, 2. injection clamp, 3. injection mount, 4. 3D motor, 5. transducer clamp, 6. transducer, 7. animal platform, 8. animal platform rail, 9. animal platform micromanipulator screws, 10. transducer mount). (**B**) Close-up of the rat positioning on the animal platform. (**C**) Transducer position with the notch directed at the right shoulder of the rat. (**D**) Precise alignment illustration of the 29 G needle with the transducer.

Figure 2: Sequence of echocardiography-guided injection B-mode images in PSLAX. (**A**) Insertion of 22 G guide needle into the thoracic cavity. (**B**) The 29 G needle positioned in the left ventricular anterior wall. (**C**) Confirmation of successful injection by visualization of the injectate (delineated by the dotted yellow circle) in the myocardium after needle withdrawal. The yellow arrows indicate the needle shaft, whereas the red arrow specifies the needle bevel. Abbreviation: PSLAX = parasternal long axis.

Figure 3: Validation of injection accuracy by *in*- and *ex vivo* bioluminescence imaging. Stem cells were transduced to express Fluc to enable tracking of the injectate following EGI. (**A**) Partial leakage of the injectate caused by excessively rapid needle withdrawal. (**B**) Whole animal imaging after fully successful EGI, immediately followed by sacrifice of the animal and (**C**) *ex vivo* imaging of the isolated, sectioned heart at the mid, mid-apex, and apex level. Abbreviations: Fluc: firefly luciferase; EGI = echocardiography-guided intramyocardial injection.

Figure 4. Spatial distribution of the injectate in tissue sections. Texas Red—dextran tracer embedded in a hydrogel matrix was injected via EGI. (**A**) Gross image of an isolated heart section with the injectate, shown in purple as indicated by the arrows, localized adjacent to the infarct site (yellow dashed circle). (**B**) Fluorescent image of Texas Red—dextran tracer with DAPI counterstaining. (**C**) Sirius Red staining, indicating the infarct site in red, whereas healthy tissue is stained in green. Scale bars = 1 cm (**A**), 1,000 µm (**B,C**). Abbreviations: EGI = echocardiography-guided intramyocardial injection; DAPI = 4',6-diamidino-2-phenylindole.

Figure 5: Safety of the injection based on strain analysis and Doppler imaging during injection. Color Doppler imaging of the left ventricle during (**A**) insertion of the needle into the left ventricle anterior wall, (**B**) during the injection process, and (**C**) after needle withdrawal following injection completion. The yellow arrows indicate the needle shaft, whereas the red arrow specifies the needle bevel. The injectate is delineated with a yellow circle.

DISCUSSION:

As preclinical research focusing on regenerative therapies to restore the heart after myocardial infarctions advances, hurdles regarding optimal therapeutic agent delivery remain[12]. Minimally invasive EGI techniques are increasingly favored over open-chest approaches because they better mimic clinical percutaneous delivery, improve animal welfare, and permit repeated administrations in longitudinal studies[8,13]. Yet, detailed, standardized protocols for rat models are scarce, and procedures are complicated by pronounced thinning of the left ventricular

anterior wall (LVAW) in chronic peri-infarct zones[14]. Here, we have significantly enhanced the safety, precision, and reproducibility of EGI in the rat myocardium through careful procedural refinements.

General precautions and critical steps

Careful control of anesthesia and continuous monitoring of physiological parameters are critical for the success of myocardial EGI procedure in rats. HR should be maintained between 300 and 450 bpm, and RR between 30 and 50 breaths per minute throughout the procedure to ensure a workable balance between adequate sedation and an optimal acoustic window for imaging[15]. Isoflurane is the anesthetic of choice for this application, offering a rapid onset and a short half-life, which allows for precise adjustments to the depth of anesthesia. This property provides a major advantage over injectable agents such as ketamine/xylazine, where modifications to anesthetic depth are slower and less predictable[16,17].

Accurate alignment of the ultrasound transducer and injection needle is crucial for reliable myocardial delivery. A common technical challenge is transducer drift or needle flexion, which can cause the needle to move out of the imaging plane. In such cases, it is recommended to adjust the alignment by either repositioning the animal platform using the micromanipulator screws or utilizing the 3D motor to precisely move the transducer and re-identify the needle. While these adjustments may temporarily hinder the visualization of cardiac structures, they are often necessary to restore proper alignment.

The use of a 29 G injection needle offers advantages in terms of minimal invasiveness, reduced risk of myocardial damage, and improved feasibility of intramyocardial injection. In our hands, this yielded a success rate of 86%, as confirmed by BLI. This is a notable improvement compared to our previous experience using 27 G needles, which resulted in an approximate success rate of 60%, primarily due to incomplete bevel insertion and consequential leakage of the injectate upon injection.

An notable property of the 29 G needle is its increased flexibility. While this makes it more prone to bending during deeper insertions, it also permits more delicate and precise navigation within the myocardium. To minimize deflection of the needle during insertion into the thoracic cavity, Operator B can perform the injection while Operator A simultaneously stabilizes the external portion of the needle and maintains its visualization. With appropriate coordination, this approach enables real-time adjustments without requiring needle withdrawal and reinsertion, thereby enhancing procedural efficiency, minimizing tissue trauma, and improving reproducibility.

Handling the injection needle demands particular attention. The 22 G guide needle provides stability when advancing the 29 G injection needle through the thoracic wall and into the myocardium. During syringe exchange, care must be taken to prevent inadvertent puncture of the heart. After injection, the needle should be withdrawn slowly over a period of approximately 10 seconds to minimize backflow of the injectate along the needle track. For operators in training, incorporating Color Doppler imaging to monitor for hemorrhage and

bioluminescence imaging to confirm injectate deposition can be invaluable in optimizing technique and building procedural confidence.

Protocol limitations and challenges

Certain model-specific factors impose limitations on the application of EGI. In acute MI models, for instance, thoracic sutures and post-surgical tissue remodeling can complicate transducer positioning and obscure visualization of the infarct zone. Chronic MI models present a different set of obstacles: progressive wall thinning restricts injection to a few viable regions, typically the anterolateral, anterior, and anteroseptal segments. It is important to recognize that not all desired injection sites will be accessible, especially when multiple injections are planned. Performing multiple injections in a single session further increases the procedural complexity. Each injection requires re-insertion of the guide needle, increasing the risk of collateral damage to adjacent structures, such as the lungs or coronary vessels. Hence, performing multiple simultaneous injections in a single procedure is demanding for the animal and requires considerable expertise from the operators. Nonetheless, this method does allow for serial injections across separate sessions, within the same animal. Thereby, it is highly applicable for investigating therapeutic agents for myocardial repair in longitudinal studies and chronic MI settings.

Lastly, it is important to note that mastering the EGI technique requires substantial practice. The procedure is initially time-consuming and associated with a steep learning curve. However, with systematic training and adherence to critical precautions, EGI becomes a reliable and reproducible method for targeted delivery of therapeutic agents or cells into the myocardium.

Clinical relevance

While the refined EGI procedure described here is not directly applicable to human patients, it models key anatomical and mechanical constraints encountered during clinical delivery of therapeutic agents for ischemic cardiomyopathy, particularly in pre-clinical models. In the clinical setting, transendocardial injections are typically performed using catheter-based systems under imaging guidance or electromechanical mapping[18,19]. In this context, non-invasive intramyocardial injection is the closest feasible approach in rodent models, as catheter-based methods are not practical in rodents[20]. Despite these differences, both clinical and preclinical strategies face common challenges, notably, targeting the peri-infarct region and navigating reduced myocardial wall thickness. The use of a 29 G needle enables injection into peri-infarct regions while respecting anatomical boundaries imposed by wall thinning. Thus, this protocol provides a standardized and reproducible platform to support the development and preclinical evaluation of novel strategies for cardiac repair intended for clinical translation.

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The authors have no conflicts of interest to disclose.

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