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Corresponding Author:	Simon Pecha University Heart Center Hamburg Hamburg, GERMANY
Corresponding Author's Institution:	University Heart Center Hamburg
Corresponding Author E-Mail:	s.pecha@uke.de
Order of Authors:	Liesa Castro Birgit Geertz Marina Reinsch Bülent Aksehirlioglu Arne Hansen Thomas Eschenhagen Hermann Reichensperner Florian Weinberger Simon Pecha
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TITLE:

Implantation of hiPSC Derived Cardiac-Muscle Patches after Myocardial Injury in a Guinea Pig Model

AUTHORS AND AFFILIATIONS:

Liesa Castro^{1,2}, Birgit Geertz³, Marina Reinsch^{2,3}, Bülent Aksehirlioglu³, Arne Hansen^{2,3}, Thomas Eschenhagen^{2,3}, Hermann Reichenspurner^{1,2}, *Florian Weinberger^{2,3}, *Simon Pecha^{1,2}

¹Department of Cardiovascular Surgery, University Heart Center Hamburg, Germany

²German Centre for Cardiovascular Research (DZHK), partner site Hamburg/
Kiel/Lübeck, Germany

³Department of Experimental Pharmacology and Toxicology, Cardiovascular Research
Center, University Medical Center Hamburg-Eppendorf, Germany

* These authors contributed equally

Email Address of Corresponding Author:

Simon Pecha (s.pecha@uke.de)

Email Addresses of Co-Authors:

Liesa Castro (l.castro@uke.de)

Birgit Geertz (b.geertz@uke.de)

Marina Reinsch (m.reinsch@uke.de)

Bülent Aksehirlioglu (b.aksehirlioglu@uke.de)

Arne Hansen (a.hansen@uke.de)

Thomas Eschenhagen (t.eschenhagen@uke.de)

Hermann Reichenspurner (reichenspurner@uke.de)

Florian Weinberger (f.weinberger@uke.de)

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Engineered heart tissue, cardiomyocytes, cryoinjury, guinea pig, cardiac muscle patch, iPS-cells

SUMMARY

Here we present a protocol for the induction of left ventricular cryoinjury followed by the implantation of a cardiac muscle patch, derived from human iPS-cell cardiomyocytes in a guinea pig model.

ABSTRACT

Due to the limited regeneration capacity of the heart in adult mammals, myocardial infarction results in an irreversible loss of cardiomyocytes. This loss of relevant amounts of heart muscle mass can lead to the heart failure. Besides heart transplantation, there is no curative treatment option for the end-stage heart failure. In times of organ donor shortage, organ independent treatment modalities are needed. Left-ventricular assist devices are a promising therapy option, however, especially as destination therapy, limited by its side-effects like stroke, infections and bleedings. In recent years, several cardiac repair strategies including stem cell injection, cardiac progenitors or myocardial tissue engineering have been investigated. Recent improvements in cell biology allow for the differentiation of large

amounts of cardiomyocytes derived from human induced pluripotent stem cells (iPSC). One of the cardiac repair strategies currently under evaluation is to transplant artificial heart tissue. Engineered heart tissue (EHT) is a three-dimensional *in-vitro* created cardiomyocyte network, with functional properties of native heart tissue. We have created EHT-patches from hiPSC derived cardiomyocytes. Here we present a protocol for the induction of left ventricular myocardial cryoinjury in a guinea pig, followed by implantation of hiPSC derived EHT on the left ventricular wall.

INTRODUCTION

The number of patients with heart failure is increasing in our aging population. For end-stage heart failure, orthotopic heart transplantation is the only curative treatment option. However, especially in European countries, there is an increasing organ donor shortage. Therefore, alternative treatment options are necessary. Recent achievements in mechanical circulatory support are promising, but especially in the long-term run, limited by its side effects like bleeding, pump thrombosis and infectious complications¹.

The endogenous regeneration capacity of the adult human heart is extremely limited. Therefore, cardiac regeneration therapies might become an alternative treatment option for end-stage heart failure patients^{2,3}. Different techniques including stem cell-based cell injection or tissue engineering approaches have been described³⁻⁵.

Human induced pluripotent stem cells (hiPSC), as well as human embryonic stem cells (hESC) can be effectively differentiated to spontaneously beating human cardiomyocytes⁶, which has been a major achievement in the field of cardiac regenerative therapies.

To replace myocardium after a myocardial infarction and to improve the function of a failing heart, survival of a suitable number of cardiomyocytes and their mechanical and electrical coupling with the native heart is essential. To investigate the potential of cardiac regenerative therapies with human iPS cell derived cardiomyocytes, a suitable research model is needed. The ideal model should be cost-effective and have a human-like physiology and electrophysiology. Large animal models like pigs would be ideal from that point of view, however, those experiments are very expensive and large amounts of cardiomyocytes would be necessary to replace a relevant number of cardiomyocytes in order to see effects on the left ventricular function in a pig infarction model.

To answer elementary biological questions towards human cell-based cardiac regeneration, e.g., cell survival, vascularization, and electrical coupling, small animal models are more suitable. From the available small animal models, the guinea pig is the most useful species, as compared to rats and mice, as their electrophysiology more closely resembles the situation in humans⁷. In this guinea pig model, we induced a transmural cryoinjury of the left ventricle. One week after induction of myocardial infarction implantation of a three-dimensional, spontaneously beating hiPS-cell derived cardiomyocyte patch was performed. Cardiomyocyte cell survival was evaluated 28 days after implantation by histological examination.

PROTOCOL

Animals received humane care in compliance with the Guide for the Principles of Laboratory Animals, prepared by the Institute of Laboratory Animal Resources, and published by the National Institutes of Health. All animal protocols were approved by the responsible local

authority ("Amt für Gesundheit und Verbraucherschutz, Hansestadt Hamburg"/ Animal protocol # 109/16).

1. Obtain Animals

1.1. Commercially obtain female Guinea pigs weighing 500 - 600 g.

1.2. House them under conventional conditions in animal cages. Feed standard rat chow and autoclaved water *ad libitum*.

2. Transthoracic Echocardiography

2.1. Place the guinea pig in an induction chamber and anesthetize the animal with isoflurane (2-3%). Check the depth of anesthesia by lack of response to the toe-pinch.

2.2. Place the anesthetized Guinea pig on a warming platform (40 - 42 °C) in a supine position. Continue anesthesia via a nose cone (isoflurane 1.5-2%)

2.3. Shave and depilate the guinea pig's thorax using an electric animal hair shaver.

2.4. Apply prewarmed (approx. 25 °C) ultrasound transducer gel. Use an echocardiography system that is equipped with a transducer frequency higher than 15 MHz.

2.5. Acquire two-dimensional parasternal long axis views by placing the transducer on the guinea's pig thorax facing from the right neck towards the left leg and record long axis B-mode images at the plane of the aortic valve with a concurrent visualization of the LV apex. Investigate the pre-operative left-ventricular function.

2.6. Turn the transducer by 90° degrees to obtain a short axis B-mode view at the mid-papillary level.

Note: The animal is then immediately transferred to the OR table. The animal is continuously anesthetized with 3% isoflurane. 0.05 mg/kg atropine (i.m.) is injected to avoid increased bronchial secretion during mechanical ventilation.

3. Surgery

3.1. Induction of Myocardial infarction

3.1.1. Inject 4-5 mg/kg carprofen and 0.05 mg/kg buprenorphine subcutaneously with a 21 G needle and a 10 mL syringe.

3.1.2. Place the guinea pig on its back and keep anesthesia with a facemask covering mouth and nose. Check the depth of anesthesia by pinching the hind feet (lack of pedal reflex).

3.1.3. Spread the guinea pigs' legs and fix the position using tape.

3.1.4. Shave the chest and the tracheal region of the anesthetized animal with an electric

145 shaver. Disinfect the area widely using iodine-based scrub, followed by 80% ethanol. Repeat
146 this disinfection steps twice.

147
148 3.1.5. Perform a 1.5 cm vertical incision in the tracheal area and bluntly dissect the muscles
149 covering the trachea until you see the trachea. Puncture the trachea with 18 G i.v. cannula
150 and insert the flexible part of the cannula as a tracheal tube.

151
152 3.1.6. Connect the tracheal tube to an animal respirator to continuously ventilate the guinea
153 pig during the procedure.

154
155 Note: Now the anesthesia is maintained with isoflurane 3% via the tracheal tube (inspiration
156 assisted ventilation with maximum inspiration pressure, respiration rate: 100-120/min, peak
157 inspiration pressure: 18-22 cm Hg using PEEP-ventilation while the chest is open).

158
159 3.1.7. Identify the 5th intercostal space by counting the rib spaces beginning at the first
160 intercostal space. Perform a 2 cm horizontal incision on the 5th intercostal space on the left
161 side of the guinea pig using scissors and a tweezer. Insert a small animal retractor. Dissect the
162 muscles with an electrocautery until the intercostal muscles are reached, which can be seen
163 after removing the subcutaneous tissue, are reached.

164
165 3.1.8. Gently dissect the intercostal muscles with tweezers until the pleural space is reached
166 and one can see the left lung in front. Insert the retractor between the ribs and open it
167 carefully until a good view of the heart is obtained.

168
169 3.1.9. Open the pericardium approximately 1 cm in the region of the anterior left ventricular
170 wall with scissors. Place a compress on the left lung to protect it from damage when inducing
171 the cryoinjury of the left ventricle.

172
173 3.1.10. Place the tip of an eroded metal stamp with a cross-sectional diameter of 0.5 cm into
174 liquid nitrogen for 3 min.

175
176 3.1.11. Press the nitrogen cooled probe onto the left anterior wall of the heart for 30 s. Then
177 separate it from the heart using an electric soldering iron (200 °C) which is placed inside the
178 stamp to warm it up. Repeat this procedure 3 times to obtain a transmural myocardial injury.
179 Observe the blanching of the myocardium.

180
181 3.1.12. Inflate the lungs with maximum pressure (by clamping the outflow tube of the
182 ventilator for 2 s), to avoid atelectasis of the lung. Remove the retractor from the intercostal
183 space.

184
185 3.1.13 Close the ribs with two 3-0 sutures. Close the muscles over the ribs with a 4-0 running
186 suture. For closure of the skin use 5-0 suture single stitches.

187
188 3.1.14. Reduce the isoflurane to 1%. When the animal is breathing spontaneously, remove the
189 tracheal tube, and continue anesthesia with a facemask (isoflurane 2-3%).

190
191 3.1.15. Assure the absence of reflexes by pinching the hind limb to monitor sufficient depth
192 of anesthesia. Then use a single 8-0 suture to close the puncture site at the trachea. Close the

wound with three single stitch 4-0 sutures.

3.1.16. Use buprenorphine (0.05 mg/kg per 12 h) and carprofen (5 mg/kg per 24 h) for pain medication for the following 5 days.

3.2. Implantation of EHT (7 days after cryoinjury)

3.2.1. Place the guinea pig in an induction chamber and anesthetize the animal with isoflurane. Check the depth of anesthesia by the lack of response to the toe-pinch.

3.2.2 Inject 4-5 mg/kg carprofen and 0.05 mg/kg buprenorphine subcutaneously with a 21 G needle and a 10 mL syringe after induction of anesthesia. Place the guinea pig on its back and keep anesthesia with a facemask covering mouth and nose.

Note: Sufficiency of anesthesia should be checked by pinching the hind feet.

3.2.3. Spread the guinea pig's legs and fix the position using tape.

3.2.4. Perform the pre-surgical preparations as described in step 3.1.4-3.1.6. Perform a 2 cm horizontal skin incision in the scar area of the left lateral side using scissors and tweezers.

3.2.5. Gently dissect the extrapleural adhesions using an electrocautery. Carefully open the pleural space with scissors. Insert a rib spreader to expose the heart.

3.2.6. Visually identify the region of the infarction by its pale color in comparison to the healthy surrounding myocardium. Place the engineered heart tissue patch over the infarction region.

3.2.7. Secure it with two 8-0 sutures at both sides. Make sure to secure the patch in the non-infarcted area of the heart (**Figure 1**). Inflate the lungs with pressure, to avoid atelectasis of the lung. Remove the retractor from the intercostal space.

3.2.8. Close the ribs with two 3-0 sutures. Close the muscles over the ribs with a 4-0 running suture. For closure of the skin use 5-0 suture single stitches.

3.2.9. Reduce the isoflurane to 1%. When the animal is breathing spontaneously, remove the tracheal tube, and continue anesthesia with a facemask (isoflurane 2-3%).

3.2.10. Assure the absence of reflexes by pinching the hind limb to monitor sufficient depth of anesthesia. Then use a single 8-0 suture to close the puncture site at the trachea. Close the wound with three single stitch 4-0 sutures.

3.2.11. Use buprenorphine (0.05 mg/kg per 12 h) and carprofen (5 mg/kg per 24 h) for pain medication for the following 5 days.

3.2.12. Four weeks after EHT implantation perform a transthoracic echocardiography (as described in step 2) to monitor LV function over time.

NOTE: The LV function is monitored before EHT implantation and 4 weeks after implantation to monitor for improvement of cardiac function by EHT implantation

3.2.13. Euthanize the animal which IACUC approved protocol and surgically explant the heart for further analysis.

RESULTS

This guinea pig model is a suitable model to investigate cardiac regeneration after implantation of hiPSC derived EHT-patches. It reproducibly leads to large transmural myocardial injuries. Scar size is evaluated by histology four weeks after cryoinjury. Mason trichrome staining reveals large transmural scars (**Figure 2**). Scar size was similar over a large number of injured animals reflecting a high degree of reproducibility⁸. On average 25% of the left ventricular myocardium was replaced by scar tissue. Dystrophin staining demonstrated large myocardial grafts that partially remuscularized the scar (**Figure 3a**). Staining for human Ku80 proved the human origin of this newly formed myocardium (**Figure 3b**). Higher magnification revealed that these grafts consisted of densely packed myocytes. Left ventricular function was monitored using transthoracic echocardiography. A successful EHT implantation is characterized by an improvement in LVEF, Fractional area shortening, and a decrease in Left ventricular end-diastolic diameter (**Figure 4**).

FIGURE LEGEND

Figure 1: hEHT-patches after implantation. (A) Patch immediately after implantation. (B) Patch four weeks after implantation.

Figure 2: Determination of infarction on Masson's trichrome stained paraffin section 28 days after cryoinjury. The infarction is determined by the blue color in Masson's trichrome staining, representing fibrotic tissue. Scale bar 2mm.

Figure 3: Staining with Dystrophin and Human Ku80. (A) Dystrophin staining shows large muscle islands (brown) in the scar area (B) Ku-Staining of paraffin sectioned heart. Ku-80 is a human-specific antibody. Therefore, the positively stained cells (brown color) are from human origin.

Figure 4: M-Mode measurement of the left ventricle using transthoracic echocardiography. (A) at baseline and (B) one week after cryoinjury. (IVS: Intraventricular septum, LV: Left ventricle, PW: Posterior wall). Measurements are performed 3 times. At baseline, 1 week after cryoinfarction and 4 weeks after EHT implantation

DISCUSSION

A variety of small animal models are available to study the effect that cell transplantation exerts on injured hearts⁹⁻¹¹. We chose a guinea pig model because of all small animal models its (electro)physiology resembles most closely that of humans. The advantages of small animal models are simple housing, manageable costs, and few workforces. In comparison with mice and rats, guinea pigs' cardiac (electro)physiology is more similar to humans. In particular heart frequency is lower (~250/min), ventricular action potential shows a plateau phase and the potassium current I_{Kr} is important for repolarization⁷. Furthermore, the larger size of the animals facilitates surgeries, which is especially important as a re-thoracotomy is required one

week after the induction of injury. However, guinea pigs are sensitive to anesthesia requiring some experience.

Shiba *et al.*, described the use of 50 mg/kg ketamine and 2 mg/kg xylazine (i.p.) to anesthetize guinea pigs for cardiac cryoinjury⁴. We have also evaluated this anesthesia regiment. One of the disadvantages is the fact that it is impossible to suspend the narcotic effect of xylazine and ketamine due to missing potent antidotes. Considering that guinea pigs are at high risk of hypoglycemia during surgical procedures, we decided to use a narcotic gas whose effect is easy to control and therefore leading to a very short anesthetic recovery phase. A tracheotomy is used to ensure a safe ventilation throughout the procedure. An oro-tracheal intubation is technically very demanding in guinea pigs, as the visualization of the opening of the trachea is often difficult and a safe intubation cannot be guaranteed.

Although cryo-injury induces a severe myocardial injury it does not precisely mirror a coronary artery disease/ myocardial infarction in humans. In this respect, the ligation of the LAD or even an ischemia-reperfusion injury model more closely recapitulates the human pathophysiology. However, the coronary artery anatomy in guinea-pigs is characterized by an extensive collateralization. Therefore, a ligation of the left anterior descending coronary artery does not reproducibly result in large myocardial injuries in guinea pigs^{12,13}. LAD-ligation in mice and rats leads to an extensive remodeling and eventually thinning of the anterior wall, a characteristic that is not seen in humans to this extent. The guinea-pig cryo-injury model induces remodeling processes and thinning of the ventricular wall, but to an extent, that more closely resembles a myocardial injury seen in humans. Therefore, a cryoinjury model resulting in a more reliable myocardial injury was chosen¹³. Periprocedural mortality is a major problem with this model. Initially, about 30% of the animals died during the first operation or within 7 days after cardiac injury. Another 30% died during EHT implantation accounting for an overall mortality of 60%. Several modifications lead to a significant decrease in mortality. Isoflurane induces heavy salivation and bronchial secretion. Atropine reduces bronchial secretion and was a major factor to decrease mortality. The second factor that heavily decreased mortality is the use of a soldering iron to release the cold metal stamp from the heart. Warm water was initially used to detach the stamp from the myocardium. However, it was difficult to remove the water from the thoracic cavity. Remaining water negatively influenced respiration and lead to the development of adhesion that worsened the animal's prognosis. Another modification that positively influenced the procedure was the protection of the left lung from the cold metal stamp with a compress. After these modifications were implemented mortality dropped to ~20% for the cardiac injury operation (including the first postoperative week) and another 25 % for EHT transplantation. Induction of cryo injury represents the technically easier operation and consistent results can be achieved after 5-10 animals have been operated under supervision. EHT-transplantation is more demanding and about 20 surgeries under supervision are recommended until reproducible results can be obtained.

In the beginning, we used warm saline to rinse the heart and, therefore, detach the frozen probe from the left ventricular wall. This procedure is easier to handle but a few milliliters of saline remain in the thorax increasing the risk of respiratory failure.

In conclusion, the depicted guinea pig model is reproducible, inexpensive and leads to consistent results. Transthoracic echocardiography offers a feasible and non-invasive tool to

evaluate the presence of cardiac injury before re-operation and graft-implantation is performed.

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DISCLOSURES

None of the authors has competing financial interests or other conflicts of interest to declare.

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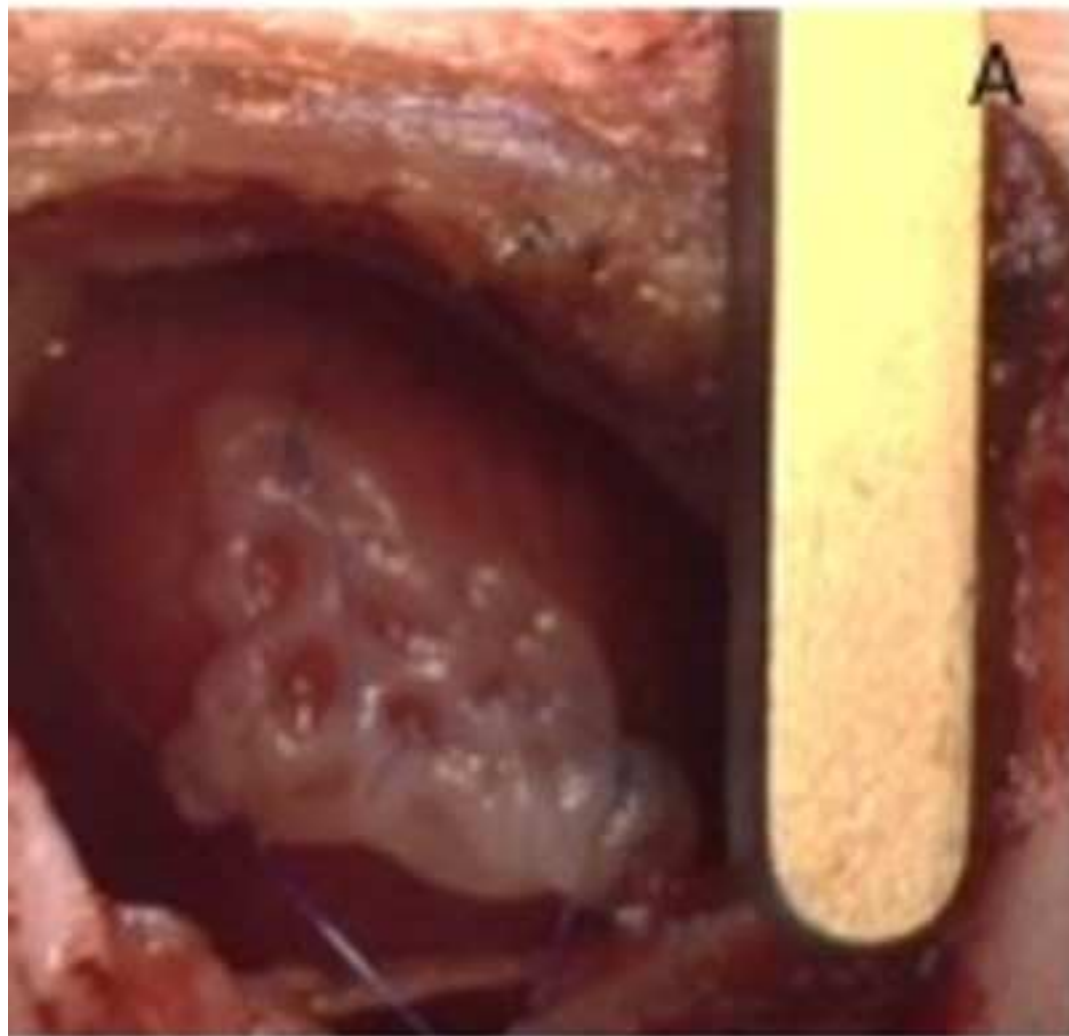
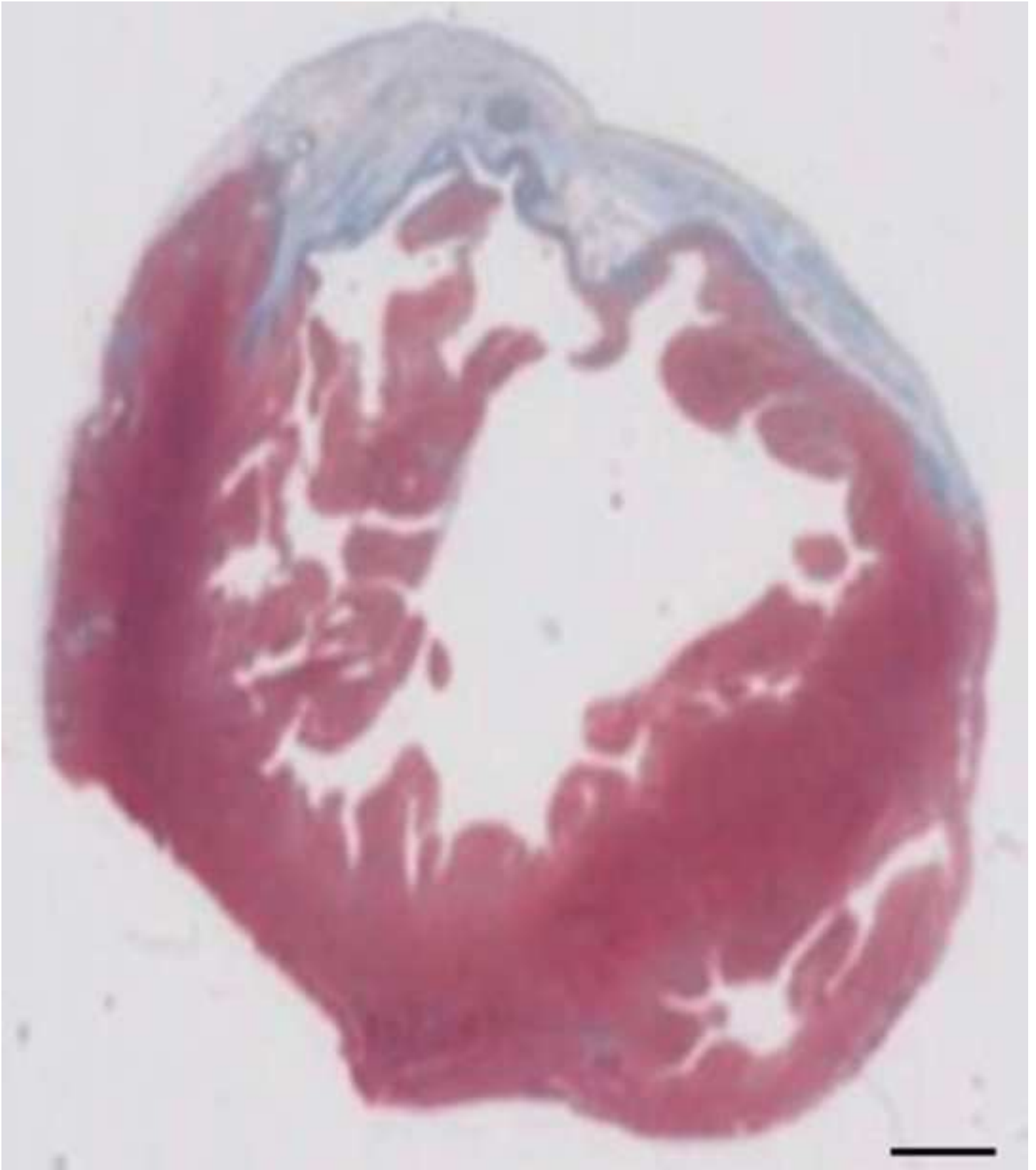


Figure 2

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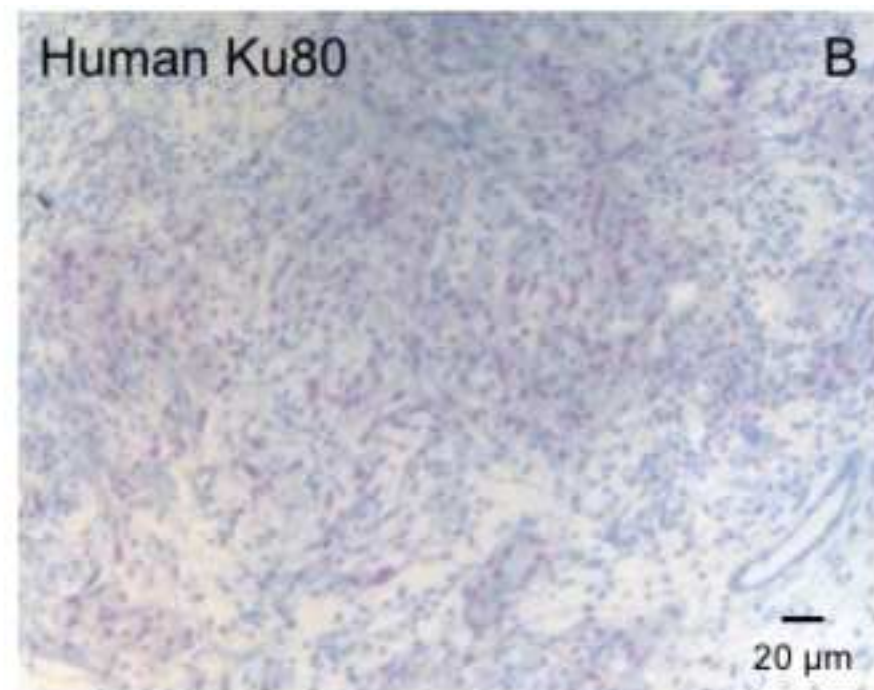
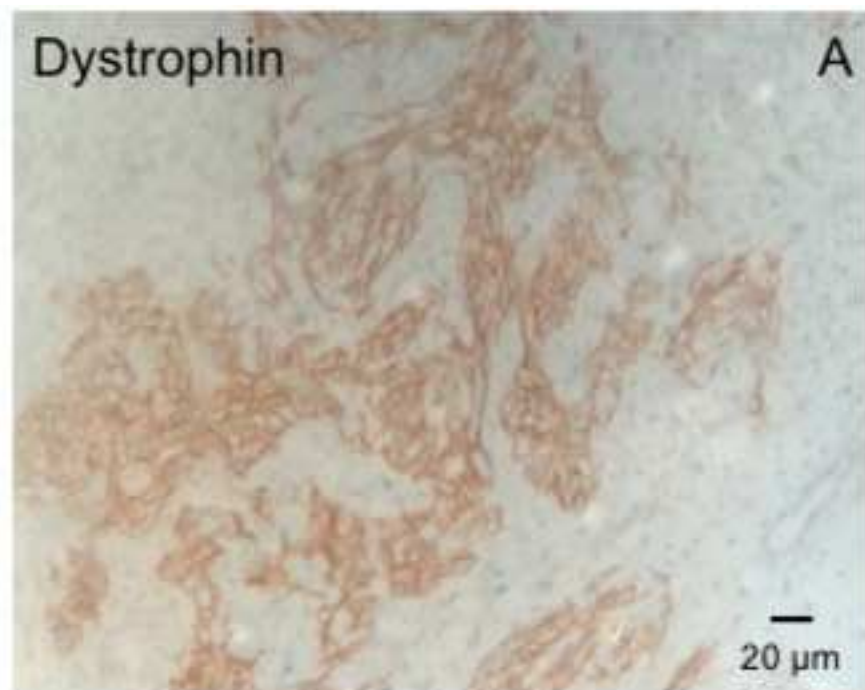
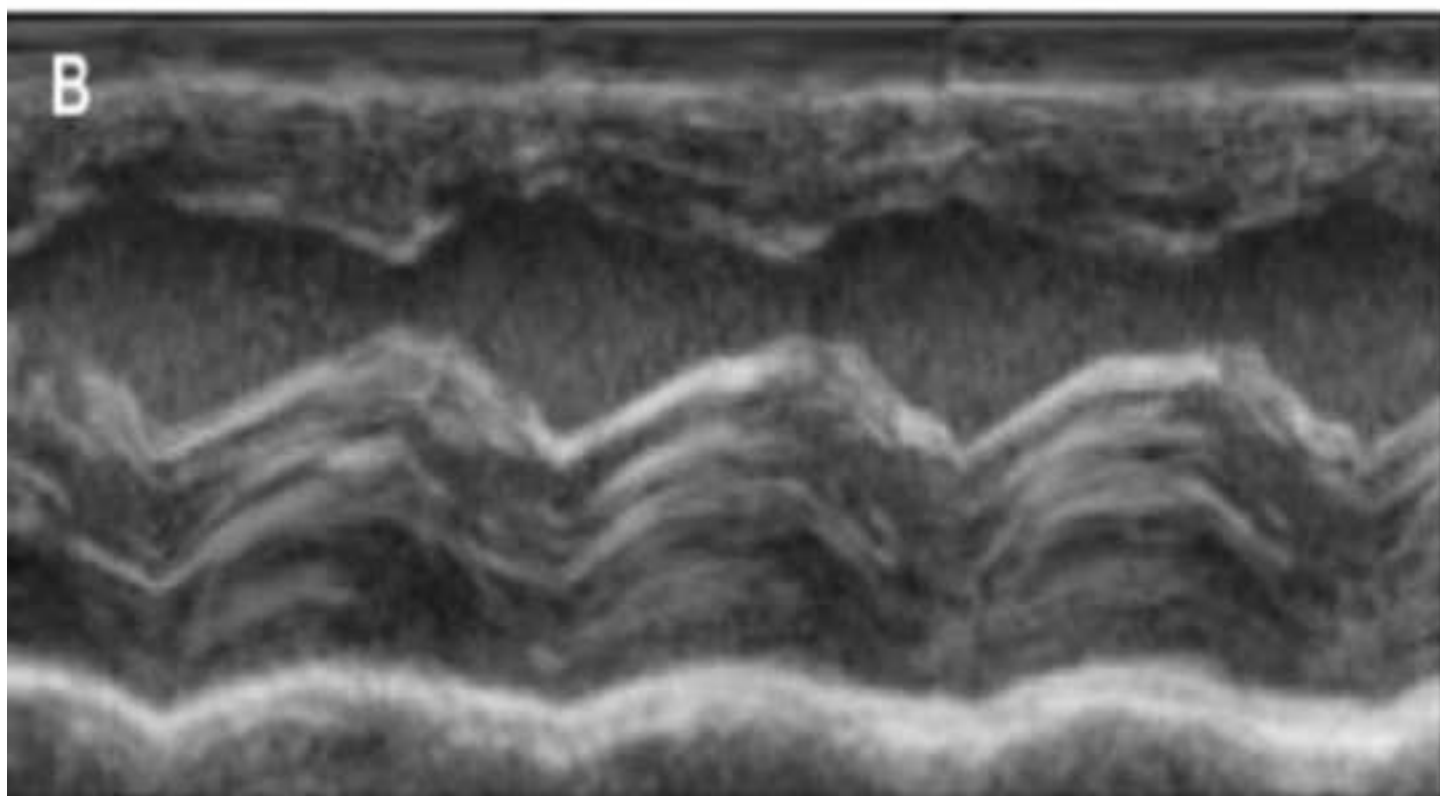
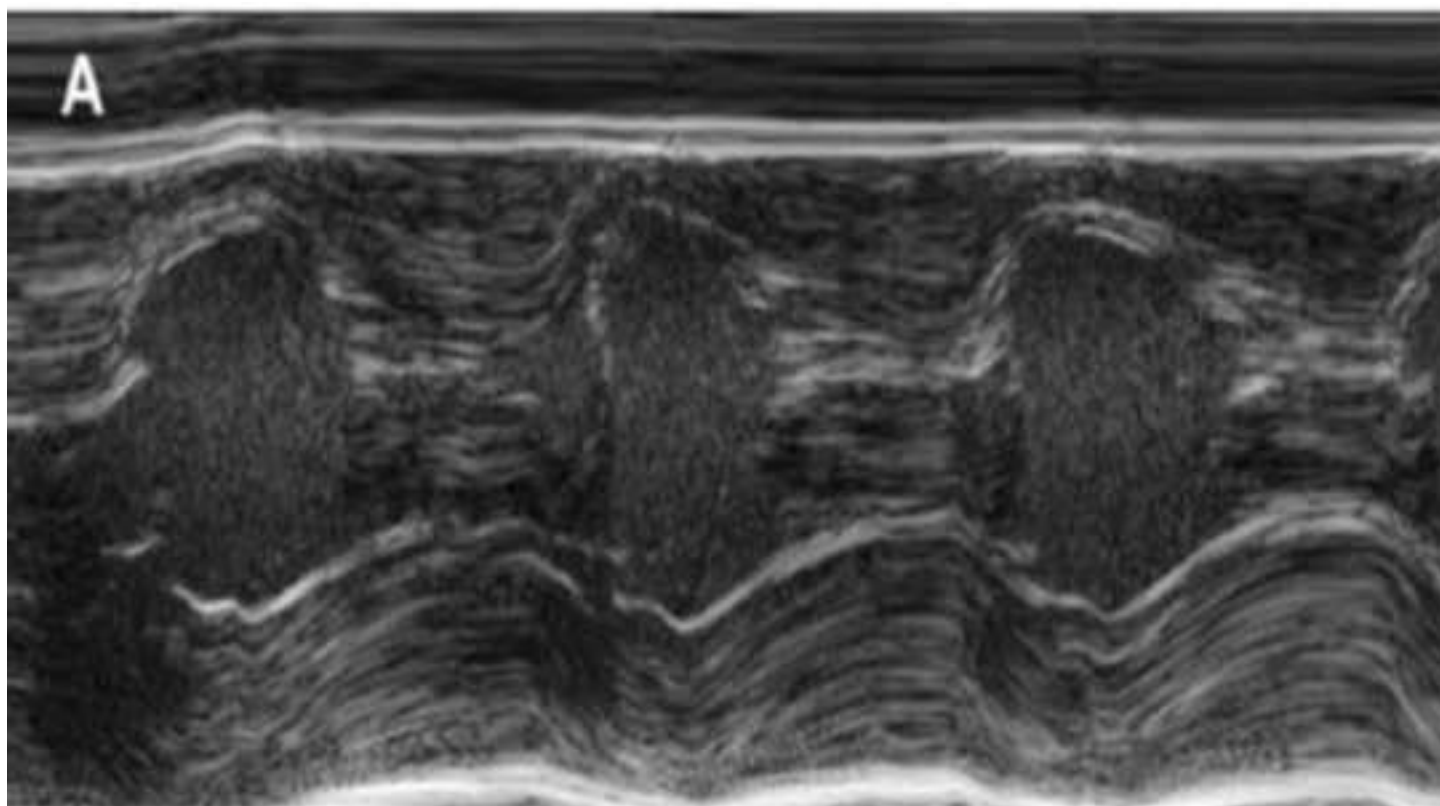


Figure 4



Name of Material/ Equipment	Company	Catalog Number
Ventilator (VetFlo Dual Mode)	Kent Scientific	
Forene	abbvie	1000009819
Carprofen	Zoetis	256692
Atropin	Braun	PZN 00648037
Buprenorphin	Sigma	
Metal stamp		
Electric soldering iron	Claytools	
3-0 prolene suture	Ethicon	
4-0 prolene suture	Ethicon	662SLH
5-0 prolene suture	Ethicon	8710H
8-0 prolene suture	Ethicon	8841H
Tungsten Carbide Scissor	FST	No. 14568-12
Stainless sterilization Container	FST	No. 20890-51
Graefe Forceps	FST	No.11652-10
Extra fine Graefe Forceps	FST	No.11150-10
Forceps	FST	No. 11022-15
Halsted- Mosquito	FST	No. 13009-12
Forceps	FST	No.13003-10
Baby Mixer	FST	No. 13013-14
Needle holder (Castroviejo with Tungsten Carbide Jaw)	FST	No. 12565-14
Needle Holder (Halsey)	FST	No. 12501-13
Alm Retractor with Blunt Teeth	FST	No. 17008-07
Spring Scissor	FST	No. 15000-00
Compress 5x5	Fink + Walter	PZN 08821417
Venflon Pro Safety	Becton Dickinson	PZN11123964
Cautery High Temp 2"	Bovie Medical Corporation	0100607151011055

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CORRESPONDING AUTHOR:

Name: Dr. Simon Pecha
Department: Cardiovascular Surgery
Institution: University Heart Center Hamburg
Article Title: MD
Signature: [Signature] Date: 16.07.18

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Dear Prof. Singh,

attached please find the revised version of our manuscript entitled "**Implantation of hiPSC derived cardiac-muscle patches after myocardial injury in a guinea pig model**".

The reviewers comments have been very helpful in improving the manuscript. Please find attached a revised version with the changes highlighted in yellow.

Editor: Converted to a note please check.

Answer: We agree with the Editor that this should be a note.

Editor: Please provide a figure for this. This is important

Answer: We have now provided a figure showing Dystrophin, as well as Ku80 Staining

Editor: Yes, monitoring is fine... please describe your observation as well.

Answer: A successful EHT implantation is characterized by an improvement in LVEF, Fractional area shortening, and a decrease in Left ventricular end-diastolic diameter.

Editor: Please expand on what is being observed with the staining. Please provide a scale bar in the figure and describe it in the figure legend.

Answer: The infarction is determined by the blue color in Masson's trichrome staining, representing fibrotic tissue. Scale bar 2mm.

Sincerely

Simon Pecha