I\textsubscript{f} Current and Spontaneous Activity in Mouse Embryonic Ventricular Myocytes

Kenji Yasui, Weiran Liu, Tobias Opthof, Kenji Kada, Jong-Kook Lee, Kaichiro Kamiya, Itsuo Kodama

Abstract—Knowledge of the initiation of electrical and contractile activity in the embryonic heart relies to a large extent on data obtained in chicken. In recent years, molecular biological techniques have raised an interest in mouse physiology, including early embryonic development. We studied action potentials and the occurrence of one of the pacemaker currents, I\textsubscript{f}, by the whole-cell voltage and current-clamp technique at the earliest stage at which a regular heartbeat is established (9.5 days postcoitum) and at 1 day before birth. We show, first, that at the early stage there is a prominent I\textsubscript{f} in mouse embryonic ventricles, which decreases by 82% before birth in concert with the loss of regular spontaneous activity of ventricular cells. Second, the decrease in I\textsubscript{f} current is associated with a slight change in channel gating kinetics and a decrease in total mRNA expression of the genes encoding for I\textsubscript{f} current. Third, the most prevalent mRNA subtype is switched from HCN4 to HCN2 during the second half of embryonic development. Fourth, the I\textsubscript{f} current may be modulated by the \(\beta\)-adrenergic cascade, although the coupling to the \(\beta\)-adrenoceptor in the sarcolemma itself is not yet mature. We conclude that I\textsubscript{f} current of the sinus node type is present in early embryonic mouse ventricular cells. In association with a loss of I\textsubscript{f} current, the ventricle tends to lose pacemaker potency during the second half of embryonic development. (Circ Res. 2001;88:536-542.)

Key Words: embryo ■ development ■ I\textsubscript{f} current ■ HCN gene ■ \(\beta\)-adrenoceptor modulation

Knowledge about the onset of electrical activity in the embryonic heart is restricted to chicken, and information available for mammalian species is still limited.\textsuperscript{1-4} Recent progress of genetic engineering has raised an interest in the early development of the mouse heart. It is not known when first electrical activity occurs, although first contractions may be seen at 8.5 days postcoitum (dpc), with regular beating at 9 dpc.\textsuperscript{5} The order by which membrane currents appear in the embryo has started to be determined from studies in mouse embryos\textsuperscript{6} and in cell cultures derived from mouse embryonic stem cells.\textsuperscript{7} The slow inward calcium current (\(I_{CaL}\)) has been demonstrated at 9.5 dpc\textsuperscript{4} and increases steadily until birth at 19 dpc,\textsuperscript{6,8} whereas the fast inward sodium current (\(I_{Na}\)) becomes prominent at a later stage.\textsuperscript{6} With respect to the repolarizing currents, the transient outward current (\(I_{to}\)) develops first\textsuperscript{6,7} with higher atrial than ventricular density.\textsuperscript{6} Other outward potassium currents develop later with different regional densities.\textsuperscript{6} Thus, cells at 11 to 13 dpc depend on \(I_{CaL}\) for the upstroke and on \(I_{Na}\) for repolarization of their action potentials, in line with the observation that these currents also develop first in cells derived from mouse embryonic stem cells.\textsuperscript{7} However, it is not clear which currents are responsible for diastolic depolarization in embryonic cells.

The membrane current I\textsubscript{f} plays a prominent role in pacemaking in the adult sinus node and Purkinje system.\textsuperscript{9,10} I\textsubscript{f} at the pacemaker potential range has been shown to be enhanced by catecholamine, whereas it is attenuated by acetylcholine via positive or negative shifts of its activation curve.\textsuperscript{11} Whatever the role of I\textsubscript{f} for basic pacemaker function,\textsuperscript{12} it is at least important for the autonomic modulation of heart rate. The pacemaker current I\textsubscript{f} has not been investigated in cells from mouse embryos. Because this current is present in early pacemaker cells derived from mouse embryonic stem cells and because all cells with I\textsubscript{f} current showed spontaneous activity,\textsuperscript{7} we investigated whether this current is associated with early electrical activity in the mouse embryo. The genes encoding for I\textsubscript{f} current (HCN1 through HCN4) were analyzed by mRNA measurement.

Materials and Methods

Isolation and Culture of Cardiac Myocytes

Single myocyte cultures were prepared from ventricles of 9.5- and 18-dpc mouse embryonic hearts by methods previously described.\textsuperscript{8} Briefly, pregnant mice were killed, and ventricles were dissected from the exposed embryos. All animal procedures were approved by the Animal Care and Use Committee, Research Institute of Environmental Medicine, Nagoya University. After incubation in
TABLE 1. Sequence of PCR Primers and Sequence-Specific Probes for HCN1–4 and GAPDH

<table>
<thead>
<tr>
<th>Target Sequence</th>
<th>Accession</th>
<th>Primer</th>
<th>Sequence (5′→3′)</th>
<th>Position</th>
<th>Amplicon Length, bp</th>
<th>Enzyme and Site</th>
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<td>HCN1</td>
<td>AJ225123</td>
<td>Sense</td>
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<td>590–614</td>
<td>464</td>
<td>CiaI, 875–880</td>
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<tr>
<td></td>
<td></td>
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<td>1029–1053</td>
<td></td>
<td></td>
</tr>
<tr>
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<td>AJ225122</td>
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<td>FP</td>
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<td>464</td>
<td>Scal, 808–813</td>
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<tr>
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<td>226–250</td>
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<td></td>
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<td>GAPDH</td>
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<td></td>
<td></td>
<td>GAPDH probe</td>
<td>CCTGCCAGGTAGCTACATCGCAGACATTTT</td>
<td>517–344</td>
<td></td>
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</table>

*In GenBank release 120.0 (December 2000).

Electrophysiological Experiments

A coverslip with attached ventricular myocytes was placed in the chamber of an inverted microscope and superfused with normal Tyrode’s solution containing (in mmol/L) NaCl 146.9, KCl 5.4, CaCl₂ 1.8, MgCl₂ 0.5, NaH₂PO₄ 0.33, and HEPES 5 (pH 7.35). Whole-cell patch clamp was performed using Axopatch 200B (Axon Instruments). Internal solution contained (in mmol/L) KOH 60, KCl 80, aspartate 40, HEPEs 5, EGTA 10, MgATP 5, Na₂-phosphocreatinine 5, and CaCl₂ 0.65 (pH 7.2, pCa 7.96). Action potentials were recorded from 9.5- and 18-dpc ventricular myocytes in a current-clamp mode in normal Tyrode’s solution. To study If current, the external solution was modified to reduce the interference of other components by adding to the normal Tyrode’s solution (in mmol/L) BaCl₂ 2, NiCl₂ 2, and 4-aminopyridine 0.5. In some experiments, 30 μmol/L tetrodotoxin was added to the solution. The amplitude of If was determined as the difference between the amplitude of If at 50 mV and fitted by the Boltzmann equation: If = Iₒ/(1 + exp[(V_m − V₅₀)/k]), where V₅₀ is the membrane voltage, Iₒ is the current at half-maximal activation, and k is the slope factor. All experiments were carried out at 37°C.

Reverse Transcriptase–Polymerase Chain Reaction and Identification of HCN Subtype

Total RNA of heart (ventricles) or brain was extracted with RNasea Mini Kit (Qagen) from 9.5-dpc mouse embryo and acid guanidinium thiocyanate-phenol-chloroform method from 18-dpc mouse embryo and adult mouse. Single-stranded cDNA synthesis was performed with total RNA using oligo dT primer and SuperScript II RNase H reverse transcriptase (Gibco BRL). Polymerase chain reaction (PCR) was carried out by 2 different methods. The GAPDH gene was used as an endogenous control. The first method was to obtain the PCR product for all HCN1 through HCN4 genes by using degenerated primers (Table 1) and AmpliTaq Gold (Roche Molecular Systems, Inc). For identification of the HCN subtype, the PCR product of degenerated primers was digested by specific restriction enzymes (CiaI for HCN1, Scal for HCN2, MluI for HCN3, and Tth111I for HCN4).

The second method is to quantify the expression of each HCN gene by a real-time fluorogenic 5′-nuclease PCR assay (Perkin-Elmer ABI Prism 7700). The respective primers and TaqMan probes are listed in Table 1. The threshold cycle (Cₘ) from the baseline to reach a statistically significant increase in fluorescence signal was
measured. The Ct value predicts the quantity of target cDNA in the sample. PCR products for HCN1 through HCN4 genes were subcloned using TA cloning (pGEM-T Easy, Promega) and were verified by sequencing. cDNA standards were obtained by digesting plasmid by EcoRI. Five different molecules of cDNA standards for HCN1 through HCN4 genes (1×10^7, 1×10^6, 1×10^5, 1×10^4, 1×10^3) were amplified to determine the linear relationship between Ct and log starting molecule number of cDNA standards. Slope factors of HCN1, HCN2, HCN3, HCN4, and GAPDH were 2.3.283, 2.3.355, 2.3.382, 2.3.582, and 2.3.525 cycles/log decade, respectively.

**Data Analysis**
Electrophysiological data were analyzed using Clampfit of the pCLAMP program (Axon Instruments). Data are presented as mean±SEM. Statistical analysis of data were performed using paired and nonpaired Student’s t test (patch-clamp data) or ANOVA (quantitative PCR). Differences were considered significant at P<0.05.

**Results**

**Action Potentials at 9.5 and 18 dpc**
Recordings were made from 13 isolated ventricular cells of 9.5-dpc mice and 18 cells of 18-dpc mice. Representative records are shown in Figures 1A and 1B. All 13 cells from 9.5-dpc mice showed regular spontaneous activity with prominent diastolic depolarization. In contrast, only 6 myocytes (33%) from the 18-dpc mice were spontaneously active, albeit at irregular cycle length. Maximum diastolic potential, maximum upstroke velocity, diastolic depolarization rate, and cycle length are summarized in Table 2. Spontaneous action potentials at 18 dpc had slower diastolic depolarization rate and higher maximum upstroke velocity than those at 9.5 dpc (P<0.05).

**Identification of If Current**
Figures 1C and 1D show membrane currents in response to hyperpolarizing voltage steps from a holding potential of −50 mV to test potentials ranging from −60 to −130 mV at 9.5 and 18 dpc. At 9.5 dpc, hyperpolarizing pulses induced substantial inward currents (Figure 1C). Current density of the time-dependent current was −7.9±4.0 pA/pF at −130 mV (n=11). At 18 dpc, hyperpolarization-induced inward current recorded from ventricular myocytes was minimal, with density at −130 mV of only −1.4±0.4 pA/pF, which constitutes an 82% decrease (Figure 1D; n=6). There was no significant difference of cell capacitance (28±3.6 pF at 9.5 dpc and 31±3.3 pF at 18 dpc). The decrease in If current might be attributable to greater rundown during the 18 to 24 hours after plating in the cell cultures from the 18-dpc ventricles than that in the cell cultures from the 9.5-dpc ventricles. Therefore, we did 2 subsets of 8 experiments each in cell cultures of 2 to 4 hours derived from 9.5- and 18-dpc ventricles. A similar reduction of If density (88%) from 9.5 dpc to 18 dpc was observed in the experiments with earlier cell cultures.

**Figure 1.** Pacemaker activity in mouse embryonic ventricular cells at 9.5 dpc (left) and 18 dpc (right). A and B, Spontaneous action potentials. C and D, Hyperpolarization-induced inward currents. Regular spontaneous firing was only observed at the 9.5-dpc stage, when the induced If currents were much larger. Spontaneous firing at the 18-dpc stage was observed in 6 of 18 myocytes (33%). Moreover, it was irregular and unstable.

**TABLE 2. Action Potential Parameters of Mouse Ventricular Cells**

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>MDP, mV</th>
<th>Vmax, V/s</th>
<th>DDR, mV/s</th>
<th>CL, ms</th>
</tr>
</thead>
<tbody>
<tr>
<td>9.5 dpc</td>
<td>13</td>
<td>−71.2±0.4</td>
<td>88.2±13.1</td>
<td>38±5</td>
<td>510.8±32.8</td>
</tr>
<tr>
<td>18 dpc</td>
<td>6*</td>
<td>−69.2±1.4</td>
<td>113.2±6.8†</td>
<td>11±4.6†</td>
<td>Irregular</td>
</tr>
</tbody>
</table>

Values are mean±SEM.

n indicates number of cells; MDP, maximum diastolic potential; Vmax, maximum upstroke velocity; DDR, diastolic depolarization rate; and CL, cycle length.

*For 18 dpc, action potentials were analyzed in 6 myocytes, because the remaining 12 were quiescent.

†P<0.05 vs control.
I$_{\text{Na}}$ despite the presence of 30 μmol/L tetrodotoxin. Normalized amplitude of the time-dependent inward current was plotted as a function of the hyperpolarizing potential (Figure 2C). The activation curves were S-shaped, with a threshold potential between −60 and −70 mV. Fitting with a Boltzmann function yielded $V_{1/2}$ at −89.5±2.7 mV with $k$ of 12.3±0.9 mV at 9.5 dpc (n=6) and $V_{1/2}$ at −88.9±3.6 mV with $k$ of 7.8±1.1 mV at 18 dpc (n=6). There was no significant difference in $V_{1/2}$ between 9.5 and 18 dpc, whereas the slope factor $k$ was significantly higher at 18 than at 9.5 dpc (P<0.05).

The reversal potential of $I_f$ in ventricular myocytes at 9.5 dpc was measured at 2 different extracellular potassium concentrations ([K$^+$])$_o$) (5.4 mmol/L and 25 mmol/L) (Figures 3A and 3B). After a hyperpolarizing pulse to −130 mV with maximal activation of inward current, the clamp potential was changed in the range from −50 to +10 mV and the tail currents were measured. The time-dependent inward current was larger at the higher [K$^+$]$_o$. Figure 3C shows the averaged current density of tail currents as a function of tail pulse potential (n=4). The reversal potential of the I-V relationship was $-24±1.5$ mV at 5.4 mmol/L and $-16±1.8$ mV at 25 mmol/L [K$^+$]$_o$. The slope of the I-V relationship was steeper at 25 mmol/L [K$^+$]$_o$. The characteristics of the time-dependent inward current induced by hyperpolarization in ventricular myocytes at 9.5 dpc are comparable to those of $I_f$ found in sinoatrial node and Purkinje fibers$^9,10$.

The hyperpolarization-induced inward current was blocked by Cs$^+$, and its blocking action was voltage-dependent$^{16}$ (data not shown). The application of 3 mmol/L CsCl completely abolished the current activated by the hyperpolarizing pulse, whereas the tail current at the more positive potentials than the reversal potential was unaffected. We conclude that $I_f$ is well developed in mouse ventricular cells at 9.5 dpc but decreases by >80% before birth.
HCN Gene Expression in Mouse Embryonic Cardiac Ventricles

The apparent loss of \( I_f \) with development was additionally substantiated by investigating mRNA expression of HCN genes that encode \( I_f \) channels. In the first series of PCR experiments, the primers were designed to amplify cDNAs of all HCN1 through HCN4 genes. Figure 4A shows a strong signal in 9.5-dpc ventricles and a weak signal in 18-dpc ventricles, with no expression in adult ventricles. In adult brain, a strong band was detected in line with the presence of the mRNAs of all HCN1 through HCN4 genes, as reported previously.\(^ {17} \) This suggests that in mouse cardiac ventricles the expression of the \( I_f \) channel decreases with development. To identify the subtypes of the HCN gene expressed in cardiac ventricles at 9.5 dpc, the PCR product was digested by a restriction enzyme for each of them (Figure 4B). In the adult brain, all HCN1 through HCN4 genes were detected after the enzyme digestion. In 9.5-dpc cardiac ventricles, the restriction enzyme for HCN4, but not those for HCN2 and HCN3, digested the PCR product. The restriction enzyme for HCN1 produced a small effect. Similar findings were obtained in 2 other experiments.

In the second series of experiments, each subtype of HCN genes in the ventricles of mouse embryo was quantified by real-time PCR. The amounts of each subtype mRNA (normalized to GAPDH) are summarized in Figure 4C. Major HCN gene subtypes detected at 9.5 dpc were HCN4 and HCN1 (HCN4>HCN1, \( P<0.05 \)), but HCN2 was also detectable with minimal amount. The HCN genes detected at 18 dpc were HCN2, HCN4, and HCN1, in order of their amounts (HCN2>HCN4 or HCN1, \( P<0.05 \)), although the total amount was much less than that at 9.5 dpc. Thus, at 9.5 dpc, HCN4 was the predominant subtype. At 18 dpc, HCN2 was more expressed than the other subtypes. For HCN4 and HCN1, there was a clear-cut downregulation between 9.5 and 18 dpc. The opposite was seen for HCN2.

\[ \beta \text{-Adrenergic Modulation of } I_f \]

\( I_f \) increases in response to \( \beta \)-adrenergic stimulation in adult myocytes.\(^ {11} \) Figure 5A shows how we tested \( \beta \)-adrenergic modulation of \( I_f \) in 9.5-dpc ventricular myocytes. From a holding potential of \(-50 \text{ mV} \), we applied a hyperpolarizing pulse to \(-70 \text{ mV} \), followed by another hyperpolarizing pulse to \(-130 \text{ mV} \). This protocol was also performed in the presence of either 3 \( \mu \text{mol/L} \) isoproterenol, a \( \beta \)-adrenoceptor agonist, or 10 \( \mu \text{mol/L} \) forskolin, a direct activator of adenylate cyclase. Obviously, isoproterenol did not affect maximum \( I_f \) current at \(-130 \text{ mV} \), although there was a minimal effect at \(-70 \text{ mV} \). In contrast, forskolin (10 \( \mu \text{mol/L} \)) induced a larger increase of \( I_f \) at \(-70 \text{ mV} \), although it did not affect maximal conductance at \(-130 \text{ mV} \). Isoproterenol increased the averaged current density at the end of the step pulse to \(-70 \text{ mV} \) from 0.94±0.25 to 1.10±0.35 pA/pF (+17%; \( n=6 \), not significant). For forskolin we observed a significant increase from 1.44±0.14 to 2.34±0.25 pA/pF (+62%; \( n=5 \), \( P<0.05 \)). Figure 5B shows the effect of isoproterenol (\( n=8 \)) and forskolin (\( n=5 \)) on the activation curve, which was obtained from normalized \( I_f \) amplitude during hyperpolarizing pulses.

Isoproterenol shifted the half-maximum activation voltage by 2 mV (not significant) in the positive direction, whereas this shift with forskolin was 7.8 mV (\( P<0.05 \)). This suggests that in mouse ventricular myocytes, the signaling cascade to stimulate \( I_f \) channel downstream from the adenylate cyclase is already evolved at 9.5 dpc, but that \( \beta \)-adrenergic receptors or their transducing mechanisms via G proteins are still immature.

\[ \text{Discussion} \]

In this study, we demonstrated substantial \( I_f \) current in spontaneously beating 9.5-dpc ventricular myocytes, whereas the current decreased by 82% in 18-dpc ventricular myocytes in association with the loss of regular spontaneous activity. As early as at the 9.5-dpc stage, forskolin dramatically increased \( I_f \) current at physiological voltage, although isoproterenol had only a minor effect.
**$I_f$ in the Embryonic Heart: Presence and Decrease**

In embryonic hearts, $I_f$ has thus far only been demonstrated in chicken ventricular myocytes, where it has a comparable density as in the present study. The current has also been observed in cell cultures derived from mouse embryonic stem cells. In a previous study on mouse embryonic heart, however, $I_f$ was not detected; no explanation was provided.

The decrease in density of $I_f$ current that we observed during embryonic development of the mouse ventricles was previously also demonstrated in the chicken heart. Because the activation curve was more or less similar at 9.5 and 18 dpc, it is suggested that downregulation of the $I_f$ channel underlies the 82% loss of current density. In the chicken heart, a change in the activation kinetics of the $I_f$ current (negative shift of the activation curve) was reported in one study at room temperature, whereas in another study at 37°C, no change in activation kinetics was observed. We additionally demonstrated a decrease of mRNA expression of the $I_f$ encoding genes during development. These findings together strongly suggest downregulation of the channel protein as explanation for the observed decrease in current density during the second half of embryonic development.

Cerbai et al. observed that in rat ventricular myocytes during the neonatal stage, $I_f$ current decreased without a shift of the activation curve, and that the number of cells in which the current could be elicited decreased from nearly 100% at 1 to 2 days to only 32% at 28 days. In contrast, Shi et al. reported a negative shift of the activation curve of $I_f$ current with aging in rat ventricle from the neonate to the adult stage. They demonstrated HCN2 and HCN4 but no HCN1 gene expression, with a ratio of HCN2 through HCN4 of 4.7 in the neonatal stage and 13.7 in the adult stage. The change in the balance of HCN gene expression may explain the more negative $I$-$V$ relationship of $I_f$ in ventricle compared with the sinus node. In the present study, we show that the isoforms of HCN gene expressed in mouse embryonic ventricle are HCN4 (dominant) and HCN1 at early stage (as in the rabbit sinus node) but that HCN2 is predominant at the late embryonic stage. The switching of the most predominant isoform from HCN4 to HCN2 during the embryonic development seems consistent with that observed in rat ventricle after birth. However, there was no appreciable negative shift of the $I_f$ activation curve from the early to the late stage of mouse embryo.

**β-Adrenergic Modulation**

We demonstrated that the $I_f$ current at 9.5 dpc may be increased >60% at physiological membrane potential by forskolin, which directly activates adenylate cyclase, although the effect of isoproterenol was much smaller (17%). An even larger increase in $I_f$ in response to isoproterenol has been reported in 3-day-old chicken cardiac myocytes. By and large, both the appearance and regulation of $I_f$ current are early events in embryonic development. We previously demonstrated β-adrenoceptor mediated modulation of the L-type Ca$^{2+}$ current at the same stage of development of the mouse embryonic heart.

**Implications**

Early pacemaking starts in the sinus venosus in the chicken heart even before the heart starts to beat. In mammals, the ventricles are the first contracting parts of the embryonic heart. It is not obvious whether this reflects early pacemaking in the ventricles or early excitation-contraction coupling. We have shown previously that in the mouse embryo, isolated ventricular myocytes beat faster than isolated ventricles or intact hearts. This observation together with the present data on the sinus-node type of mRNA of the genes encoding for $I_f$ in the mouse embryonic ventricle raises the question of whether pacemaking may start in the ventricle rather than in the sinus venosus in mouse.

$I_f$ has been demonstrated in ventricular myocytes of the adult rat, guinea pig, and dog but not in rabbit. The presence of the current in these adult cells probably is without functional consequence, because the threshold for activation seems to be far more negative from the normal resting membrane potential. Recently, it was demonstrated in the adult rat that the HCN2 mRNA isoform gene product probably encodes for this adult type of $I_f$ channel. Interestingly, $I_f$ current has been reported in ventricular cells of patients...
with ischemic or dilated cardiomyopathy and also in atrial cells of patients who underwent a coronary bypass graft procedure or open heart surgery. The relevance of this increase has previously been discussed. In the hypertensive rat, the \( I_f \) current has also been shown to increase as a function of the degree of hypertrophy caused by the hypertension, and this density becomes even larger if heart failure is involved. It has been speculated that this increase in \( I_f \) density in hypertrophy and heart failure may involve a recapitulation of a fetal gene program. Our data on \( I_f \) current in the mouse embryonic heart are relevant to this hypothesis. The threshold for activation of \( I_f \) in atrial and ventricular cells from human patients and in rats with hypertrophy or heart failure seems more negative than in the mouse embryonic heart (this study) but far more positive than in the normal adult rat, guinea pig, and dog ventricular myocyte. The molecular characterization of the type of HCN mRNA underlying the fetal \( I_f \) current and the upregulated \( I_f \) currents under pathophysiological conditions within the same species shall establish whether this fetal recapitulation hypothesis is correct.

**Limitations**

In this study, we recorded \( I_f \) only by the ruptured whole-cell patch-clamp technique. We selected cells showing little rundown of \( I_f \) for data analysis. Still, we cannot neglect the possibility that rundown could distort the results.

**Acknowledgments**

This study was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, and Culture, Japan. We thank Drs M. Takano and T. Ishii (Kyoto University, Japan) for technical advice for the molecular study.

**References**

If Current and Spontaneous Activity in Mouse Embryonic Ventricular Myocytes
Kenji Yasui, Weiran Liu, Tobias Opthof, Kenji Kada, Jong-Kook Lee, Kaichiro Kamiya and Itsuo Kodama

Circ Res. 2001;88:536-542
doi: 10.1161/01.RES.88.5.536

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the
World Wide Web at:
http://circres.ahajournals.org/content/88/5/536

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