Mini Review

Creations of biological Pacemaker

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The heart is one of the most important targets of regenerative medicine. The pacemaker, which controls the rate of beating, is also a target of regeneration. Until today, various researches have been aiming at generating biological pacemakers, but there are only a few reports about detailed analyses of candidates of cells for a really functional pacemaker. Recently, we reported novel induction methods of spontaneous beating cardiomyocytes from mouse embryonic stem (ES) cells, and detailed analysis of ion channel dynamics during their differentiation. This review briefly overviews up to date researches about biological pacemaker and our results achieved recently.

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Introduction

The heart provides blood to every tissue, and is one of the most important organs. Although many researches aiming at cardiac regeneration have been reported, their target is mainly ventricular cardiomyocytes after myocardial infraction, i.e. regeneration of pump function[1)]. Moreover, severe heart failure often supervene bradyarrhythmia and conduction disturbance, which induces exacerbations of symptom. Thus, regeneration of pacemaker function is another important target[2)].

Initiation and regulation of heart beat arise in the sinoatrial (SA) node where pacemaker cells exist. The pacemaker activity in this node is driven by a spontaneous change in the membrane potential, called the slow diastolic depolarization. The slow diastolic depolarization results in the formation of pacemaker potentials, thereby triggering the contraction of the heart. Mice pacemaker cells express various kinds of ion channels to generate pacemaker activity[3,4)]. Pacemaker activity seems to be generated by a balance of inward (Ih/I*f*, $I_{Ca\text{-}T}$, $I_{Ca\text{-}L}$, Ist)/ outward (I_{Kr}, Ik,slow) currents (Fig.1A,B). The most important current in this depolarization is I*f* (Ih) current, which believed to be generated by hyperpolarizaion-activated cyclic nucleotide-gated (HCN) channels[5)]. HCN channel activity is controlled by cAMP binding generated by acetylcholine (Ach)-muscarinic receptor II (M2) or norepinephrine-β-adorenergic receptor (B-AR). Other important channels regulating pacemaker activity is Cav3.1, 3.2 which generate IcaT current. The low threshold activation po-

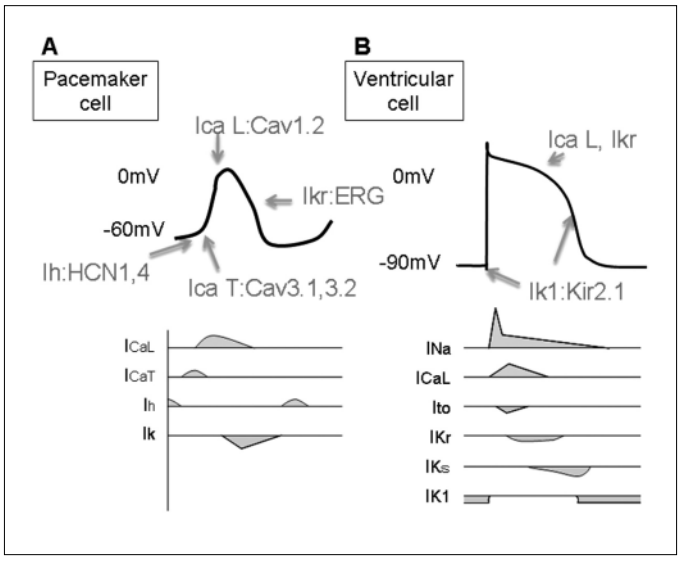

Fig.1
Representative action potential (AP) of pacemaker cells (A) and ventricular cells (B). Arrows indicate responsible currents and channels during AP maintenance.

tential of T-type channels should provide an inward depolarizing current during the slow diastolic depolarization and contribute to cell automaticity[6]. Not only I*f* current and IcaT but also many inward/outward currents control pacemaking[7].

Functional disorder of pacemaker cells leads to severe arrhythmia. At present, implantation of electronic pacemaker is a major treatment for patients, but still has problems in costs, need for frequent battery exchange (especially for younger people of congenital heart disease), and complications (pulmonary collapse, infection and device failure). To improve such problems, various trials aiming at generating biological pacemakers and their transplantation have been performed.

Genetic Modification of Cardiomyocytes

1) Over expression of dominant negative Kir2.1

Working cardiomyocytes of atrium and ventricle have abundant Ik1 current (generated by Kir2.1channel). Ik1 plays an important role in repolarization and stabilization of the maximum diastolic potential (MDP). Knockdown of Kir2.1 channel-mediated depolarization of MDP causes endogenous pacemaker activity. Miake et al. constructed a dominant negative form of Kir2.1 by modification of pore amino acid residue GYG to AAA and overexpressed it in ventricular myocytes[8]. Transduced cells increased their pacing rate in response to B-AR stimulation, and premature ventricular beats occurred. This study suggests a possibility that ventricular myocytes can be a source of pacemaker cells.

2) Overexpression of HCN

I*f* current is believed to be the most important current to generate pacemaker activity. Thus, another approach to make biological pacemakers was performed by overexpression of HCN channel to generate I*f* current. Qu et al. first reported that overexpression of HCN2 in neonatal ventricular myocytes increased beating frequency[9]. Moreover, induction of the same vector to dog left atrium caused beating that originated form left atrium after Vagal stimulation[5].

3) Overexpression of beta-adrenergic receptor

The approach reported by Edelberg et al. is that induction of B-AR to cardiomyocytes enables them to control beatings[10]. They report that induction of B-AR to mouse cardiomyocytes resulted in increase of beating rate[11], and transplantation of transfected cardiomyocytes to right atrium of mice and swine *in vivo* caused up regulation of beating rate[10,11]. These experiments first showed that genetic modification could control pacemaking rates.

Induction of Pacemaker Cells from Stem Cells

Recent progress in stem cell biology is facilitating generation of biological pacemakers by inducing differentiation from stem cells. Now, many groups showed heart stem/progenitor cell population with potentials to generate cells with pacemaker properties[1,9].

1) ES cells

The abilities of ES cells to self-renew indefinitely and to differentiate into cells of all three germ layer types (termed pluripotency), makes them one of the most promising sources to study regenerative medicine. Xu et al. reported in 2002 that ES cell-derived cardiomyocytes differentiated using embryoid body (EB) method could electronically integrate with co-cultured rat neonatal cardiomyocytes[12].

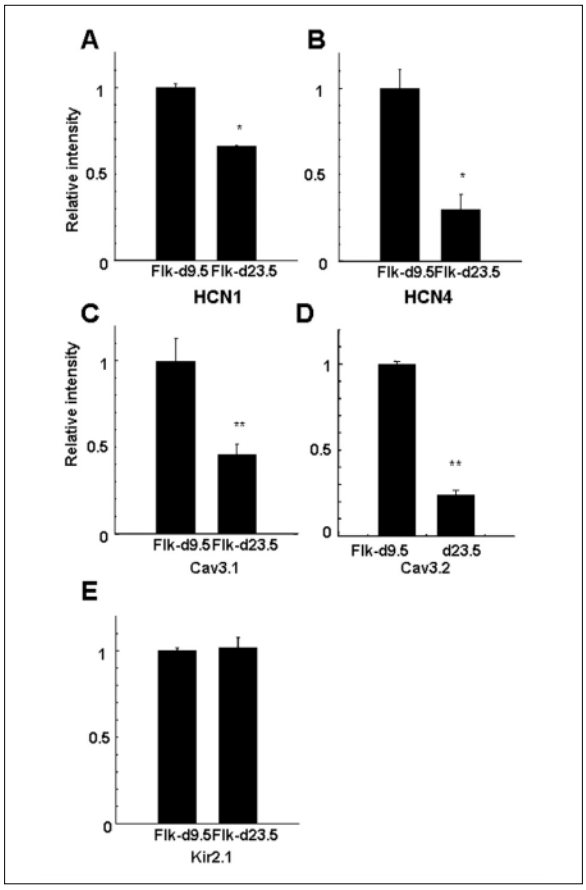

Fig.2

Western blot analyses for ion channel expressions in cardiomyocytes. (A-E): Quantitative evaluation of ion channel expressions in cardiomyocytes. Relative intensities normalized with beta-actin expressions are shown. (A): HCN1; (B): HCN4; (C): Cav3.1; (D): Cav3.2; (E): Kir2.1; $*p<0.05$, $**p<0.01$ versus Flk-d9.5 ($n=3$). (quote from ref.14 with revision)

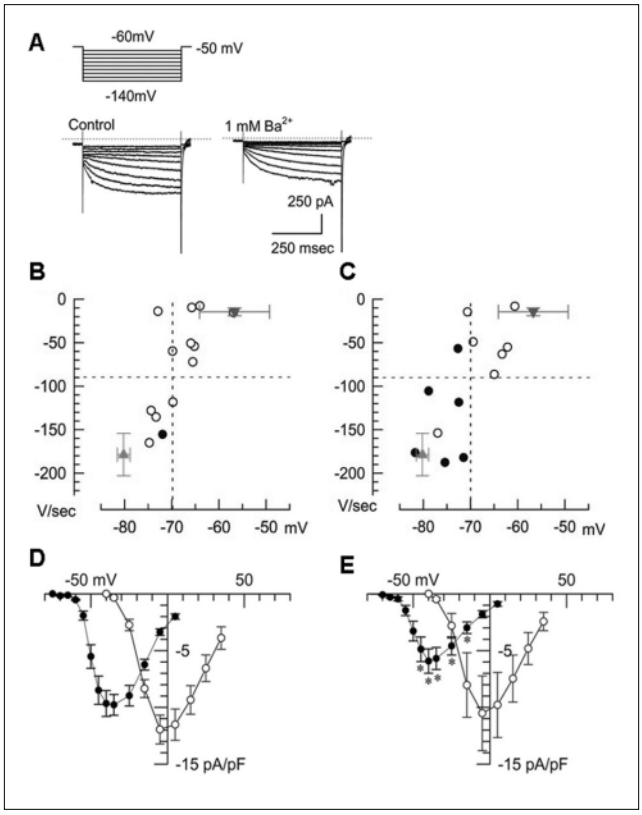

Fig.3

(A): The membrane current elicited by hyperpolarization at Flk-d9.5. left, in control bathing solution; right, in the presence of 1 mM BaCl2. Note that the initial current jump at the onset of test pulse became smaller. (B-C): Action potential parameters at Flk-d9.5 (B) and Flk-d23.5 (C). Each symbol represents the data from individual myocytes: ○, spontaneously beating myocytes; ●, quiescent myocytes; ▲, adult mouse ventricular myocyte; ▼, adult mouse pacemaker cells. Dotted lines indicate -70 mV of maximal diastolic potential (MDP) (or resting membrane potential [RMP]) and -90 V/sec dV/dt $_{max}$. (D-E): Changes of $I_{Ca\text{-}T}$ and $I_{Ca\text{-}L}$ during differentiation. Flk-day9.5 (D), day23.5 (E). ●, the amplitude of $I_{Ca\text{-}T}$ was defined as the difference between peak inward current and the steady-state current at the end of test pulse. ○, the amplitude of $I_{Ca\text{-}L}$. The amplitude of $I_{Ca\text{-}T}$ from -45 to -15 mV in Flk-d23.5 was significantly smaller than those in Flk-d9.5 ($n=5$, $*p<0.05$ vs. Flk-d9.5). (quote from ref.14 with revision)

Though many studies aiming at cardiac pacemaker regeneration have been undergoing, there are only a few evidence of characterization of induced biological pacemaker candidates as a real pacemaker.

We recently established a 2-demensinal cardiomyocyte induction system from mouse ES cells which allows us to trace differentiation step. Targeted differentiation enabled us to obtain many beating and non-beating cardiomyocyte[13]. By using this novel induction system, we induced cardiomyocytes and analyzed ion channel dynamics during long term culture[14].

ES cells were cultured in the absence with leukemia inhibitory factor to induce differentiation into Flk1+ mesodermal progenitor cells. To induce cardiomyocyte differentiation, Flk1+ cells were co-cultured on OP9 stroma cells (designated as Flk-day0). Spontaneous beating cardiomyocytes appeared at Flk-day4. Similar to previous results reported in EB method[15], number of beating colonies was decreased during long term culture also in 2D differentiation system. Parallel to this phenomenon, protein expression of HCN and Cav3 were decreased (Fig.2A-D). On the other hand, expression of Kir2.1, abundantly expressed in ventricular type cardiomyocytes, were not changed during 1 month

culture periods (Fig.2E). Moreover, patch clamp analysis for ion channel properties using single cardiomyocyte revealed that (1) only beating cells but not quiescent cells had functional HCN channels (Fig.3A). (2) Though most of the cells were spontaneously beating at Flk-day9.5, quiescent cells were increased to approximately 50% of total cardiomyocytes at Flk-day23.5 (Fig.3B,C). Quiescent myocytes showed shallower MDP and slower dV/dtmax than those of native ventricular myocytes (Fig.3C). (3) Despite the long term culture, ES cell-derived beating cardiomyocytes showed deeper MDP and faster dV/dtmax than those of native mouse pacemaker cells (Fig.3C). (4) Cav3 channels were significantly decreased in quiescent cells (Fig.3D,E). These data suggest that ES cell-derived cardiomyocytes represent intermediate differentiation stages of cardiomyocytes diversifying into two populations, pacemaker cells and non-beating ventricular or atrial cells. To generate fully differentiated pacemaker cells, further processes for the maturation as pacemakers would be required[14].

Though induction and transplantation of cardiomyocytes with pacemaker-like features induced from human ES cells using EB method were reported[16], those studies are still required to more precisely evaluate properties of induced cells as pacemaker and improve transplantation strategies.

2) iPS cells (induced pluripotent stem cells)

Recently, novel pluripotent stem cell lines (i.e. iPS cells) have been established from mouse and human adult tissues. iPS cells have brought novel possibilities and options to cell-based regenerative medicine. Recently, by applying our ES cell differentiation system to iPS cells, we succeeded in inducing various cardiovascular cells from mouse iPS cells[17]. Cardiomyocytes were successfully induced from mouse iPS cells as a mixture of various cardiac cell types, such as ventricular, atrial, conduction system and pacemaker cells. Moreover, we have already succeeded in inducing self-beating cells from human iPS cells (unpublished data). Further studies on iPS cells would largely contribute to generate biological pacemakers.

Discussion

Our study provided novel insights to create biological pacemakers from stem cells. One is that HCN and Cav3 channels are important to generate automaticity in ES cell-derived cardiomyocytes. Another is that self-beating cardiomyocytes with the automaticity do not always suffice the electrophysiological property as complete adult pacemakers. Previous genetic modification studies aiming to generate pacemaker cells just manipulated single ion channels. Inferring from our data, these single ion channel-manipulated cardiomyocytes should not satisfy complete properties as pacemaker cells. To generate more mature biological pacemaker cells, manipulation of multiple ion channels, such as HCN and Cav3, would be required. If a master transcription factor for pacemaker cells that regulate several ion channel expression cooperatively were identified, that would be a promising target to make complete biological pacemakers. There are reports that overexpression of the dominant negative form of neuron-restrictive silencer factor (NRSF) transcription factor in cardiomyocytes led to increase of HCN2 and -4 and Cav3.2 mRNA expressions[18], and NRSF regulated HCN4 transcription *in vitro*[19]. Recently, a T-box transcriptional repressor, Tbx3, was reported to be involved in SA-node development and to control various ion channel expressions[20]. Manipulation of multiple ion channel expressions toward pacemaker induction may be possible by controlling these regulatory machineries. However, because genetic modifications always involve risks for generation of mutant cells, it should be better to explore induction methods without genetic modifications, such as using small molecules.

Even after establishment of an efficient method to induce pacemaker cells from ES/iPS cells, there are many hurdles still remain. For example, (1) how to purify pacemaker cells from all induced cardiomyocytes? Specific molecular markers for pacemaker cells applicable to cell sorting or other purification methods should be identified. (2) How to transplant these pacemaker cells? Ectopic pacemaker could become arrhythmic foci. (3) How long do the induced pacemaker cells keep their pacemaker capacities? *In vivo* tracing methods to evaluate long-term behavior of transplanted cells should be also explored.

Regeneration of pacemaker function by biological pacemaker would largely improve the *Quality of Life* of patients. Progressing stem cell technologies including iPS cell research would explore and establish methods for the creation of biological pacemakers in the near future.

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