Supplement Material

Expanded Materials and Methods

Plasmid construction and lentivirus preparation

Lentiviral vectors (LVs) based on the human immunodeficiency virus Type 1 were used throughout this study. The third-generation constructs used in this study include the self-inactivating (SIN) long terminal repeat (LTR), the central polypurine tract (cPPT) and the woodchuck hepatitis virus post-transcriptional regulatory element (Wpre). The four plasmids required for lentivirus vector (LV) production were kindly supplied by Professor Inder Verma from the Salk Institute, San Diego, USA. The cDNAs for mouse Kir2.1 or dominant-negative mutant, Kir2.1AAA,[1] were subcloned into the LV plasmid, pRRLsin18.cPPT.CMV.eGFP.Wpre following the removal of eGFP.[2] A control lentiviral plasmid devoid of a heterologous expression cassette was also constructed to produce empty LVs (LV-Empty). LVs encoding eGFP (LV-eGFP), Kir2.1 (LV-Kir2.1), dominant-negative mutant Kir2.1AAA (LV-Kir2.1AAA) and LV-Empty were produced by calcium-phosphate co-precipitation transfection of the four LV plasmids into human embryonic kidney (HEK) 293T cells as previously described.[2] The supernatant from HEK cell flasks containing LVs was collected 48 and 72 hours after transfection, filter sterilized using 0.2 μm cellulose acetate filter units (Corning, Cambridge, MA) and concentrated by ultra-filtration (100,000 MWCO, Centricon Plus-70, Millipore, Milford, MA). Transduction titer was assigned on concentrated viral stock by assessing transgene expression in HEK 293T cells using a limiting dilution assay in the presence of 8 μg/mL of Polybrene (Sigma-Aldrich, St. Louis, MO) three days after transduction. For LV-

Empty, the titer was assigned by performing an enzyme-linked immunosorbent assay (ELISA) for HIV-1 p24 core antigen (PerkinElmer Life Sciences, Inc., Boston, MA).

Cell culture

NRVMs were enzymatically dissociated from the ventricles of 2-day-old Sprague-Dawley rats (Harlan, Indianapolis, IN) with the use of trypsin (Amersham Biosciences, Piscataway, NJ) and collagenase (Worthington Biochemical Corporation, Freehold, NJ).[2] Freshly isolated NRVMs were resuspended in M199 culture medium (Gibco) supplemented with 10% FBS, glucose, 2 mM L-glutamine, penicillin, vitamin B12, HEPES buffer and MEM non-essential amino acids (Gibco). Two 90-minute pre-plating steps were performed to reduce fibroblasts and enrich cardiac myocyte content in the culture. The final cell suspension was collected, counted for NRVMs and diluted at the desired plating concentration. For patch-clamping studies, 10^5 cells were plated on 12-mm glass coverslips coated with fibronectin at a concentration of 25 μg/mL. For mapping studies, 10^6 cells were plated on 21-mm fibronectin-coated plastic coverslips. The time of plating is considered day 0 for the NRVM cultures. After 24 hours, the coverslips were washed with warm phosphate buffered saline (PBS) and fresh medium with 10% serum was added. Starting from day 2 (two days after plating), the cultures underwent media change on every second day and were maintained in medium containing 2% serum.

All animal experiments were performed in accordance with guidelines set by the Johns Hopkins Committee on Animal Care and Use and were in compliance with all federal and state laws and regulations. The animals, neonatal rat pups, were only used for harvesting cardiac tissue and were sacrificed thereafter.

Lentiviral transduction of NRVMs

For all transduction experiments, the concentrated LV stock was applied at the indicated multiplicity of infection (MOI; i.e., the number of active vector particles per target cell) in the presence of 8 μg/mL of Polybrene (Sigma-Aldrich) as previously described.[2]

Stenciling and preparation of monolayers with heterogeneous I_{K1} expression

Two populations of NRVMs isolated on two consecutive days were used to obtain confluent monolayers with regions of altered Kir2.1 expression. In this modified stenciling technique, 200-300 μL of fibronectin (Sigma-Aldrich) solution (50 μg/mL) in deionized water was carefully transferred to the surface of the PDMS stencils with a central hole 6 mm in diameter made by a punch. The stencils were left undisturbed for 1-2 h. After this period, stencils with adsorbed fibronectin were cast against UV-treated, circular, plastic coverslips (21 mm diameter) and fibronectin solution at a lower concentration (25 μg/mL) was added to the circular well region formed by the stencil and the coverslip. After about 1 hour, excess fibronectin solution was aspirated, and a mixture of freshly isolated NRVMs (from first day of isolation) and concentrated lentiviral vectors encoding target genes of interest (empty, Kir2.1, or Kir2.1AAA) were plated into the wells. After 20-24 h, the excess medium was aspirated, and PBS was added to wash off any unattached cells. Without much delay, the PDMS stencils were gently peeled off the coverslips, which were immediately left in culture plates containing warm Tyrodes solution. Fibronectin transferred to the plastic coverslips via the stencils was wetted by this procedure and enabled subsequent plating and attachment of non-transduced NRVMs (from second day of isolation). Non-transduced NRVMs were added to already adherent and transduced NRVMs to form an *in vitro* model of cardiac myocytes with spatially-localized functional heterogeneity.

After 20-24 h, the preparation was washed twice with warm PBS to remove unattached NRVMs. At this time, a well-defined monolayer of non-transduced NRVMs with a spatially (centrally) localized population of transduced NRVMs was obtained. To obtain a monolayer with an inverse pattern of heterogeneity, non-transduced NRVMs from first day of isolation were plated inside the stencil region. After 24 h, NRVMs from second day of isolation were transduced with LVs in suspension for 2-3 h, pelleted by centrifugation at 4^{o}C and 750 rpm for 3 minutes and resuspended in fresh cell culture medium. The process of pelleting and resuspending was repeated three more times so that the final cell suspension was devoid of free LVs. This suspension was then added to already adherent and centrally localized non-transduced NRVMs from previous day to obtain confluent monolayers with inverse pattern of I_{K1} heterogeneity.

CellTracker labeling

CellTracker Red CMTPX (Invitrogen) was dissolved to 10 mM in DMSO (Sigma-Aldrich) and further diluted to 25 μM in serum-free medium. Freshly isolated non-transduced NRVMs were centrifuged to form a pellet, supernatant was aspirated, and cells were resuspended in staining solution pre-warmed to 37^{0}C. The cells were incubated for 30 mins under normal growth conditions. Stained cells were pelleted, resuspended in culture medium containing 10% serum, plated on fibronectin-coated plastic coverslips and allowed to adhere over a period of 24 h.

Immunohistochemistry

Six-day old non-transduced, LV-Empty transduced and Kir2.1 gene-modified NRVMs were characterized thoroughly for their myocyte content, morphology, and levels of Kir2.1 and gap junction protein expression using an immunostaining assay with antibodies against cardiac

Troponin I (cTnI), actin, Kir2.1 and connexin43 (Cx43). Cultures were washed with PBS and fixed in 4% paraformaldehyde for 10 minutes at room temperature. Cells were washed again with Tris-buffered saline (TBS) and permeabilized using TBS with 0.1% Triton X-100 for 20 minutes. Cells were blocked overnight at 4^0C with 5% non-fat dry milk in TBS with Tween-20 (TBS-T). Primary antibodies against cTnI (1:50, SC-15368, Santa Cruz Inc., Santa Cruz, CA), Kir2.1 (1:50, SC-28633, Santa Cruz Inc.) and Cx43 (1:200, C6219, Sigma-Aldrich, St. Louis, MO) were diluted in the blocking medium. Cells were incubated with primary antibodies for 1 h at room temperature then washed with blocking reagent. Cells were incubated with Alexa Fluor conjugated secondary antibodies (1:200, Invitrogen) for 1 h at room temperature and washed extensively with TBS-T. Actin was stained using Alexa Fluor 647 phalloidin (1:200, A22287, Invitrogen) and nuclei were stained using Hoechst (1:10000, Invitrogen). Coverslips were mounted with ProLong Gold antifade reagent (P36930, Invitrogen), sealed with nail polish, and imaged.

Fluorescence imaging

Confocal fluorescence imaging of immunostained 6-day old NRVM cultures was performed on a Zeiss LSM 510 META confocal laser scanning microscope (Carl Zeiss Inc., Thornwood, NY), and all images were processed with the Zeiss LSM software (Carl Zeiss Inc.) and ImageJ software (National Institutes of Health, Bethesda, MD).

Western Blot

Six-day old non-transduced and transduced cultures were characterized for their Kir2.1, Cx43 and tubulin levels using standard Western blotting techniques. Cultures were lysed with SDS-

PAGE sample buffer and boiled for 5 minutes. Proteins were separated with 4-12% bis-tris gels (Invitrogen) using MES running buffer and transferred onto 0.45 μm nitrocellulose membranes using an iBLOT apparatus (Invitrogen). Equal protein loads were confirmed by Ponceau-S stain. After transfer to nitrocellulose, membranes were blocked overnight at 4°C then incubated with primary antibodies against Kir2.1 (1:1000, Santa Cruz), Cx43 (1:1000, Sigma-Aldrich) and α-tubulin (1:1000, Abcam) for 1 hour at room temperature. Membranes were incubated with alkaline phosphatase–conjugated secondary antibodies (1:20000, Jackson ImmunoResearch, West Grove, PA) for 1 hour at room temperature. Proteins were visualized with chemiluminescent substrate and x-ray film, and images were analyzed using ImageJ.

Whole-cell patch clamping

Experiments were carried out using standard microelectrode whole-cell patch-clamp techniques with an Axopatch 200B amplifier (Axon instruments, Union City, CA).[3] Data was sampled at 20 kHz and low-pass Bessel-filtered at 5 kHz. All experiments were performed at room temperature. Six-day old cells were washed with a normal Tyrode's solution containing (mM) NaCl 138, KCl 5, $CaCl_2$ 2, glucose 10, $MgCl_2$ 0.5, and HEPES 10; pH 7.4. The micropipette electrode solution was composed of (mM): K-glutamate 130, KCl 9, NaCl 8, $MgCl_2$ 0.5, HEPES 10, EGTA 2, and Mg-ATP 5; pH 7.2. For the measurement of I_{K1} density, 138 mM Na^+ in the external bath solution was replaced with 140 mM K^+ (total). Microelectrodes had tip resistances of 2 to 4 MΩ when filled with the internal recording solution. Voltage-clamp recordings were obtained with an inter-episode interval of 2.5 seconds. Action potentials were either initiated by short depolarizing current pulses (Kir2.1-overexpressed NRVMs) or were spontaneous (eGFP-

transduced control and Kir2.1AAA-overexpressed myocytes). Data was corrected for the measured liquid junction potential of -14.3 mV.[4,5]

Optical mapping

Optical mapping was performed on NRVM monolayers 6-7 days post-transduction as previously described.[2] Bipolar line stimulation via platinum electrodes was applied just above one edge of the monolayer. To determine APD_{80} and CV, cells were stimulated with monophasic, 10ms pulses at 2 Hz delivered by the stimulus electrode at twice diastolic threshold. A 3-s recording was taken after a ten beat drive train. To initiate reentry, a rapid pacing protocol (with a pacing frequency of 5 Hz or above) was used.[6]

Data analysis

For patch clamping, the dV/dt_{max} for eGFP-transduced and Kir2.1 gene-modified NRVMs was equal to the maximum positive value of the first derivative of the action potential. For optical mapping, the raw optical signals were detrended by subtracting a fitted second-order polynomial curve and then low-pass filtered with a fourth order elliptical filter. Isopotential and isochrone maps were generated from the processed signals using custom-written MATLAB (The MathWorks Inc., Natick, MA) scripts. APD_{80} was defined as the interval from the activation time to the time in the repolarization phase where the AP amplitude dropped to 20% of its maximum, and APD_{80} values were averaged across all channels and over 3 to 4 APs. CV was defined as the inverse of the distance along a line normal to the wavefront that crossed isochrones spaced 10 ms apart for paced waves and 3 ms apart for reentrant waves. For paced waves, CV was measured along 4 to 5 manually selected paths and averaged spatially across these paths and temporally

over 3 to 4 APs. For reentrant waves, CV was measured at a fixed distance from the wave tip measured along the wave front. Paths were chosen to be sufficiently far away from the stimulus site so that latency delays associated with excitation could be neglected. MCR was defined as the maximum pacing rate at which each stimulus evoked a tissue response in at least 90% of recording sites. To track the spiral wave tip, we analyzed the system in phase space and tracked the phase singularity, the point at which wavefront and waveback meet.[7]

Expanded Results

Characterization of non-transduced and Kir2.1 gene-modified cultures

Immunostain images of cTnI (Online Figure I, first column) show NRVMs in non-transduced (Online Figure IA) and in LV-Empty (Online Figure ID), LV-Kir2.1 (Online Figure IG) and LV-Kir2.1AAA (Online Figure IJ) transduced cultures. Immunohistochemistry against actin (Online Figure I, second column) and Cx43 (Online Figure I, third column) confirmed that in a given field of view both non-transduced and transduced cultures were morphologically similar (Online Figure IB, IE, IH and IK for non-transduced, LV-Empty, LV-Kir2.1 and LV-Kir2.1AAA transduced, respectively) and had similar levels of gap junctional protein expression (Online Figure IC, IF, II and IL for non-transduced, LV-Empty, LV-Kir2.1 and LV-Kir2.1AAA transduced, respectively) that was primarily localized at cell-to-cell appositions. No striking differences in the levels and distribution of Cx43 expression were apparent among the cultures.

Characterization of single-cell electrophysiological properties of Kir2.1-transduced NRVMs

Spontaneous APs were observed in eGFP-transduced NRVMs (Online Figure IIA). While spontaneous APs were absent in Kir2.1-overexpressed NRVMs, single APs could be triggered by a short depolarizing current stimulus (Online Figure IIB). Kir2.1AAA-transduced NRVMs fired spontaneous APs resembling those of genuine pacemaker cells (Online Figure IIC).

Impulse propagation in monolayers with islands of Kir2.1 gene-modification

Representative isopotential maps of AP propagation in monolayers with islands of LV-Empty transduced, Kir2.1-overexpressed and Kir2.1-suppressed NRVMs are shown in Online Figure IIIA, IIIB and IIIC, respectively.

Dynamics of induced reentrant spiral waves in monolayers with heterogeneous I_{K1} expression

After 1 minute of superfusion with Tyrode's solution containing 50 μM Ba^{2+}, the frequency of reentry in a monolayer with a central island of Kir2.1 overexpression gradually decreased (Online Figure IVA) to 7±1 Hz (n=9; Online Figure IVB). The stability of the spiral wave was also disturbed, as reflected by a transition from a circular wavetip pattern (as seen in Figure 6C of the main text) to a meandering pattern of almost the same size (Online Figure IVC).

References

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Figure Legends

Online Figure I. Characterization of non-transduced and Kir2.1 gene-modified NRVM monolayers. Immunostain images of cTnI (first column), actin (second column) and Cx43 (third column) in non-transduced (A-C), LV-Empty transduced (D-F), LV-Kir2.1 transduced (G-I) and

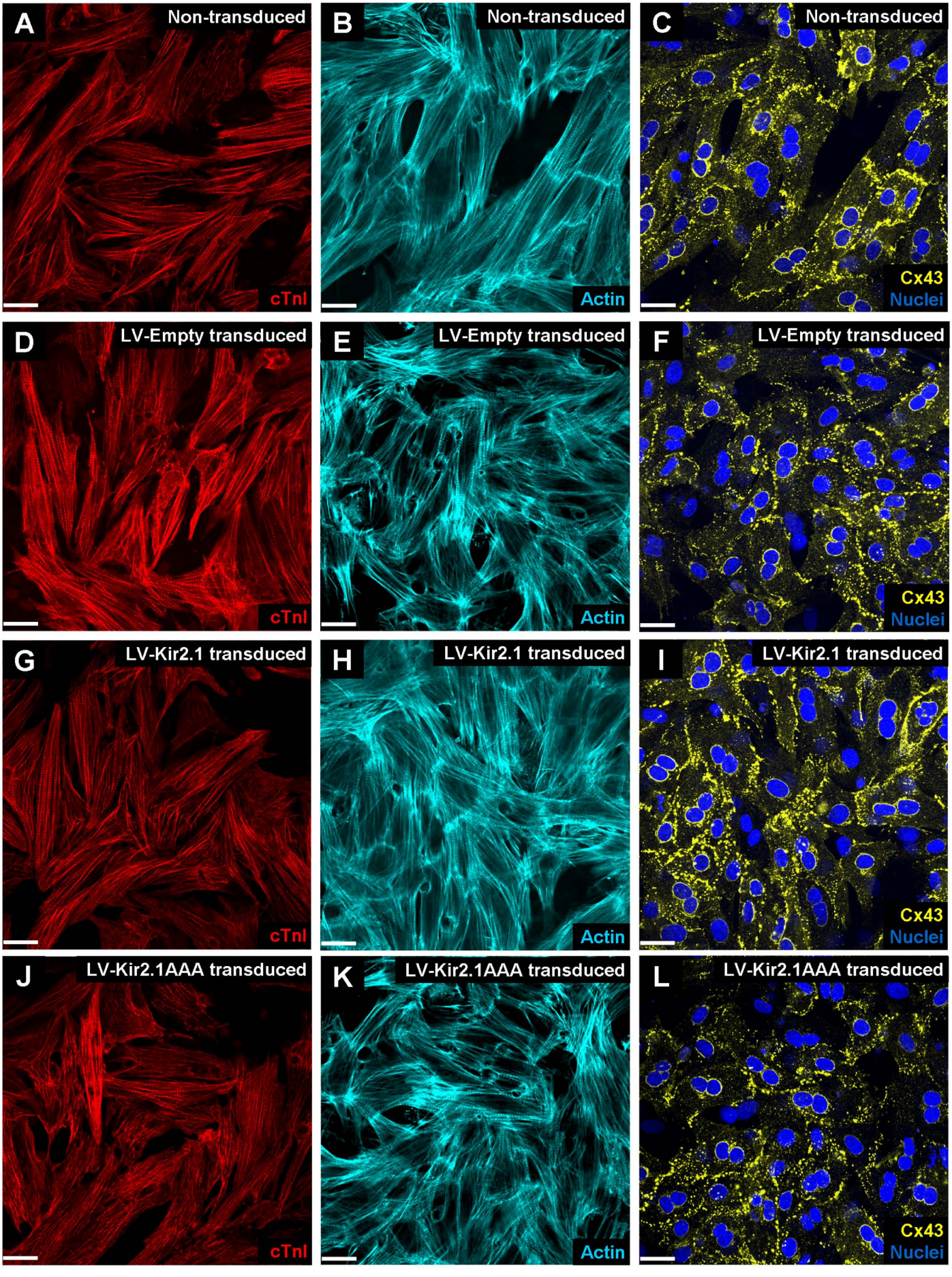
LV-Kir2.1AAA transduced (J-L) monolayers. Hoechst (blue) was used to label the nuclei in Cx43 immunostain images. All scale bars, 50 μm.

Online Figure II. Representative action potentials obtained by whole-cell current clamp of Kir2.1-transduced NRVMs. (A) Spontaneous APs in eGFP-transduced control myocytes. (B) Electrically stimulated APs in I_{K1}-overexpressing myocytes. (C) Spontaneous APs in I_{K1}-suppressed NRVMs.

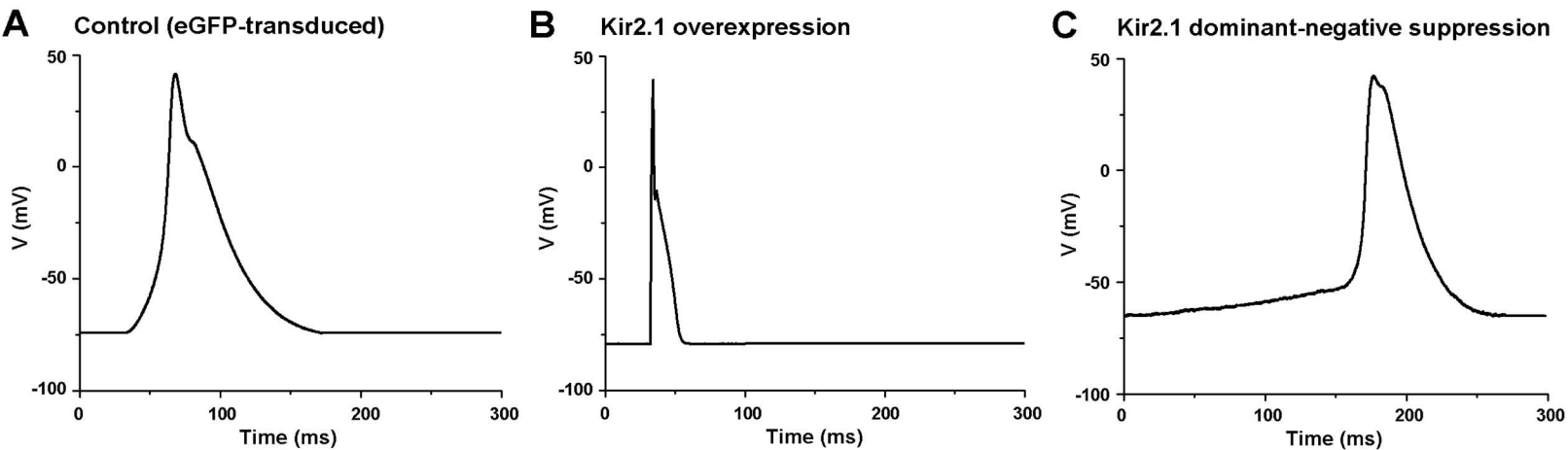
Online Figure III. Impulse propagation in monolayers with LV-Empty transduced or Kir2.1 gene-modified NRVM islands. Representative isopotential maps of AP propagation in monolayers with a central island of LV-Empty transduced (A), Kir2.1-overexpressed (B) or Kir2.1-suppressed (C) NRVMs. Representative sites in the transduced and non-transduced regions of the monolayer are indicated by "x" and "+" symbols, respectively. The direction of impulse propagation is indicated by black arrows, and islands are shown by white circles.

Online Figure IV. Dynamics of induced reentrant spiral waves in monolayers with a central island of Kir2.1 overexpression. (A) Spiral wave after 1 min of superfusion with Tyrode's solution containing 50 μM Ba^{2+}. (B) Five sec and 1 sec acquisitions of the spiral wave shown in (A) from site 224 marked by "x" in A. (C) Tip trajectory of the spiral wave after 1 min of Ba^{2+} superfusion. The X- and Y- axes are position on monolayer in mm.

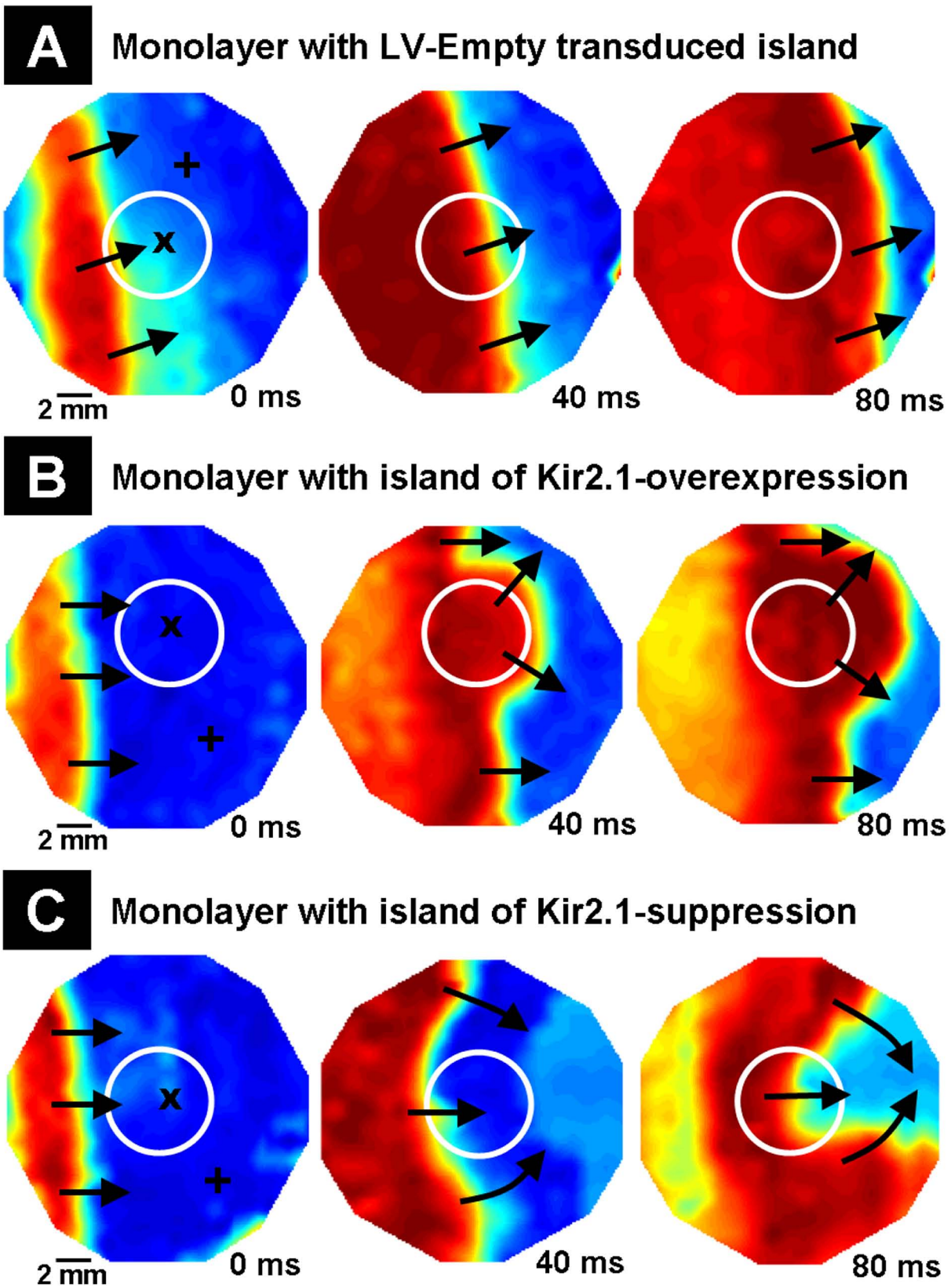
ONLINE FIGURE I



ONLINE FIGURE II



ONLINE FIGURE III



ONLINE FIGURE IV