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Original Article

Virulence factors released from *Porphyromonas gingivalis* induce electrophysiological dysfunction in human pluripotent stem cell-derived cardiomyocytes

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KEYWORDS
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Abstract *Background/purpose:* Periodontal disease development correlates with the occurrence of systemic diseases. The present study investigated the association between periodontal disease and the development of cardiac arrhythmia.
Materials and methods: Human embryonic stem cell-derived cardiomyocytes (hESC-CMs) were treated with *Porphyromonas gingivalis* (Pg). Cardiotoxicity and electrophysiological properties of hESC-CMs were measured using the cell counting kit-8 assay and a multi-electrode array, respectively. Reverse-transcription-quantitative polymerase chain reaction (RT-qPCR) revealed the mRNA expression of S100 calcium binding protein A1 (*S100A1*), calsequestrin 2 (*CASQ2*), troponin I3 (*TNNI3*), myosin light chain 2 (*MYL2*), integrin subunit beta 1 (*ITGB1*), and cadherin 2 (*CDH2*) in hESC-CMs.
Results: Treatment with Pg broth significantly decreased the beat period, field potential duration, spike amplitude, and conduction velocity without affecting the viability of hESC-CMs. In addition, the mRNA expression of *CASQ2*, *TNNI3*, and *MYL2*, which are all associated with calcium handling, were downregulated by Pg broth treatment.

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Conclusion: These findings indicate that Pg may induce cardiac arrhythmia mediated by virulence factors.

Introduction

Periodontal disease (PD) is one of the most common chronic inflammatory diseases affecting the gingival tissues that support the teeth in humans. Epidemiological studies have revealed that PD is prevalent in nearly 50% of the world's population and that its occurrence increases with age.[1,2] PD begins with gingivitis, an inflammatory condition of the gingiva that is primarily caused by bacteria in dental plaque. *Porphyromonas gingivalis* (Pg), a key periodontopathogen in dental plaque, has been proposed to be closely correlated with the progression of PD.[3,4]

Pg is a gram-negative, rod-shaped, obligate anaerobe that constitutes a mature subgingival biofilm, and is considered a major etiological component in the pathogenesis of periodontal inflammation.[5] Approximately 79% of patients with periodontitis harbor Pg colonies in their oral cavity, and the levels of Pg are positively correlated with the depth of the periodontal pocket.[6] Throughout evolution, Pg has developed the ability to survive in the commensal bacterial community and persist in host tissues.[7] Its survival strategies largely depend on potential virulence factors, including cysteine proteases (gingipains),[8] lipopolysaccharide (LPS),[9] fimbriae,[10] and outer membrane vesicles.[11] Furthermore, virulence factors confer Pg with the ability to be involved in the occurrence and development of many systemic diseases such as cancer,[12] Alzheimer's disease,[13] and atrial fibrillation.[14]

Cardiac arrhythmia (or dysrhythmia), specifically nonsustained ventricular tachycardia and sustained atrial fibrillation, refers to irregularities in the heartbeat caused by disease-related changes in the conduction system of the ventricular and atrial myocardium. Arrhythmia can be induced by systemic inflammation. Persistent local infection induced by PD stimulates circulating levels of inflammatory molecules, such as IL-1, IL-6, TNF-α, CRP, and IFN, which have been associated with a higher risk of arrhythmia.[15] The detection of periodontal bacterial DNA in the atrium and myocardium further supports the possibility that PD is associated with myocardial inflammation.[16] However, the exact mechanisms underlying the link between PD and cardiac arrhythmia remain unclear.

Due to the lack of a human cardiomyocyte cell line and difficulties in acquiring primary human cardiomyocytes, we differentiated functional cardiomyocytes (CMs) from human embryonic stem cells (hESCs). hESC-CMs have been demonstrated to be a good alternative for cardiac disease modeling, drug screening, and cardiotoxicity testing, as they are known to have electrophysiological properties similar to those of native cardiomyocytes.[17,18] In this study, we investigated the effects of Pg culture broth on the survival, electrophysiological properties, and transcriptional activity of hESC-CMs to ascertain the association between periodontopathogenic bacteria and cardiac arrhythmia.

Materials and methods

hESC maintenance and cardiac differentiation

H9 embryonic stem cells were purchased from the WiCell Research Institute (Madison, WI, USA). hESCs were maintained in a feeder-free culture on ESC-Matrigel in mTeSR1 medium (STEMCELL Technologies, Vancouver, BC, Canada). All hESC-CM experiments were performed using H9 cells between passages 30 and 40. Cardiac differentiation was performed via small molecule-based modulation of Wnt signaling, as previously described.[19,20] To induce cardiac differentiation, hESCs were detached using accutase and allowed to grow for 3 days. The media were switched to RPMI-1640 supplemented with B27 minus insulin (Thermo Fisher Scientific, Waltham, MA, USA) on day 0. Cells were treated with CHIR99021 (12 μM, Selleck Chemicals LLC, Houston, TX, USA), recombinant activin A (50 ng/mL, PeproTech, East Windsor, NJ, USA), and L-ascorbic acid (50 μg/mL, Sigma–Aldrich, Burlington, MA, USA) for 24 h, followed by endo-IWR 1 (5 μM, Tocris Bioscience, Bristol, UK) for 5 days, of which ascorbic acid (50 μg/mL) was supplemented during the first 3 days. Thereafter, cells were cultured in RPMI-1640 supplemented with B27. For cardiac purification, metabolic selection was performed between days 10 and 14 using glucose-free RPMI medium supplemented with 4 mM lactate, 0.5 mg/mL recombinant human albumin, and 213 μg/mL L-ascorbic acid 2-phosphate. All experiments were conducted with day 30 hESC-CMs in the present study.

Flow cytometry

hESC-CMs on day 30 from the start of their differentiation were fixed in 4% paraformaldehyde in PBS. For permeability, cells were incubated in 0.1% Triton X-100 in PBS for 20 min and blocked in 5% BSA containing PBS. Cells were stained with fluorescently-conjugated cardiac troponin T (cTnT) antibody (abcam, Cambridge, UK) in PBS containing 5% BSA and 0.2% Tween 20 for 30 min at room temperature. Stained cells were washed three times in PBS containing 0.2% Tween 20. The samples were analyzed using an Attune NxT Flow Cytometer (Thermo Fisher). Data were collected and analyzed using the FlowJo software.

Immunofluorescence

For immunofluorescence staining, hESC-CMs were fixed with 4% paraformaldehyde for 15 min and permeabilized with 0.2% Triton X-100 for 10 min. After blocking with 5% BSA, cells were incubated with anti-sarcomeric α-actinin (SAA) anti-cardiac troponin T (cTnT) antibodies for 16 h, followed by incubation with secondary antibodies (Alexa Fluor 488 goat anti-mouse IgG and Alexa 647 donkey anti-rabbit IgG) for 1 h. The cells were mounted with Vectashield medium (Vector Laboratories. Inc, Burlingame, CA, USA) containing 4′,6-diamidino-2-phenylindole (DAPI) for visualization of the nuclei. A confocal laser scanning microscope (LSM 700, Zeiss, Oberkochen, Germany) was used to collect images.

Bacterial culture and broth preparation

Pg strain W83 was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in gifu anaerobic medium broth (Nissui Pharmaceutical, Tokyo, Japan), which contained vitamin K (5 μg/mL) and hemin (5 μg/mL) at 37 °C in an anaerobic chamber in an atmosphere containing 90% N_2, 5% H_2, and 5% CO_2. For culture broth collection, freshly grown bacterial cultures (OD 660 = 1.0, equivalent to 1×10^9 CFU/mL) were centrifuged twice at 8000×*g* at 4 °C for 15 min) and the supernatant was collected, followed by filtration with a 0.2 μm syringe filter (Sartorius, Goettingen, Germany). All experiments were conducted with 0.5% of culture broth in the present study.

Cell counting kit 8 assay

hESC-CMs were seeded in 96-well plates (Nunc, Roskilde, Denmark) at a density of 1×10^4 cells/well. On days 0, 1, and 2 after changing the medium containing either control (CTL) or Pg broth, cell viability was assessed by adding 20 μL of cell counting kit-8 (CCK-8) solution (Dojindo, Rockville, MD, USA). The absorbance was measured at 450 nm using an Opsys MR microplate reader (DYNEX Technologies Inc., Denkendorf, Germany).

Reverse-transcription-quantitative polymerase chain reaction (RT-qPCR)

Total RNA was purified using the RNeasy Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions, and 2 μg of RNA was reverse-transcribed under standard conditions using Superscript II (Invitrogen, Waltham, MA, USA). For qPCR analysis, 50 ng of cDNA was mixed with SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) and amplified for 40 cycles using an AB7500 (Applied Biosystems). Experiments were performed in triplicate and the data were normalized to β-actin. Data were analyzed using the $2^{-\Delta\Delta Ct}$ method. The primer sequences used were as follows: S100A1: 5′-TTCCTGGATGCCCAGAAGGATG-3′, 5′-CCGTCTCCATTCTCGTCTAGC-3′; CASQ2: 5′-TTGCCATCCCCAACAAACCT-3′, 5′-AGAGTGGGTCTTTGGTGTTCC-3′; TNNI3: 5′-CCTCACTGACCCTC-CAAACG-3′, 5′-GAGGTTCCCTAGCCGCATC-3′; MYL2: 5′-TTGGGCGAGTGAACGTGAAAA-3′, 5′-TCCGCTCCCTTAAGTTTCTCC-3′; ITGB1: 5′-GGATTCTCCAGAAGGTGGTTTCG-3′, 5′-TGCCACCAAGTTTCCCATCTCC-3′; CDH2: 5′-CCTCCAGAGTTTACTGCCATGAC-3′, 5′-GTAGGATCTCCGCCACTGATTC-3′; β-actin: 5′-ACTCTTCCAQGCCTTCCTTCC-3′, 5′-TGTTGGCGTACAGGTCTTTG-3′.

Electrophysiological measurement

Field potential duration (FPD) and action potential duration were analyzed using microelectrode arrays (MEAs) following the procedure described in manufacturer's instructions (Maestro Edge, Axion Biosystems, Atlanta, GA, USA). The cells were plated on fibronectin-coated (50 μg/mL, Sigma–Aldrich) electroarray plates. Cells were maintained in the absence or presence of either CTL or Pg broth medium for 48 h. The electrophysiological events of hESC-CMs were recorded every 3 h using Axion cardiac analysis. Plots were generated using GraphPad Prism software and MATLAB.

Statistical analysis

All data were obtained from at least three independent experiments conducted in triplicate. Results of multiple observations are presented as mean ± SEM. To analyze multivariate data, group differences were assessed using one-way ANOVA followed by Bonferroni post-hoc test.

Results

Characterization of CM differentiated from hESCs

We first differentiated human embryonic stem cells into CMs by sequential treatment with CHIR99021, IWR-1, and activin A. Metabolic selection was applied to isolate high-purity CMs (Fig. 1A). The CM purity assessed by flow cytometry for cTnT ranged from 90% to 96% (n = 8 independent differentiation experiments; Fig. 1B). CMs showed normal cardiac sarcomere organization, as verified by immunofluorescence staining of SAA and cTnT (Fig. 1C). These results indicate that our protocol can efficiently achieve CM differentiation with high purity.

Effects of Pg broth on hESC-CM viability

To examine the stimulatory effect of Pg on CMs, culture supernatants were prepared by culturing broth in the absence (CTL broth) or presence of Pg (Pg broth) under anaerobic conditions. Control CMs received no treatment (wild type [WT]; Fig. 2A). To evaluate whether Pg broth could stimulate the cytotoxicity of CMs, cell viability was measured using the CCK-8 assay. After 48 h of incubation, the viability of CMs was not changed by Pg broth treatment compared with WT or CTL broth-treated CMs.

Pg broth stimulates abnormalities of electrophysiological properties in hESC-CMs

To test the effects of Pg broth on the electrical properties of CMs, we utilized MEAs, which provide high throughput by recording extracellular field potentials.[21] We assessed the beat period, FPD, and spike amplitude in CMs after CTL or Pg

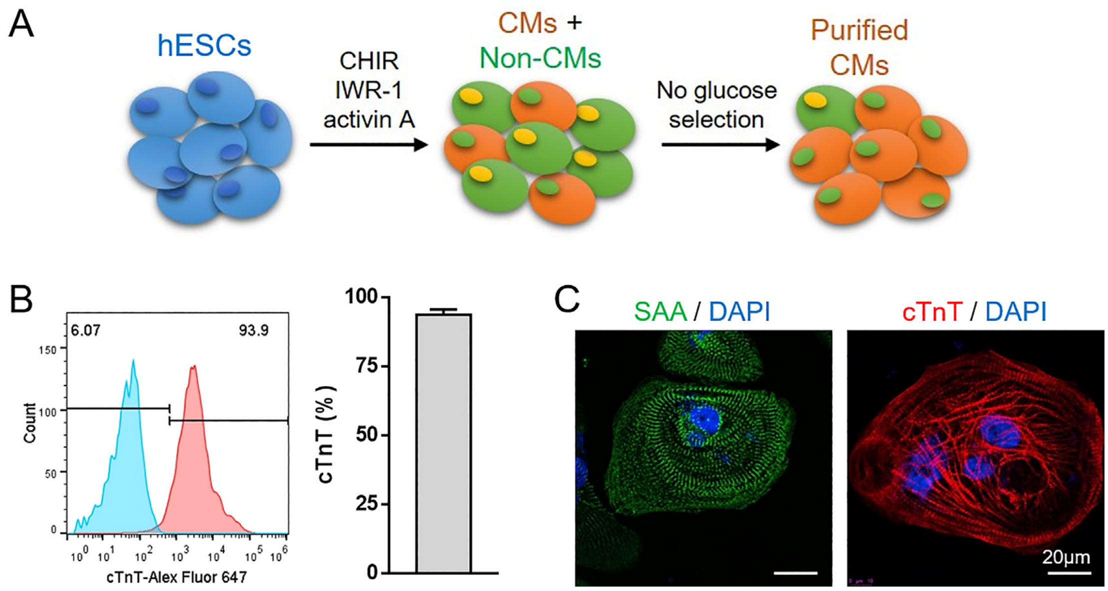

Figure 1 Characterization of CM differentiation from H9 hESCs. (A) Schematic diagram showing the protocol for CM differentiation from hESCs. (B) Representative flow cytometry readout (left) and quantitative analysis (right) of $cTnT^+$ hESC-CM population ($n = 8$). (C) Representative immunofluorescence images of SAA and cTnT in hESC-CMs. Scale bars = 20 μm. CM, cardiomyocyte; cTnT, cardiac troponin T; hESC, human embryonic stem cell.

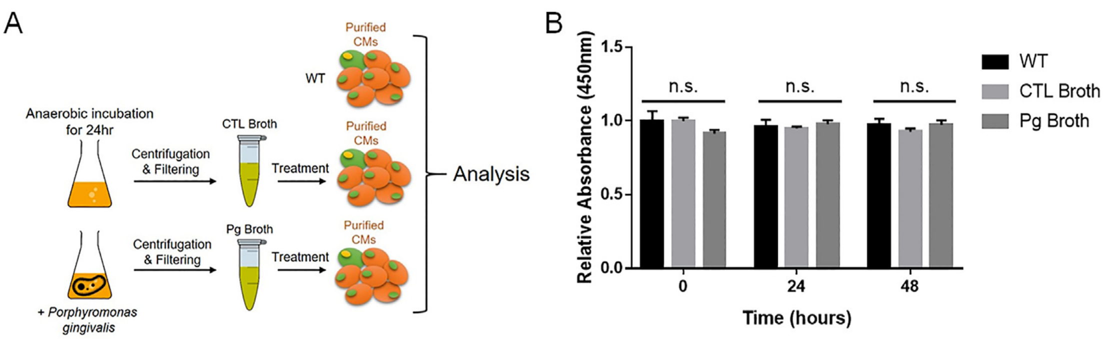

Figure 2 Effects of Pg broth on hESC-CM viability. (A) Schematic diagram showing the preparation of culture broth. (B) hESC-CMs were cultured with or without either CTL broth or Pg broth for the indicated period. Cell viability of cells was analyzed by the CCK-8 assay. Data represent the mean ± SEM. *$P < 0.05$ by one-way ANOVA. n.s., not significant. CCK-8; counting cell kit-8; hESC-CM, human embryonic stem cell-derived cardiomyocytes; Pg, *Porphyromonas gingivalis*.

broth treatment for 48 h by analyzing the electrophysiological signal from the electrode array (Fig. 3A). Fig. 3B shows the typical field potential waveforms of WT CMs or CMs exposed to CTL or Pg broth. Treatment with Pg broth resulted in a significant decrease in beat period (Fig. 3C), FPD (Fig. 3D), and spike amplitude (Fig. 3E). Furthermore, the conduction velocity of CMs was dramatically inhibited by the Pg broth treatment (Fig. 3F and G). These results suggest that Pg broth can affect the electrophysiological properties of CMs, resulting in cardiac arrhythmia.

Effects of Pg broth on the expression of transcription factors in hESC-CMs

To assess the potential changes in the molecular signatures underlying cardiac arrhythmia induced by Pg broth, we performed qRT-PCR. Compared to WT and CTL broth-treated CMs, Pg broth treatment altered the mRNA expression of S100 calcium binding protein A1 (*S100A1*) and calsequestrin 2 (*CASQ2*), which affect excitation-contraction (E-C) coupling. The expression of *S100A1* increased, whereas that of *CASQ2* decreased by 2-day treatment with Pg broth (Fig. 4A). Moreover, the mRNA expression of structure-related genes, including troponin I3 (*TNNI3*) and myosin light chain 2 (*MYL2*), were significantly downregulated by Pg broth treatment (Fig. 4B). We found no significant changes in the mRNA expression of adhesion-related genes such as integrin subunit beta 1 (*ITGB1*) and cadherin 2 (*CDH2*) (Fig. 4C). These results suggest that Pg broth may induce cardiac arrhythmia by modulating the transcriptional activity of E-C coupling- and structural-related genes.

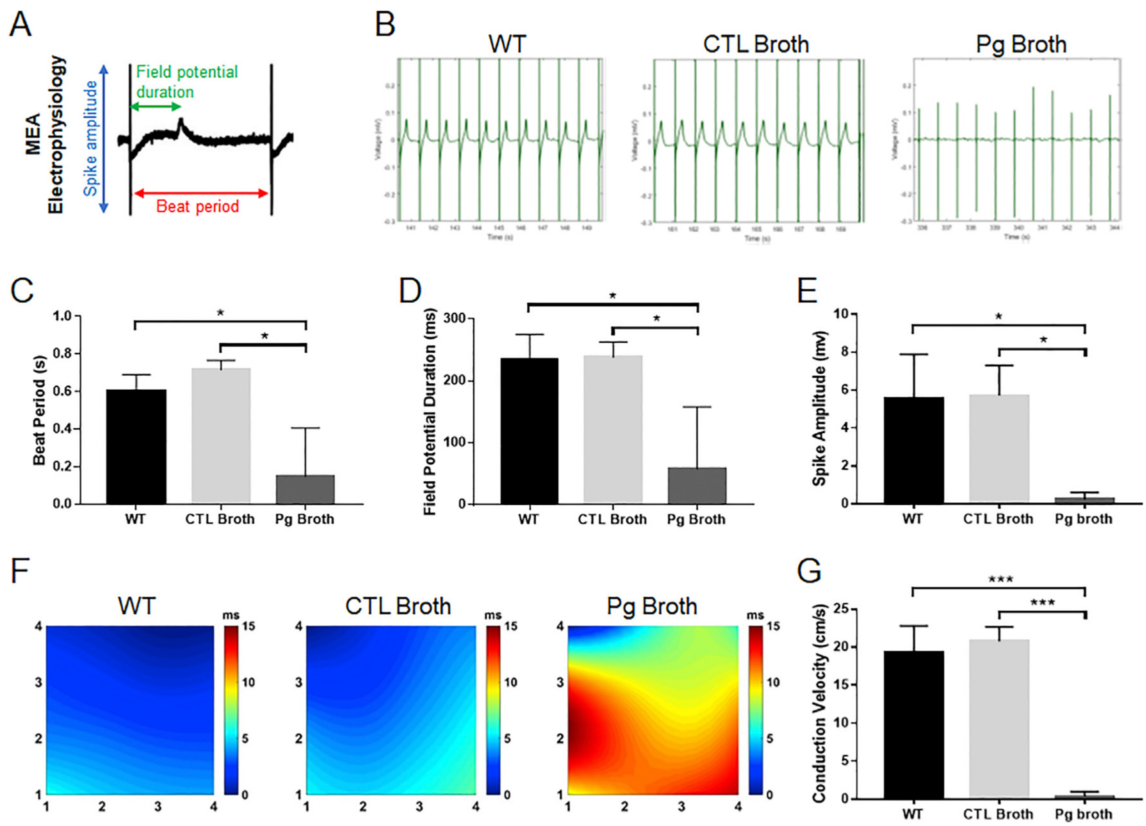

Figure 3 Abnormalities of electrophysiological properties were increased in response to Pg broth in hESC-CMs. Cells were cultured with or without either CTL broth or Pg broth for 48 h and electrophysiological properties were analyzed by MEA. (A) A parameter of interest for analysis of electrophysiological properties recording with MEA. (B) Representative images of field potential record in hESC-CMs (C—E) Quantitative analysis of beat period, FPD, and spike amplitude in hESC-CMs. (F, G) Representative images of isochrones activation maps and quantitative analysis of conduction velocity in hESC-CMs. Data represent the mean ± SEM. *$P < 0.05$ and ***$P < 0.001$ by one-way ANOVA. FPD, field potential duration; hESC-CM, human embryonic stem cell-derived cardiomyocyte; MEA, multielectrode array; Pg, *Porphyromonas gingivalis*.

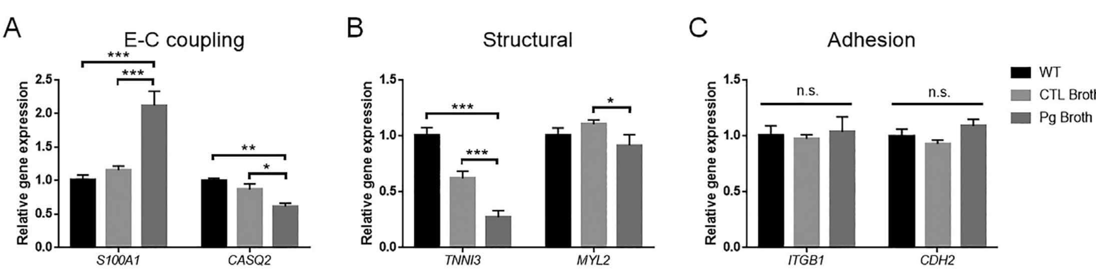

Figure 4 Effects of Pg broth on transcriptional activity in hESC-CMs. Cells were cultured with or without either CTL broth or Pg broth for 48 h mRNA expression levels of (A) E-C coupling (*S100A1* and *CASQ2*), (B) structural (*TNNI3* and *MYL2*), and (C) adhesion (*ITGB1* and *CDH2*) related genes were determined by qPCR and normalized to β-actin. Data represent the mean ± SEM. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ by one-way ANOVA. n.s., not significant. E-C, excitation-contraction; hESC-CMs, human embryonic stem cell-derived cardiomyocytes; Pg, *Porphyromonas gingivalis*.

Discussion

Mounting evidence demonstrates that Pg is closely correlated with multiple systemic diseases. In approximately 82.61% of cases, Pg DNA was observed in atherosclerotic plaques from patients with atherosclerotic cardiovascular diseases; Pg also aggravates atherogenesis in apolipoprotein E-deficient mice.[22] In addition, Pg bacteremia induces IL-17A-mediated myocarditis and/or myocardial infarction in mice.[23] To our knowledge, the present study is the first to provide evidence that virulence factors released from Pg can induce cardiac arrhythmia. We revealed that administration of Pg broth to CMs resulted in a reduction in the beat period, FPD, spike amplitude, and conduction velocity, which are all associated with arrhythmogenic features of hESC-CMs. Arrhythmogenic events were likely not mediated by cardiotoxicity since the administration of Pg broth did not affect CMs viability in our experimental conditions.

Abnormalities in calcium (Ca^{2+}) homeostasis play a key role in the pathogenesis of cardiac arrhythmia.[24] Calsequestrin (encoded by *CASQ2*) is a high-capacity Ca^{2+}-binding protein located in the sarcoplasmic reticulum of CMs.[25] In *CASQ2*-null mice, ryanodine receptor type-2, which is the principal Ca^{2+} release channel, opens spontaneously without being mediated by L-type Ca^{2+} channels, and mutations in cardiac *CASQ2* have been linked to ventricular arrhythmia in humans.[26,27] In addition, mutations of sarcomeric proteins, including troponin I-3 and myosin light chain-2 (encoded by *TNNI3* and *MYL2*, respectively), are involved in electrophysiological abnormalities through the Ca^{2+} handling defects, thereby inducing both hypertrophy and arrhythmia in patient-specific iPSC-CMs.[28] In the present study, treatment with Pg broth decreased the FPD in CMs, indicating blockade of L-type Ca^{2+} channels and downregulation of the mRNA expression of *CASQ2*, *TNNI3*, and *MYL2*. Although the mRNA expression of *S100A1* was upregulated by Pg broth treatment, the effect of *S100A1* on electrophysiological abnormalities may not be significant, as high myocardial *S100A1* overexpression does not cause cardiac arrhythmia or impairment of contractile function *in vivo*.[29] Collectively, these results suggest that Pg broth induces cardiac arrhythmia by altering Ca^{2+} homeostasis.

In recent years, many researchers have focused on diverse virulence factors of Pg to investigate its pathogenicity. By mediation of fimbriae, Pg significantly upregulates the expression of various adhesion molecules, such as vascular cell adhesion molecule-1, intercellular adhesion molecule-1, and P- and E-selectins in endothelial cells.[30] In addition, Pg LPS exacerbates macrophage-derived foam cell formation by regulating cholesterol efflux and lipid accumulation through a heme oxygenase-1-dependent mechanism.[31] Endothelial dysfunction and foam cell formation are essential steps in atherosclerosis pathogenesis. Gingipains are the primary virulence factors of Pg that are released into the extracellular milieu in soluble or outer membrane vesicle forms. Increasing evidence shows the involvement of gingipains in several systemic diseases, including rheumatoid arthritis,[32] Alzheimer's disease,[33] and cardiovascular diseases.[34] More recently, Dominy et al. designed and synthesized inhibitors targeting gingipains;[13] these compounds prevented gingipain-induced neurotoxicity and rescued neurons in the hippocampus, suggesting the implication of gingipains in Alzheimer's disease. Here, we have not addressed which virulence factors in Pg broth are responsible for inducing cardiac arrhythmia; elucidation of these factors requires further investigation.

In summary, virulence factors released from Pg may induce cardiac arrhythmia. The results demonstrated that treatment with Pg broth caused aberrant electrophysiological properties and downregulated the transcriptional factors associated with Ca^{2+} signaling in hESC-CMs. Overall, this study provides a novel link between PD and cardiac arrhythmia, although further studies are needed to assess the exact mechanism underlying Pg-induced arrhythmia.

Declaration of competing interest

The authors declare that they have no conflicts of interest.

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