Online Supplement

Materials and Methods

Animals were either treated immediately following creation of complete AV block through radiofrequency ablation (RFA) and followed for a period of 2 months (Group A), or were treated 1 month following AV node RFA in an effort to recover myocardial function from the detrimental effects of long term RV pacing[1] (Group B). In the long term follow up group A, 10 female Yorkshire-SPF farm pigs (35.8±1.2kg) were used. Animals were randomized to receive either Ad.TBX18 (n=6) or Ad.GFP (n=4) by a minimally invasive delivery system previously described by us[2, 3]. Like group A, in group B, 10 female Yorkshire-SPF farm pigs (36.4±1.4kgs) were randomized to receive either Ad.TBX18 (n=6), or phosphate buffered saline (PBS) (n=4) as control **(Figure 1A**). The sample size was estimated to achieve a power of 0.8 and a p value of 0.05 using a two-tailed t test. Different physiological variables were evaluated including local and systemic safety profiles.

Large animal model of PICM and gene delivery.

All experiments were approved by the Cedars-Sinai Institutional Animal Care and Use Committee and performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Animals were premedicated with intramuscular acepromazine (0.25 mg/kg), atropine (0.05 mg/kg), and ketamine (20 mg/kg). Anesthesia was induced with intravenous Propofol (4 to 6 mg/kg), followed by intubation and maintenance on isoflurane during positive pressure ventilation. A baseline 12-lead electrocardiogram (ECG) was recorded. The right internal jugular vein was cannulated by open cut-down, and blood was sampled through a venous catheter before any drug infusion or experimental manipulation. A

ventricular pacemaker lead was advanced and positioned under fluoroscopic guidance into the RV apex. A single-chamber electronic pacemaker (St. Jude Medical) was inserted and the pacemaker was placed in a submandibular pocket. The backup pacemaker was programmed in ventricular demand pacing (VVI) mode at a rate of 50 beats/min. RFA was performed at the atrioventricular nodal region to achieve complete heart block using a non-irrigated RF ablation catheter (Boston Scientific). Complete AV block was confirmed by electronic pacemaker dependence immediately following ablation (VVI at 50 beats/min) and persistent AV dissociation for 30 minutes. During ablation, 2% lidocaine was administered intravenously (IV) (33 µg/kg/min) temporarily to prevent ventricular tachyarrhythmias. Ad.TBX18 or Ad.GFP was delivered into the high RV septum using a NOGA Myostar™ injection catheter[2, 4] (200ul per injection, 5 injections total). Before the injections, contrast was used (Omnipaque™) to confirm retention of the needle within the myocardium. Telemetry devices (Data Sciences International) were implanted in the left caudal neck area in the suprascapular region as to not interfere with the range of motion of the neck or shoulder. Subcutaneous leads from the telemetry device were placed near the heart base at the level of the xyphoid process with a distal lead advanced to the anterior chest near the LV apex. Animals in protocol A were monitored by implanted telemetry continuously for 8 weeks. In protocol B**,** animals underwent the same procedures utilizing the same techniques as described above. Similarly, protocol B animals received an electronic pacemaker followed by AV node ablation. Animals became pacemaker dependent and were back-up paced at 50 beats/min for the entire 8-week protocol. During week 4, animals were injected with either Ad.TBX18 or PBS (1ml). Following injection, a telemetry device was implanted and the animals were followed for an additional 4 weeks. In protocols A and B, animals received an MRI exam at 4 and 8 weeks post AV block at 8 weeks post AV block in

both protocols, a non-survival surgery was conducted. At endpoint, invasive hemodynamic studies were performed using a Swan-Ganz catheter at baseline, and following infusion of 8µg/kg/min of isoproterenol for 10 minutes. After waiting for the isoproterenol washout (10 minutes)., hemodynamic measurements were made after a fluid challenge (1000cc bolus over 10 mins) of normal saline. One animal from each experimental group(A and B) were excluded from the study due to device related infection with systemic symptoms requiring euthanasia.

Adenovirus production, and purification.

Human TBX18 was sub cloned into an expression vector containing ZsGreen1 (referred in the manuscript as GFP for clarity) as previously described[2, 5]. The expression vector was then inserted into an adenoviral vector backbone (Invitrogen) to create the final adenoviral expression construct, pAd-CMV-TBX18-IRES-GFP. An adenoviral vector expressing GFP alone under the cytomegalovirus (CMV) promoter (Ad.GFP) was used as control. A total of 5.0×10^{11} viral particles of each vector (Ad.GFP and Ad.TBX18) in the buffer (Puresyn Inc.) were injected into each animal.

Continuous ECG and activity recordings.

Continuous digital ECG and physical activity recordings were performed and analyzed at a sampling frequency of 500Hz using a Ponemah Physiology Platform (Data Sciences International). Heart rate in each protocol was monitored daily following delivery of TBX18 or control for 14 days, then each animal was evaluated for a 24-hour period once a week for the duration of the study. The telemetry implants included a built-in triaxial accelerometer and ECG

recorders. Single-lead ECG and physical activity data could be recorded simultaneously and subsequently analyzed. Activity data were obtained from a triaxial accelerometer (x, y, and z axes), and the activity value, in arbitrary units (AU) was assessed as previously described[2]. Heart rate and activity data are represented as a summation of the average daily values acquired from implanted telemetry devices from each animal, following injection of TBX18 or control.

Evaluation of electro-mechanical dyssynchrony and global ventricular function by Magnetic Resonance Imaging, ECG, and Swan-Ganz cardiac catheterization.

Electrical dyssynchrony was evaluated by measuring the mean daytime QRS duration of 10 non-paced beats from the implanted telemetry device. QRS duration was measured by averaging 10 consecutive beats acquired at 24 hours post telemetry implantation, and each week following intervention. Additionally, at the endpoint non-survival surgery (2 months following AV block), QRS duration and morphology was evaluated by 12 lead ECG and measured as the mean of 10 beats (EP Workmate™, St. Jude Medical) while animals were under general anesthesia. Cardiac MRI was performed in both protocols (A and B), 4 and 8 weeks following AV node ablation. All studies were done with a 3-Tesla, Siemens Magnetom Verio (Erlangen, Germany). ECG-gated, breath-hold cine MRI sequences in short axis, vertical axis, and horizontal long axis were acquired to assess function. In the short axis view, the LV was completely encompassed by contiguous 6-mm-thick slices beginning just distal to the mitral annulus up to the LV apex. Late-gadolinium-enhanced (LGE) images (i.e., 8-10 min after injection of 0.2 mmol/kg body weight of gadopentate dimeglumine (Magnevist, Bayer Healthcare Pharmaceuticals) were used to evaluate focal areas of fibrosis near the injection site. All MRI studies were analyzed

independently on an offline workstation (CVi42, Circle Cardiovascular Imaging Inc., Calgary, Canada). Global LV function, ejection fraction (LV end-diastolic volume [LVEDV] and LV end-systolic volume [LVESV]) were measured. Chamber volumes were normalized to body surface area BSA ($0.121 \text{ X } BW^{0.575}$). Additionally, the time to peak radial strain was used to evaluate septo-lateral segment differences from 2 septal chords, and 2 chords from the LV free wall using the tissue tracking analysis function of Cvi42. The mean segment differences from 3 mid ventricular short axis slices were utilized for the analysis. Following the endpoint MRI, each animal underwent a diagnostic procedure to evaluate hemodynamics (via Swan Ganz thermodilution catheter), and the extent of autonomic response on animals previously treated with TBX18 compared to those who received PBS as a control. Cardiac function was evaluated through cardiac output (CO); left ventricular end-diastolic pressure, (EDP) were each evaluated under anesthesia at baseline conditions, following infusion with isoproterenol (8ug/kg/min) for 10 minutes and finally after a 1L bolus fluid challenge. After discontinuing isoproterenol the animals were allowed to recover for 15 minutes until a baseline heart rate was achieved.

Immunohistochemistry, confocal microscopy

Frozen sections of heart tissue (2 months after adenoviral transduction) were fixed with 4% paraformaldehyde and permeabilized with 0.1% TritonX-100 and then incubated with the appropriate primary antibody—sarcomeric a-actinin (Abcam, ab9465, 1:200), Cx43 polyclonal antibody (Sigma, C6219, 1:100) anti-wheat germ agglutinin antibody (abcam, ab20528), Alexa Fluor–conjugated secondary antibodies (Invitrogen). Counterstaining with DAPI (Molecular Probes) was performed. Sections were imaged using a confocal laser microscope (Leica

Microsystems), and images were processed by Leica LAS software. Cardiomyocyte hypertrophy was quantified by measuring the cell area of 100 representative cells in 10 randomly selected fields at different anatomic locations under 63x. Masson's Trichrome staining (Sigma Aldrich) was used to evaluate fibrosis. (2 months after adenoviral transduction). Image J software was used to evaluate % fibrosis per 10 hpf (63X) per animal. Normally oriented Cx43 was quantified as those cells which were double positive for α-SA and Cx43 per 10 fields.

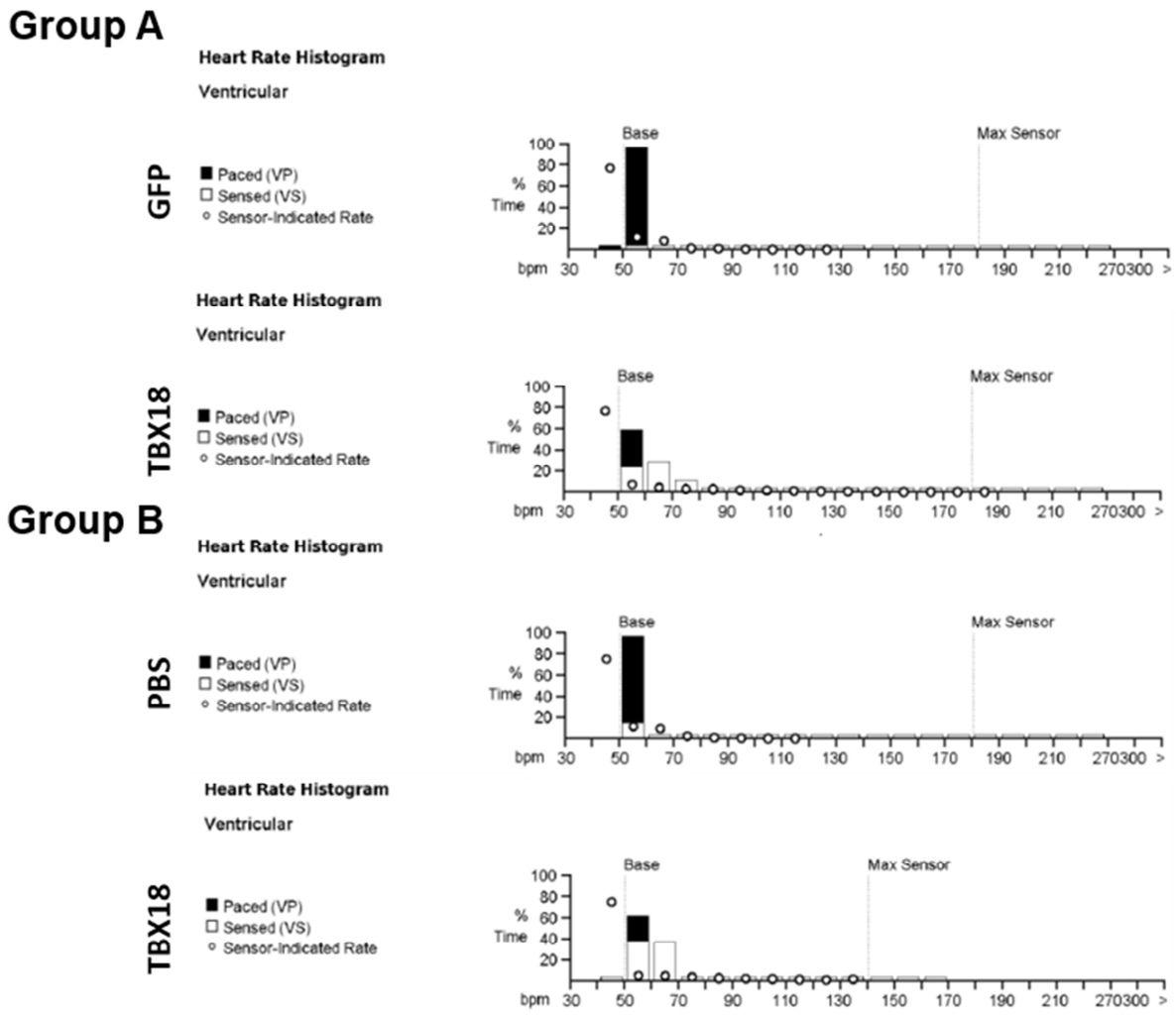
Real-time quantitative PCR

For biodistribution analysis of the adenoviral vector, postmortem heart samples were obtained, including a ~0.5-cm × 0.5-cm endocardial sample at the injection site (identified by the needle tracks) and another from the left ventricular epicardium (remote site). Tissue samples were also obtained from lung, liver, spleen, kidney, and brain. Total DNA was isolated using DNeasy (Qiagen) per the manufacturer's instructions. Real-time PCR primers designed to amplify human adenovirus type 5 genome at E2 region were as follows: forward primer (5′-GGCTAGGACGGGTTACAACA-3′), reverse primer (5′-ACGAGGAGGCACTAAAGCAA-3′), and probe (56-FAM/GACACCCAG/ZEN/CAGAAACCTGT/3IABkFQ). Real-time PCR was performed by using the TaqMan assay and a 7900HT Fast Real-Time PCR System (Applied Biosystems/Life Technologies Corp.) per the manufacturer's recommendations. To perform absolute quantification of viral vector, viral DNA extracted from TBX18 adenoviral vector was serially diluted to create an appropriate standard curve. The viral DNA standard curve, tissue DNA samples, and no template controls were simultaneously expanded and subjected to analysis.

References

1. Dreger H MK, Bondke H, Baumann G, Melzer C. Pacing-induced cardiomyopathy in patients with right ventricular stimulation for 15 years. *Europace*. 2012:238-42.
2. Hu YF, Dawkins JF, Cho HC, Marban E and Cingolani E. Biological pacemaker created by minimally invasive somatic reprogramming in pigs with complete heart block. *Sci Transl Med*. 2014;6:245ra94.
3. Cingolani E YK, Shehata M, Chugh SS, Marban E, Cho HC. Biological pacemaker created by percutatneous gene delivery via venous cathters in a porcine model of complete heart block. *Heart Rhythm*. 2012;9:1310-1318.
4. Garbayo E, Gavira JJ, de Yebenes MG, Pelacho B, Abizanda G, Lana H, Blanco-Prieto MJ and Prosper F. Catheter-based Intramyocardial Injection of FGF1 or NRG1-loaded MPs Improves Cardiac Function in a Preclinical Model of Ischemia-Reperfusion. *Sci Rep*. 2016;6:25932.
5. Kapoor N, Liang W, Marban E and Cho HC. Direct conversion of quiescent cardiomyocytes to pacemaker cells by expression of Tbx18. *Nat Biotechnol*. 2013;31:54-62.

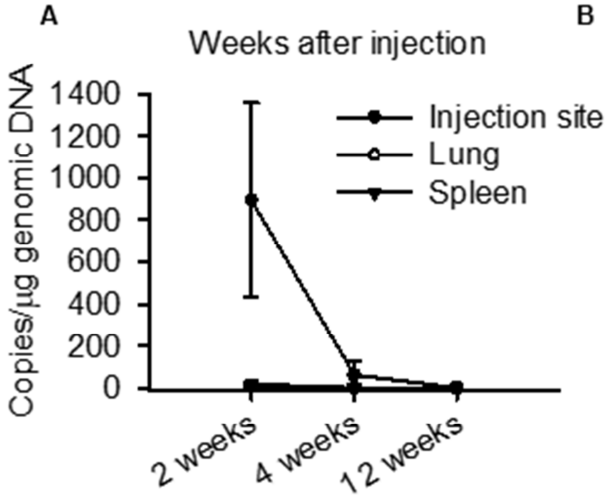
Online figures



Online Figure 1: Reduced backup electronic pacemaker utilization in TBX18 antegrade biological pacemaker-treated animals compared to controls. Top- Group A, representative endpoint electronic pacemaker histogram data showing % pacing in a GFP vs. TBX18 injected pig at 2 months post injection. Bottom-Group B, representative endpoint electronic pacemaker

histogram showing % pacing in PBS vs TBX18 injected pig 1-month post injection, 2 months post av nodal block.

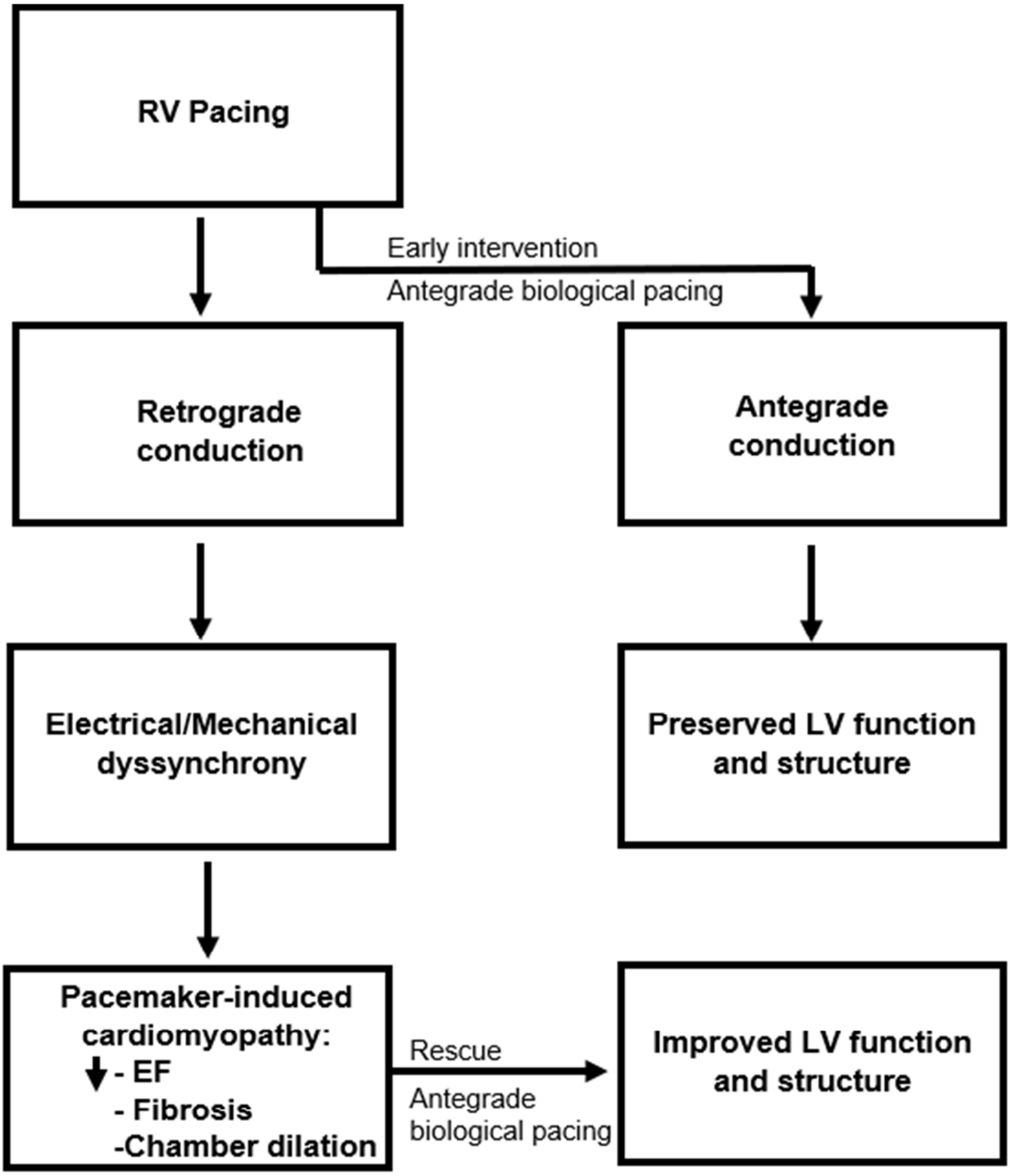
A



B

	Control (n=3)	TBX18 (n=3)
Liver function		
AST (IU/L)	14±1	19±1
ALT (IU/L)	33±3	23±9
Total Bilirubin (mg/dl)	0.1±0.1	0.1±0
GGT (IU/L)	43±6	26±2
Alk Phosphatase	110±28	110±31
Renal function		
BUN (mg/dl)	11±1	9±1
Creatinine (mg/dl)	2±0.1	1.8±0.1
Pancreas		
Amylase (IU/L)	1896±844	1685±601
Cardiac		
Troponin I (ng/ml)	0.03±0.01	0.04±0.04
Hematology		
WBC (10^3/ul)	14±2	11±2
HGB (g/dl)	8.1±0.2	7.7±0.8
Platelet Count (10^3/ul)	314±75	235±15
Lymphocytes (/ul	6731±866	6939±1305
Neutrophils (/ul)	6484±2221	4015±937

Online Figure 2: Systemic biodistribution and serum biochemistry in TBX18-treated and controls. (A) Viral distribution following injection over 8 weeks evaluated at the injection site, lung, and spleen. (B) Serum biochemistry profile at 2 months in control vs TBX18.



Online Figure 3: Schematic diagram outlining the changes seen by right ventricular single chamber pacing, ameliorated by TBX18 antegrade biological pacing.