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Generation of cardiomyocytes by atrioventricular node cells in long-term cultures

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ABSTRACT

Turnover of cardiac pacemaker cells may occur during the lifetime of the body, and we recently raised the hypothesis that specialized cardiac cells have in common the potential to generate cardiomyocytes from fibroblasts. To examine this hypothesis, we analyzed the ability of atrioventricular node cells (AVNCs) to generate functional cardiomyocytes in long-term culture. AVNCs were isolated from adult guinea pig hearts and cultured for up to three weeks. Under phase-contrast microscopic observation over time, it was found that within a week, a number of fibroblasts gathered around the AVNCs and formed cell clusters, and thereafter the cell clusters started to beat spontaneously. The nascent cell clusters expanded their area gradually by three weeks in culture and expressed specific cardiac genes and proteins. Maturation of newly formed cardiomyocytes seems to be slow in cultures of AVNCs compared with those of sinoatrial node cells. Stimulation of muscarinic receptors with acetylcholine induced a beating rate decrease which was blocked by atropine, and activation of adenylate cyclase activity with forskolin increased the beat rate, while stimulation of beta adrenoceptors by isoproterenol had no effect. These results indicate that AVNCs form a cluster of cells with properties of functional cardiomyocytes and provide evidence to support the hypothesis.

1. Introduction

Cardiac pacemaker cells, such as sinoatrial node cells (SANCs) and atrioventricular node cells (AVNCs), are specialized cardiomyocytes, which exist in a quite limited number, and thus may require efficient cell turnover *in vivo* during the lifetime of the body. The adult heart is generally understood to be an organ with poor regenerative capacity, and cardiomyocytes do not proliferate since they are highly differentiated cells. However, recent evidence indicates that a physiological renewal of cardiomyocytes takes place, although its rate is very slow [1]. The involvement of various cardiac progenitor cells has been intensively discussed as the source of this restoration of cardiomyocytes [2–8].

We recently reported that SANCs can generate cardiomyocyte clusters from the cardiac fibroblasts in their vicinity [9]. The generated cardiomyocyte clusters expressed atrial myocyte type genes and proteins, formed syncytium, and contracted synchronously. The formation of beating clusters was dependent on the electrophysiological activity of centered pacemaker cells, and the stimulation of membrane receptors for endogenous modulators affected the beating rate of the cardiomyocyte clusters.

From these results, we raised the hypothesis that specialized cardiac cells that can produce spontaneous action potentials have in common the potential to generate cardiomyocytes. In addition, if our hypothesis stands, it is possible that the beat rate, or firing rate, of the specialized cells affects the generation process of cardiomyocytes. *In vivo*, AVNCs spontaneously generate action potentials when stimulation from the sinoatrial node is absent, and their intrinsic firing rate is slower than that of SANCs [10–14]. To examine our hypothesis, we determined the ability of AVNCs obtained from adult guinea pig hearts to generate cardiomyocytes in long-term cultures and further characterized the newly generated cell clusters.

Abbreviations: ANOVA, analysis of variance; AVNC, atrioventricular node cell; SANC, sinoatrial node cell.

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2. Materials and methods

2.1. Cell isolation and culture

All animal procedures were approved by the Teikyo University Chancellor's Animal Research Committee (Permit Number 12–031). Atrioventricular node and sinoatrial node were dissected out [15] from adult male guinea pig hearts (6–8 weeks old, 450–500 g body weight, SANKYO Labo Service Inc. Japan) and digested with collagenase and elastase in 0 Ca^{2+} Tyrode's solution (135 mM NaCl, 5.4 mM KCl, 0.33 mM NaH_2PO_4, 5.0 mM HEPES, 0.5 mM $MgCl_2$, and 5.5 mM glucose) as previously described [9,16]. Thereafter, the digested tissues were resuspended in high-glucose Dulbecco's Modified Eagle's Medium (0.45% glucose) supplemented with 10% fetal bovine serum (MP Biomedicals, Inc.) and triturated to obtain single cells. The cell suspension contained atrioventricular node cells as well as other types of cells naturally existing in the dissected tissue. 2 mL of the cell-containing medium was placed in a 35 mm culture dish (Greiner Bio-one) at a density of 2–5 $\times$ 10^4 cells/cm^2. Isolated AVNCs and SANCs were cultured at 37 °C in a CO_2 (5 %) incubator. The culture medium was replaced every 3 days.

2.2. Measurements of beating cell areas

Total of 25 culture dishes, obtained from 4 animals (giving 6, 6, 6 and 7 dishes), were examined. On each culture dish, the area and the number of spontaneously beating cell cluster was measured weekly. Microscopic images of the spontaneously beating cell clusters were obtained on an inverted phase-contrast microscope (NIKON) and recorded on a digital video recorder in the culture medium at 37 °C. Still photo images of each beating cluster were taken from the movie frame data and stored as JPG images, from which the area of the spontaneously beating cell clusters was quantified, using 'ImageJ' software (National Institutes of Health). The outline of each beating cluster was determined by referring to the movement of each cluster on the original movie. For every culture dish, the average beating area was calculated out by dividing the total area of the beating clusters by the number of the beating clusters on the dish.

2.3. Quantification of beating rates of cell clusters

To measure the basal beating rate of spontaneously beating cell clusters during culture, video images were recorded as above in the culture medium at 37 °C. To examine the effects of pharmacological agents, the video recording was made in normal Tyrode's solution (135 mM NaCl, 5.4 mM KCl, 0.33 mM NaH_2PO_4, 5.0 mM HEPES, 0.5 mM $MgCl_2$, 1.8 mM $CaCl_2$, and 5.5 mM glucose), which was kept at 37 °C with 100 % O_2 continuously bubbled through it. Movements of the rims of the beating clusters were converted to analog waveform signals using a video dimension analyzer (VDA; model 303, Instrumentation for Physiology and Medicine) and recorded digitally on PowerLab (AD Instruments) to count the beat rates.

2.4. RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from cell clusters that were grown in AVNC cultures for 3 weeks and was reverse transcribed using PrimeScript Reverse Transcriptase (Takara Bio Inc., Cat.No. 2680A). PCR analyses of the resulting cDNA were performed using specific primers (Table 1A) with 27–35 cycles. Primers for glyceraldehyde-3-phosphate-dehydrogenase (*GAPDH*) or cardiac troponin T (*cTnT*) were used as internal controls.

2.5. Immunocytochemistry

Cultured cells were fixed with 2 % paraformaldehyde in 100 mM phosphate buffer solution (NaH_2PO_4 23.0 mM, Na_2HPO_4 77.0 mM) for 30 min at 4 °C. After permeabilization with 0.1% Triton X-100, primary antibodies were applied overnight at 4 °C in phosphate buffer. After washing out the primary antibodies, the Alexa 488-conjugated secondary antibody was applied for 60 min at room temperature (Table 1B). Non-specific binding of antibodies was blocked with 1 % bovine serum albumin. Fluorescent cell images of the immunocytochemically stained cells were obtained using an inverted confocal laser scanning microscope (Leica CLSM TCS SP5).

2.6. Statistical analysis

All values are expressed as mean ± standard errors of the mean (S.E.M.). Time-dependent changes in beating areas and the number of beating clusters were statistically evaluated using repeated measures two-way ANOVA using "R" software, and then analyzed post-hoc with Tukey's tests, using JMP software (version 8, SAS Institute Inc.). Total of 25 dishes, obtained from 4 animals, were examined. Since two-way ANOVA indicated that no significant difference existed among the animals from which the 25 dishes derived, data from those dishes were regarded as belonging to a homogeneous population. Once the cells were placed into culture dishes, the properties of these cells heavily depend on each culture dish rather than their originating animals. We therefore treated the values with $n = 25$ for statistical analysis for their time-dependent changes. Differences were considered significant when $p < 0.05$.

Table 1
Specific primers for target genes and the PCR conditions (A) and antibodies (B) used in the present study.

(A)

Target gene	Sequence (5′–3′) of Forward and Reverse primers	Annealing temperature	Product size
Nkx2.5	CTAAGGACCCCAGAGCGGATAAGGACAGGTACCGCTGTTGCTTG	60 °C	184 bp
GATA4	AGGGGATTCAGACCAGAAAACGCTGCCATGCCCATAGTGAGAG	60 °C	199 bp
cTnT	GCAGACAGAGAGGGAAAAGAAAAAGCCGGAGAACATTGATTTCGTATTTC	60 °C	193 bp
MLC3	CAGCACATCTCCAAGAACAAGGTTCTCCACCTCGTCTTCAGTCAG	60 °C	158 bp
MLC4	AGTTCAAGGAGGCCTTTTCATTGGAAGTCCAGCATCTTGGTGTTC	60 °C	176 bp
RYR2	TCCGTTCTCTGCTGAGTGTAAGGACTCTCCACCTCCAAGGACATTC	60 °C	174 bp
ANF	GATGCCGTTAGAAGACGAGGTGCGCTCTCAGCTTGCTTTTCAG	60 °C	202 bp
GAPDH	ATACGATGACATCAAGAAGGTGGTGATACCAAGAAACGAGCTTGACAAAG	60 °C	181 bp

(B)

Antibody	Company	Dilution
Mouse anti-Troponin T monoclonal antibody	Thermo	1:100
Mouse anti-Desmin monoclonal antibody	IMMNOTECH	1:10
Alexa Fluor 488 goat anti-mouse IgG	Invitrogen	1:500

3. Results

3.1. Generation of beating cell clusters in vitro

Isolated adult guinea pig AVNCs continued to beat spontaneously for about 24 h in the culture medium, similar to SANCs [9] but with a lower beat rate. Thereafter, a number of fibroblast-like cells moved around the AVNCs and in 3 days were gradually attracted to the AVNCs. This gathering of cells continued and resulted in the construction of cell clusters around the original AVNCs by 7 days (Fig. 1A-a and Supplementary Video 1), which then started to beat spontaneously and contracted synchronously. The beating clusters gradually expanded in their total size as the culture continued, up to 3 weeks (Fig. 1A-a and A-b, 1B-a, and Supplementary Video 2). The average size of each beating cluster increased with time (Fig. 1B–b), whereas the number of clusters remained almost unchanged (Fig. 1B-c).

Supplementary video related to this article can be found at https://doi.org/10.1016/j.bbrep.2021.101018

3.2. Characterization of the spontaneously beating cell clusters

To characterize the spontaneously beating cell clusters, we examined the expression of cardiomyocyte-specific and/or cardiomyocyte-essential genes. RT-PCR analysis revealed that the beating cell clusters expressed mRNAs of cardiac proteins such as cardiac transcription factors (*Nkx2.5* and *GATA4*), a contractile protein (*cTnT*), a Ca^{2+} handling protein (ryanodine receptor 2 (*RYR2*)), and atrial natriuretic factor (*ANF*). Neither myosin light chain 3 (*MLC3*), a ventricle type contractile protein, nor myosin light chain 4 (*MLC4*), an atrial type contractile protein, were apparently detected in the newly developed cell clusters (Fig. 2A–a). However, when *cTnT* was used as an internal standard, it was found that the cell clusters did express *MLC4* at a considerable level (Fig. 2A-b).

Consistent with the expression of cardiac genes, immunocytochemical examination revealed that the cell clusters expressed typical cardiac proteins such as cTnT and desmin after 1 week in culture (Fig. 2B–a and b). These proteins gradually expanded in their distribution area by 2 weeks. (Fig. 2B–c and d). These results suggest that AVNCs generate cardiomyocytes of atrial, but not ventricular, type in long-term culture, as we have recently reported in SANC culture [9].

3.3. Beating properties and pharmacological features of newly constructed beating cardiomyocyte clusters

Right after dispersing the cells in the culture media at 37 °C, the basal beating rate of SANCs and AVNCs varied so much that we could not determine their basal beating rate; the apparent beat rate seemed, however, to be faster in SANCs than in AVNCs [14]. After culturing, the basal beating rate of the newly formed cardiomyocyte clusters converged to 68.4 ± 4.2 beats per minute (bpm, n = 100) at 1 week and increased during the culture period. The beat rate of the SANC cultures was 79.8 ± 5.2 bpm (n = 100) at 1 week. After 3 weeks, the beating rate of clusters in AVNC cultures finally reached at a similar level as that observed in the SANC culture (Fig. 3A).

Pharmacological properties of the newly grown cardiomyocyte clusters were examined by recording their contractile activities with a VDA. Isoproterenol (0.1 μM) failed to change the rate of spontaneous beating. In contrast, acetylcholine (1 μM) stopped the spontaneous beating, and its negative chronotropic effect was blocked under pre-treatment with atropine (0.1 μM). On the other hand, forskolin (3 μM), a direct activator of adenylate cyclase, slightly but significantly increased the beating rate of the newly formed cardiomyocyte clusters (Fig. 3B).

4. Discussion

Our recent finding that SANCs generate cardiomyocyte-like clusters [9] led us to hypothesize that specialized cardiomyocytes have in common the ability to transdifferentiate fibroblasts to cardiomyocytes. In the present study, fibroblast-like cells gathered around isolated single AVNCs, and spontaneously beating cell clusters were newly formed within 7 days when they were kept in culture at relatively low densities. The beating clusters gradually expanded in area over the time of culture up to 3 weeks and expressed cardiomyocyte-specific genes and proteins.

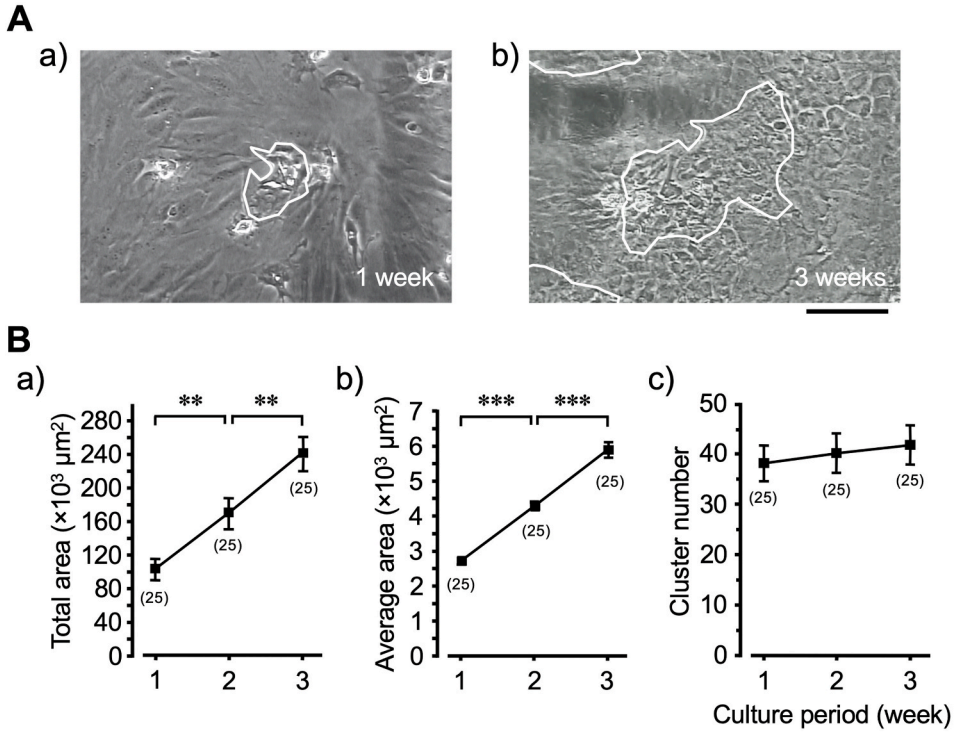

Fig. 1. Emergence of spontaneously beating cell clusters during long-term culture of adult AVNCs. (A) Typical phase-contrast images of an AVNC and its surrounding area after culture for (a) 1 and (b) 3 weeks. (a) Cell clusters with spontaneous beating formed around an isolated AVNC by 1 week of culture and (b) continuing the culture resulted in gradual expansion of cell cluster sizes. White outlines over the video images indicate the borders of the beating clusters; *bar*, 100 μm. (B) Time course of growth of the spontaneously beating cardiomyocyte clusters: (a) total beating area in a culture dish, (b) average area of the clusters in a culture dish, and (c) number of the beating cell clusters in each culture dish. Values were obtained from 25 dishes, originated from 4 animals (suppled cells for 6, 6, 6 and 7 dishes) and are expressed as mean ± S.E.M. Vertical bars denote S.E.M. (n = 25 dishes). (two-way ANOVA and Tukey's test, **, $p < 0.01$; ***, $p < 0.001$). Results were the same when the data were pre-averaged for each animal (6–7 dishes), on which statistical treatments were made (N = 4).

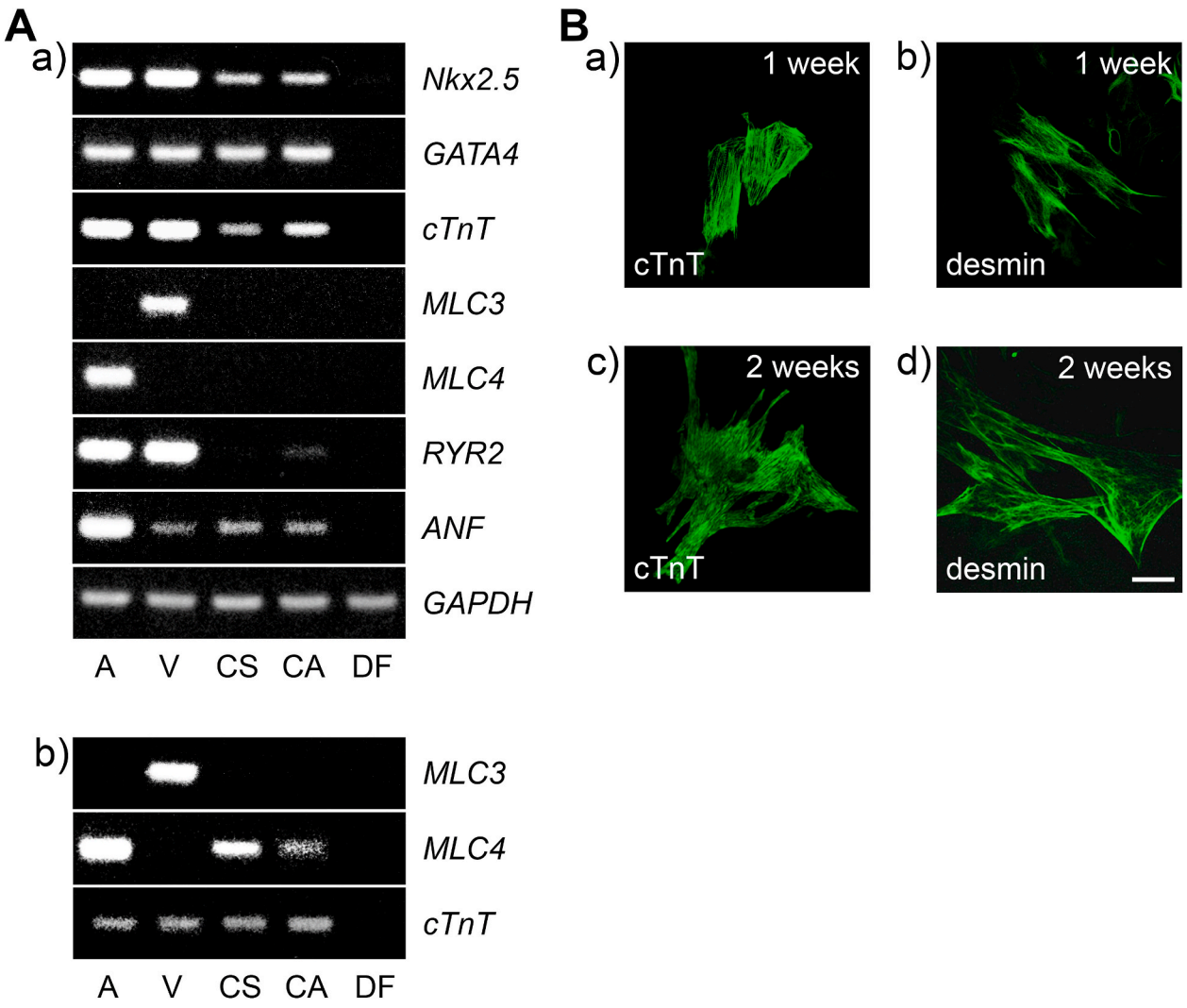

Fig. 2. Characterization of the spontaneously beating cell clusters formed during AVNC culture. (A) (a) RT-PCR analysis of cardiac gene expression in cell clusters formed after culturing isolated AVNCs for 3 weeks; *Nkx2.5*, *GATA4*, *cTnT*, *RYR2*, and *ANF* transcripts, but not *MLC3* or *MLC4* transcripts, were detected in beating cell clusters during the culture of AVNCs (lane "CA"). (b) To further detect the expression of cardiac genes in the cell clusters, *cTnT* was used as an internal control, where *MLC4*, but not *MLC3*, was found to be expressed in the newly grown cell clusters. A, atrial cell suspension; V, ventricular cell suspension; CS, cultured SANCs; CA, cultured AVNCs; DF, dermal fibroblasts. (B) Immunocytochemical detection of cardiac proteins at (a and b) 1 or (c and d) 2 weeks of AVNC culture; cell clusters that had grown around isolated AVNCs expressed (a and c) cTnT and (b and d) desmin. The bar represents 50 μm.

These findings indicate that, at least in our experimental conditions, AVNCs can generate cardiomyocytes around them during primary culture, which supports our hypothesis.

In the RT-PCR study, the spontaneously beating cell clusters expressed cardiomyocyte-specific and cardiomyocyte-essential genes after 3 weeks, including *Nkx2.5*, *GATA4*, *cTnT*, *MLC4*, *RYR2*, and *ANF*. The immunocytochemical analysis showed the presence of cTnT and desmin in the clusters as early as 1 week in culture and showed an increase in these markers with culture time. In addition, genes characteristic of atrial type myocytes, *MLC4* and *ANF*, but not a gene characteristic of ventricular type, *MLC3*, were expressed in the newly formed cell clusters. This indicates that the cell clusters include either atrial or nodal type cardiomyocytes [9,17], and these cells may have not only the contractile function but also the endocrine function of cells found *in vivo*.

In physiological conditions, the heart rate is regulated by plasma membrane receptors for endogenous modulators, such as β_1 adrenoceptors and muscarinic M_2 receptors. It is reported that β_2 adrenoceptor is also possibly involved, at least partly, in regulating heart beat [18]. Our observation showed that muscarinic M_2 receptor and the downstream sets of intracellular signal pathways were constructed and operated properly to regulate the beating rate in the nascent cardiomyocyte clusters, since acetylcholine decreased the beating rate and atropine blocked this response in the cardiomyocytes clusters. In contrast, no increase in beating rate was observed in response to isoproterenol, a nonselective stimulant for β adrenoceptor, whereas forskolin increased the beat rate. This indicated that the intracellular signaling pathway involving adenylate cyclase and its downstream molecules had developed and was operating normally at this stage. Comparing this with our previous finding that isoproterenol did increase the beat rate of nascent cardiomyocyte clusters in the SANC culture [9], it is indicated that either expression β_1, or possibly β_2, adrenoceptor or the maturation of their downstream signaling system to activate adenylate cyclase is slow in AVNC cultures, compared with the cardiomyocytes grown in the SANC cultures.

Although long-term culture of AVNCs generated cardiomyocyte clusters similar to those in SANCs [9], the detailed characteristics were not the same in the two cell types. Firstly, the cluster area, which continued to expand throughout the 3 weeks' culture period, was smaller in the AVNC culture. Secondly, the beat rate of clusters in AVNC

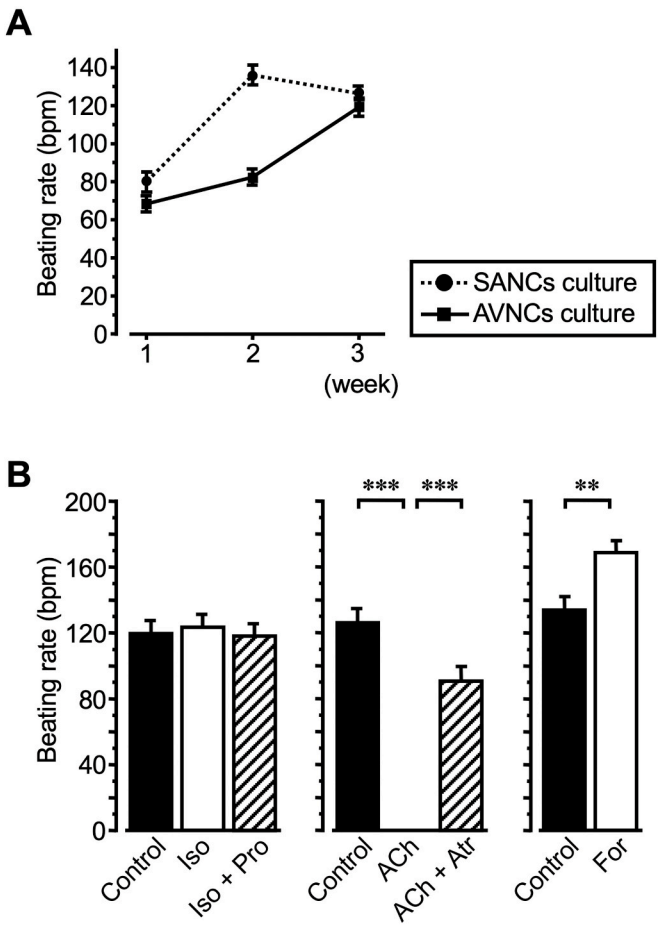

Fig. 3. Beating rate of the newly developed cardiomyocyte clusters in AVNC cultures. (A) Changes over time in the basal spontaneous beating rate of the cardiomyocyte clusters formed during the culture of AVNCs or SANCs (n = 100 for each). (B) Pharmacological characterization of the nascent cardiomyocyte clusters developed in AVNCs culture for 3 weeks. Left panel: response to isoproterenol (ISO, 0.1 μM) . Center panel: negative chronotropic response to acetylcholine (ACh, 1 μM) and its blockade by the antagonist atropine (Atr, 0.1 μM). Right panel: increase in the beating rate by forskolin (For, 3 μM). Drugs were dissolved in Tyrode's solution to the final desired concentration and applied to the dish by replacing the medium with drug-containing solutions. Data were summarized from 16 independent experiments and expressed as mean ± S.E.M. Vertical bars denote S.E.M.; Tukey's test; **p < 0.01, ***p < 0.001.

cultures continuously increased up to 3 weeks in culture, whereas the beat rate in SANC culture reached its maximum level at 2 weeks of culture. Thirdly, as discussed above, whereas the SANC culture clusters expressed functional β_1 adrenoceptor system, the AVNC culture clusters did not fully develop it during the 3 weeks culture period at least. It seems likely that maturation of the nascent cardiomyocyte clusters is slower in AVNC culture compared with that in SANC culture.

In our previous study, beating SANCs but not non-beating atrial myocytes generated cardiomyocyte clusters in their vicinity, and we found that the generation of action potentials in SANC was critical for the cluster formation. The spontaneous firing rate of AVNCs is generally lower than that of SANCs in the heart [10–14]. As shown in the present study, the beating rate of clusters in AVNC culture was lower than that in SANC culture in the culture period studied. Since structural and functional cluster formation is slower in AVNC cultures, it is possible that the maturation of myocyte clusters depends, in part, on the beat rate. We have preliminary results showing that culture of isolated cells from the His bundle also generates surrounding beating cell clusters, with a slower growth rate and lower beating rate. Therefore, future confirmatory studies on the role and/or effect of beating rate on the maturation of cardiomyocytes are warranted.

The beat rate observed in the present study seems to be under the control of the originally seeded nodal cells. Pharmacological evaluation of acetylcholine and isoproterenol in generated clusters revealed the functional integrity of the formed cardiomyocytes. However, contraction of the clusters depends on the formation of electrical syncytium between the cells in the clusters as well as the presence of pacemaker cells. A limitation of the study, therefore, is that the action site of the pharmacological effect was not specified on either the initially seeded AVNCs or the newly formed cell clusters grown around the AVNCs during the culture.

In summary, the results demonstrate that AVNCs from adult guinea pig hearts generate cardiomyocytes in long-term primary cultures, which supports our hypothesis that specialized cardiomyocytes can form cardiac myocytes in common. Quite similar to our previous study using SANCs [9], intrinsic electrical activity seems to be critical to the formation of cardiomyocyte clusters. Furthermore, the results suggest a possible role for beating rate in the structural and functional maturation of clusters. Both SANC and AVNC are specialized cardiomyocytes localized in the conduction system of the heart, with limited numbers in limited areas. This may imply that such specialized cardiac cells can contribute to intrinsic turnover of cardiac cells *in vivo* during one's lifetime. These findings may also be applicable to the development of regenerative therapies for the adult heart, by proposing a novel way to generate cardiomyocytes other than previously reported approaches such as the direct gene transfer to and chemical reprogramming of fibroblasts to obtain cardiomyocytes [19–21].

Author contributions

S.K. and K.O. conceived and designed the study and wrote the manuscript. S.K. and A.U. performed most of the experiments and data analysis. T.S. provided suggestions for molecular biological experiments. S.S. provided suggestions for pharmacological experiments. F.O. participated in the project and provided suggestions for molecular biological experiments. K.O. directed the project and supervised the experiments.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.The authors declare no conflicts of interest.

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