

## Original Article

# Ouabain facilitates cardiac differentiation of mouse embryonic stem cells through ERK1/2 pathway

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**Aim:** To investigate the effects of the cardiotonic steroid, ouabain, on cardiac differentiation of murine embryonic stem cells (mESCs).

**Methods:** Cardiac differentiation of murine ESCs was enhanced by standard hanging drop method in the presence of ouabain (20 μmol/L) for 7 d. The dissociated ES derived cardiomyocytes were examined by flow cytometry, RT-PCR and confocal calcium imaging.

**Results:** Compared with control, mESCs treated with ouabain (20 μmol/L) yielded a significantly higher percentage of cardiomyocytes, and significantly increased expression of a panel of cardiac markers including Nkx 2.5, α-MHC, and β-MHC. The α1 and 2- isoforms Na<sup>+</sup>/K<sup>+</sup>-ATPase, on which ouabain acted, were also increased in mESCs during differentiation. Among the three MAPKs involved in the cardiac hypertrophy pathway, ouabain enhanced ERK1/2 activation. Blockage of the Erk1/2 pathway by U0126 (10 μmol/L) inhibited cardiac differentiation while ouabain (20 μmol/L) rescued the effect. Interestingly, the expression of calcium handling proteins, including ryanodine receptor (RyR2) and sarcoplasmic reticulum Ca<sup>2+</sup> ATPase (SERCA2a) was also upregulated in ouabain-treated mESCs. ESC-derived cardiomyocytes (CM) treated with ouabain appeared to have more mature calcium handling. As demonstrated by confocal Ca<sup>2+</sup> imaging, cardiomyocytes isolated from ouabain-treated mESCs exhibited higher maximum upstroke velocity (P<0.01) and maximum decay velocity (P<0.05), as well as a higher amplitude of caffeine induced Ca<sup>2+</sup> transient (P<0.05), suggesting more mature sarcoplasmic reticulum (SR).

**Conclusion:** Ouabain induces cardiac differentiation and maturation of mESC-derived cardiomyocytes via activation of Erk1/2 and more mature SR for calcium handling.

**Keywords:** ouabain; embryonic stem cells; cardiac differentiation

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## Introduction

Embryonic stem cells (ESCs) have the ability to self-renew and differentiate into virtually all cell types of the three embryonic germ layers including cardiomyocytes. They thus represent an unlimited *ex vivo* cell source for cardiac regenerative therapy<sup>[1–4]</sup> as well as an ideal *in vitro* model to investigate complex developmental processes. Spontaneous differentiation of ESCs towards cardiac lineage is generally poor<sup>[1, 5, 6]</sup>. To date, protocols have exploited transcription factors involved in embryonic heart development to direct ESC differentiation

into cardiomyocytes<sup>[5–8]</sup>. In contrast, transcription pathways crucially involved in post-natal hypertrophic growth of cardiomyocytes have not been investigated to improve the efficacy of cardiac differentiation of ESCs.

The cardiotonic glycoside ouabain is a specific inhibitor of the ubiquitous Na<sup>+</sup>/K<sup>+</sup>-ATPase that is responsible for the active transport of Na<sup>+</sup> and K<sup>+</sup> across the plasma membrane of most animal cells. In adult cardiomyocytes, Na<sup>+</sup>/K<sup>+</sup>-ATPase inhibition results in a modest increase in intracellular Na<sup>+</sup>, sufficient to affect the sarcolemmal Na<sup>+</sup>/Ca<sup>2+</sup> exchange and cardiac contractility<sup>[9–11]</sup>. Alterations in concentrations of endogenous cardiotonic glycosides have been reported in various human conditions such as essential hypertension<sup>[12]</sup>, asymptomatic left ventricular dysfunction<sup>[13]</sup> and dilated cardiomyopathy<sup>[14]</sup>. In experimental models, cardiotonic glycoside has cardioprotective effects against ischemia not associated

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with Na<sup>+</sup>/K<sup>+</sup>-ATPase inhibition<sup>[15, 16]</sup>. They also cause transcriptional regulation of several cardiac-growth related genes resulting in hypertrophy of adult cardiomyocytes<sup>[17]</sup>. Extensive subsequent studies of various cell types have revealed that binding of cardiotonic glycosides to Na<sup>+</sup>/K<sup>+</sup>-ATPase in fact activates multiple pathways including cytoplasmic tyrosine kinase Src/epidermal growth factor receptor (EGFR)<sup>[18]</sup>, phosphatidylinositol 3-kinase (PI3K)-Akt<sup>[19]</sup>, phospholipase C kinase, and increased mitochondrial production of reactive oxygen species<sup>[20]</sup>. The downstream signaling pathway nonetheless appears to be cell-type specific. A previous report demonstrated the functional expression of Na<sup>+</sup>/K<sup>+</sup>-ATPase in undifferentiated ESCs as well as ESC-derived cardiomyocytes<sup>[5]</sup> although the effect of ouabain on cardiac differentiation and maturation of ESCs remains unclear. The aims of the present study were thus to determine whether ouabain, the prototypic Na<sup>+</sup>/K<sup>+</sup>-ATPase inhibitor and potent hypertrophic stimulus of adult cardiomyocytes, affects cardiac differentiation and maturity of ESCs. In order to validate the cardiac differentiation of ESCs, we quantified the number of troponin-positive cells using flow cytometry and the expression of a panel of cardiac specific markers in differentiated ESCs. We also studied the effects of ouabain on the extracellular signal-regulated kinase (ERK), c-Jun NH<sub>2</sub>-terminal protein kinase (JNK), and p38 mitogen activated protein kinase (MAPK) during cardiac differentiation of ESCs. Treatment with a specific MAPK inhibitor would be useful to investigate the specific role of MAPK in cardiac differentiation. In addition, the maturity of calcium handling properties of differentiated ESCs was assessed using confocal calcium imaging.

## Materials and methods

### Murine embryonic stem cell culture and *in vitro* cardiac differentiation

Murine (m) ES cell-line D3 (CRL-1934, American Type Culture Collection, Manassas, VA) was used and cultured as previously described<sup>[21]</sup>. Briefly, undifferentiated mESCs were cultured on an irradiation-inactivated monolayer of mouse embryonic fibroblast feeders in Dulbecco's modified Eagle's minimal essential medium (DMEM, Gibco BRL, Karlsruhe, Germany), supplemented with 15% fetal bovine serum (FBS, Gibco BRL, Karlsruhe, Germany), 0.1 mmol/L mercaptoethanol (Sigma-Aldrich, St Louis, MO), non-essential amino acids (stock solution diluted 1:100; Hyclone, Logan, UT) and 1000 U/mL of recombinant mouse leukemia inhibitory factor (LIF) (Chemicon, Hofheim, Germany). To induce cardiac differentiation, embryoid bodies (EBs) were generated from hanging drops of approximately 800 mESCs in 20 µl of culture medium in the absence of leukemia inhibitory factor and feeder cells for two days and then grown in suspension or five more days<sup>[21]</sup>.

### Effect of cardiotonic glycoside, ouabain, on cardiac differentiation

Embryoid bodies were plated on gelatin-coated plate following five day suspension and cultured with 20 µmol/L ouabain dissolved in phosphate-buffered saline (PBS) at a

stock concentration of 10 mmol/L (Sigma-Aldrich, St Louis, MO) for a further seven days. No drug or vehicle was added in the control group. When counting the number of beating EBs, number of day refers to time from plating of EBs onto gelatin-coated plates. For Erk1/2 inhibitory experiments, EBs were treated with U0126 in combination with ouabain for 7 days before FACS counting of cardiomyocytes. Some EBs pretreated with 20 µmol/L ouabain for 7 days were further incubated with 10 µmol/L U0126 (Cell-Signaling Technology, Danvers, MA) (dissolved in DMSO) in serum-free condition for 2 h prior to harvest for Western blotting. All experiments were performed using EBs generated from different passages of <10. Medium with corresponding drug was refreshed every 2 to 3 days.

### Cell viability

Cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) staining method described by Mosmann T and Hansen in a 96-well microtiter plate<sup>[22]</sup>. This method is based on the ability of viable, but not dead cells, to convert MTT to a blue colored formazan. Stock MTT solution (5 mg/mL) was prepared in PBS. Ten EBs were plated onto each 96-well coated plate with 0.1% gelatin following 5-day suspension and differentiation medium served as control. At the end of incubation, 20 µL of MTT solution was added to each well containing 180 µL medium with various concentrations of ouabain. Following 4 h incubation at 37 °C, dark crystals formed and the reaction was stopped by adding 100 µL of DMSO. The optical density (OD) of each well was read on a Bio-Rad 550 Microplate Reader (Bio-Rad Laboratories, Hercules, CA) at 570 nm. The viability values obtained in the presence of various dosages of ouabain were subsequently normalized against control values.

### Isolation of beating mESC-derived cardiomyocytes

The beating outgrowths were microsurgically dissected with a glass knife from D3 mESC-derived 7-day differentiated EBs, and incubated in collagenase B (1 mg/mL) with DNase I (60 U/mL, Roche Applied Sciences Penzberg, Germany) at 37 °C for 30 min with occasional dispersion by pipetting up and down. Isolated cells were recovered in Kraftbrühe (KB) solution containing (mmol/L) 85 KCl, 30 K<sub>2</sub>HPO<sub>4</sub>, 5 MgSO<sub>4</sub>, 1 EGTA, 2 Na<sub>2</sub>-ATP, 5 pyruvic acid, 5 creatine, 20 taurine, and 20 D-glucose at room temperature for 1 h. The cells were subsequently plated on 0.1% gelatin coated glass cover slips in 24-well culture plates with corresponding differentiation medium. Calcium imaging of isolated cells or cell clusters was performed within 2 days; some of the cells were fixed in 4% paraformaldehyde for immunocytological staining at 4 °C.

### Immunocytological staining

The 7-day differentiated EBs were microdissected and fixed with 2% paraformaldehyde for 20 min at 4 °C, followed by washing with wash buffer [DPBS with 0.1% Triton X-100 (Sigma-Aldrich, St Louis, MO)] once for 5 min. Cells were incubated overnight at 4 °C with anti-troponin-T (1:100, Lab

Vision, Fremont, CA) primary antibodies, then rinsed three times for five minutes with wash buffer. Further incubation for one hour at room temperature was performed with secondary antibodies, goat anti-mouse FITC antibodies (1:100, Molecular Probes), diluted in wash buffer. The cells were rinsed three times, counterstained and mounted with Slow-Fade® Gold antifade reagent with DAPI (Invitrogen, Life Technologies, Carlsbad, CA). Fluorescent immunostaining for troponin-T was examined and photographed under a fluorescent microscope (green). The images of troponin-T positive cells were captured by Laser Scanning Systems LSM 510 (Carl Zeiss, Inc, Oberkochen, Germany).

#### Assessment of cardiac differentiation using reverse transcription-polymerase chain reaction

Total RNA from 7-day old EBs was extracted with Trizol® reagent (Invitrogen, life technologies, Carlsbad, CA). Reverse transcription was then performed using 1 µg of total RNA in a final volume of 20 µL, using the QuantiTect® reverse transcription kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Cardiac-specific genes, alpha isoforms of sodium-potassium ATPase and calcium handling proteins

were compared in EBs in the presence or absence of ouabain with quantitative real-time polymerase chain reaction (qPCR). Primer sequence and annealing temperature are depicted in Table 1. GAPDH served as an internal control. Quantitative PCR analysis was performed with a real-time PCR Detector (Opticon 2 DNA Engine, MJ Research, MN, USA) using the iQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA). For amplification, after initial holds for 5 min at 95 °C, 50 cycles of 95 °C for 15 s followed by 57 °C for 30 s and 72 °C for 30 s, melt curve analysis was performed. The relative quantification of PCR products was performed according to the  $2^{-\Delta\Delta Ct}$  method, using mouse GAPDH as an internal control. Where  $\Delta\Delta Ct = [(Ct_{\text{target gene}} - Ct_{\text{GAPDH}})_{\text{control group}} - (Ct_{\text{target gene}} - Ct_{\text{GAPDH}})_{\text{ouabain group}}]$ .

#### Confocal calcium imaging

mESC-derived cardiomyocytes were loaded with 1:1 (*v/v*) amount of 20% Pluronic®-F127 (Invitrogen, Life Technologies) and 5 µmol/L Fluo-3 AM (Sigma-Aldrich, St Louis, MO) dissolved in DMSO with stock concentration of 5 mmol/L for 45 min at 37 °C in Tyrode solution consisting of (mmol/L): 140 NaCl, 5 KCl, 1 MgCl<sub>2</sub>, 1.8 CaCl<sub>2</sub>, 10 glucose and 10 HEPES

**Table 1.** PCR primers and PCR conditions.

Gene	Accession No	Forward/reverse (5'-3')	Annealing temperature (°C)	Reference
Nkx2.5	NM_008700	5'-GCTACAAGTGCAAGCGACAG-3' 5'-GGGTAGGCGTTGTAGCCATA-3'	60	Designed by Primer3
GATA 4	NM_008092	5'-TCTCCAGGAACATCAAACC-3' 5'-GTGTGAAGGGGTGAAAAGG-3'	60	Designed by Primer3
GATA 6	NM_010258.3	5'-CAAAGCTTGCTCCGGTAAC-3' 5'-TGAGGTGGTCGCTTGTGTAG-3'	60	Designed by Primer3
MLC2V	NM_010861	5'-GACCCAGATCCAGGAGTTCA-3' 5'-AATTGGACCTGGAGCCTCTT-3'	60	Designed by Primer3
α-MHC	GI 191623	5'-GATGCCAGATGGCTGACTT-3' 5'-GGTCAGCATGGCCATGTCCT-3'	57	[38]
β-MHC	NM_080728.2	5'-GCCAACCAACCTGTCCAAGTTC-3' 5'-TGCAAAGGCTCCAGGTCTGAGGGC-3'	64	[39]
Na <sup>+</sup> /K <sup>+</sup> ATPase α1	BC021496	5'-CTCCAGCAACAGGACCCGGCG-3' 5'-GATCTCAGCGCCCTTGCAGG-3'	57	[5]
Na <sup>+</sup> /K <sup>+</sup> ATPase α2	BC013561	5'-GAATGGGTTTCTACCATCGCG-3' 5'-GCACAGAACCACCACGTGAC-3'	57	[5]
Na <sup>+</sup> /K <sup>+</sup> ATPase α3	BC020177	5'-GGCAGCCCAGGAACCCACGC-3' 5'-AGGACAGGAAGGAGCGAGG-3'	57	[5]
RyR2	NM_023868.2	5'-TGAGTTCTGCTGTCCTGTG-3' 5'-CTCTGCCAACTCCAAGAAGG-3'	60	Designed by Primer3
NCX-1	NM_011406	5'-TGTGTTTACGTGGTCCCTGA-3' 5'-CTCCACAACCTCCAGGAGAGC-3'	60	Designed by Primer3
SERCA-2a	NM_001110140	5'-AAGCTATGGGAGTGGTGGT-3' 5'-GCAATGCAAATGAGGGAGAT-3'	60	Designed by Primer3
GAPDH	NM_011406	5'-ACATCAAGAAGGTGGTGAAGCAGG-3' 5'-CTCTTGTCTCAGATCCTTGTGG-3'	60	Designed by Primer3

Abbreviation: Nkx2.5, NK2 transcription factor related, locus 5; GATA 4, GATA binding protein 4; GATA 6, GATA binding protein 6; MLC-2v, myosin light chain 2v; α-MHC, alpha-myosin heavy chain; β-MHC, beta-myosin heavy chain; Na<sup>+</sup>/K<sup>+</sup> ATPase, sodium potassium ATPase; RyR2, ryanodine receptor 2; NCX, sodium calcium exchanger; SERCA-2a, sarcoplasmic reticulum Calcium ATPase 2a; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase.

at pH 7.4. The calcium transient of single ESC-derived cardiomyocytes was recorded with a confocal imaging system (Olympus Fluoview System version 4.2 FV300 TIEMPO) mounted on an upright Olympus microscope (IX71) with temporal resolution of the line scan at 274 frames per second (2000 scan per 7.3 s). They were then quantified as the background subtracted fluorescence intensity changes normalized to the background subtracted baseline fluorescence using Image J. The amplitude, maximal upstroke and decay velocity of calcium transient were analyzed by Clampfit version 9.2.0.09. (Axon Instruments, Inc, Foster City, CA).

#### Quantification of cardiac differentiation by flow cytometry

Beating clusters first appeared on day 2 after plating. The percentage of mESC-derived cardiomyocytes was quantified by FACS analysis on day 7 of mESC differentiation. Briefly, 10-cm dishes of EBs were dissociated to a single-cell suspension by collagenase B (1 mg/mL) with DNase (60 U/mL) (Roche Applied Sciences, Penzberg, Germany) treatment, and washed by DPBS twice. Cells were permeabilized for 15 min using a Cytotfix/Cytoperm permeabilization kit (BD Biosciences, San Diego, CA), treated with 1% fetal bovine serum to block non-specific antigens and incubated overnight at 4 °C. Cells were then stained with monoclonal anti-Troponin T [dilution 1:100; Cat no: MS-295-P0; Cardiac Isoform Ab-1 (Clone 13-11)], NeoMarker, Fremont, CA) for 1 h. After twice rinsing in washing buffer, anti-mouse IgG H+L-PE was used for secondary antibody staining (dilution 1:100; Beckman Coulter, Fullerton, CA, USA) for one more hour. Analysis was performed with a Beckman Coulter FC500 flow cytometer in which 10 000 events were counted. The background signal was determined using IgG<sub>1</sub> isotypic control as the primary antibody.

#### Measurement of ERK1/2, JNK, and p38 Western blot analysis

Cells were washed with PBS, and collected in RIPA buffer (Cell Signaling Technology, Danvers, MA) containing 0.2% Triton X-100, 5 mmol/L EDTA, 1 mmol/L PMSF, 10 µg/mL leupeptin, 10 µg/mL aprotinin, with additional 100 mmol/L NaF and 2 mmol/L Na<sub>3</sub>VO<sub>4</sub> and lysed for 30 min on ice. Protein assay was performed using a Bio-Rad protein assay kit (Hercules, CA): 20 µg of protein was loaded per well on a 12.5% sodium dodecyl sulphate (SDS)-polyacrylamide gel. Proteins were subsequently transferred onto 0.45 m pore size nitrocellulose membranes and blocked with 5% non-fat dry milk in TBS (pH 7.4) with 0.5% Tween-20 at 4 °C. The blots were challenged with primary antibody (1:1000) overnight at 4 °C, followed by washing three times with TBST (0.1% Tween-20), then challenged with HRP-conjugated goat anti-rabbit (dilution 1:2000; Cell Signaling Technologies) respectively, followed by detection with enhanced chemiluminescent substrate (Millipore, Billerica, MA). As primary antibodies, the rabbit polyclonal anti-p38 MAPK, anti-ERK, anti-JNK, directed against the phosphorylated form of the proteins (dilution 1:1000; Cell Signaling Technology, Danvers, MA).

#### Statistical analysis

Continuous variables are expressed as mean±standard deviation. Statistical comparisons were performed using Student's *t* test. Calculations were performed with SPSS (version 14.0). A *P* value <0.05 was considered statistically significant.

#### Results

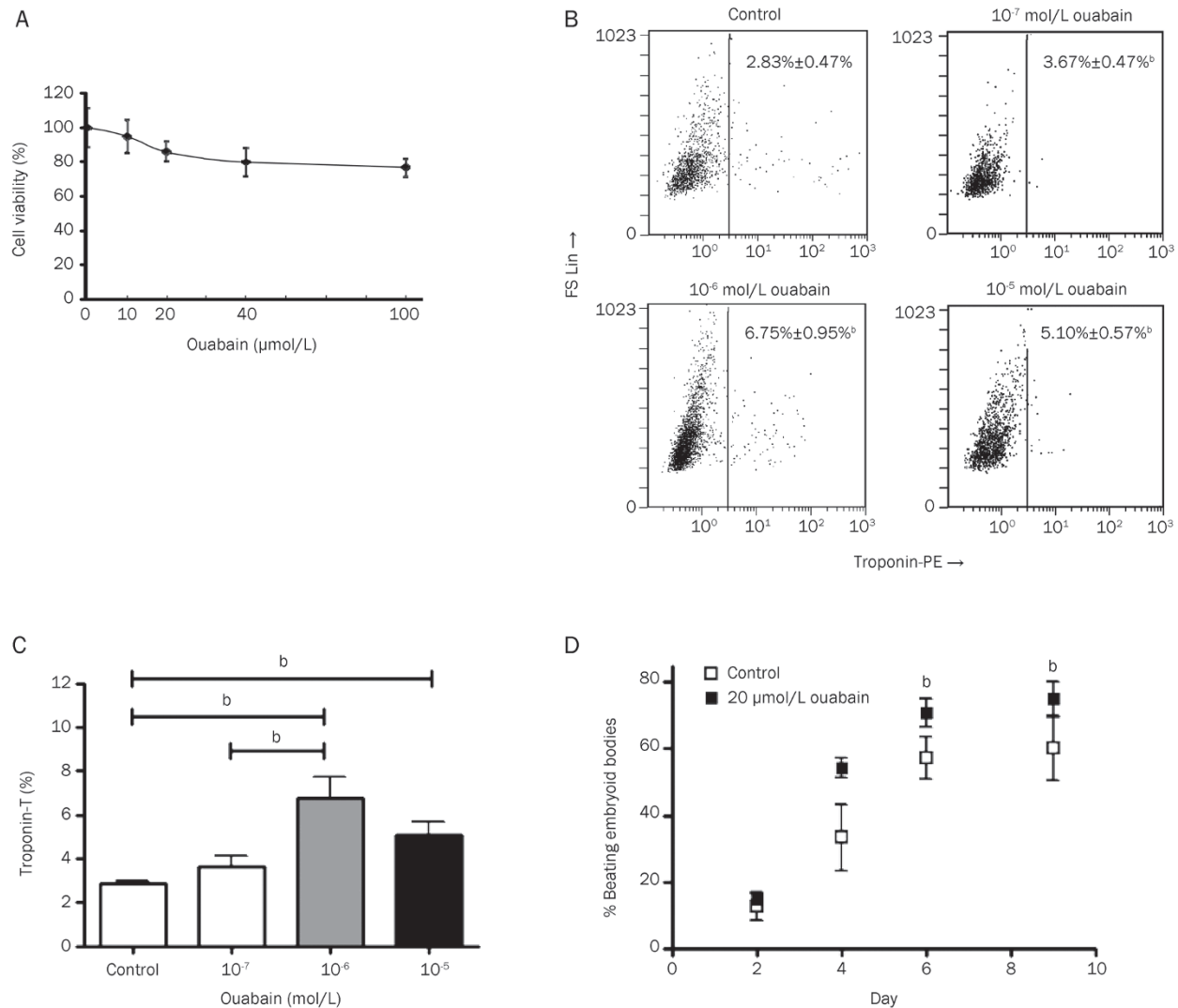
##### Ouabain enhanced cardiac differentiation of mESCs

The effect of ouabain on the viability of differentiating mESCs was determined by MTT assay at different concentrations of ouabain for 72 h. Ouabain was well tolerated even in relatively high concentrations in differentiating mESCs (Figure 1A). To assess whether ouabain treatment enhances cardiac differentiation of mESCs and to determine the optimal range ouabain dosage in enhancing cardiac differentiation, flow cytometry to determine the percentage of cardiomyocytes as identified by troponin-T positive cells was performed. As depicted in Figure 1B and 1C, the optimal dosage of ouabain to enhance cardiac differentiation of mESC was about 10 µmol/L (Figure 1A and 1B). Taken together with the viability test, ouabain at concentration of 20 µmol/L was selected for subsequent experiments. Cardiac differentiation of undifferentiated mESCs was assessed by the percentage of spontaneous beating EBs under the microscope and troponin-T positive cells using flow cytometry at d 7. In this study, spontaneous beating outgrowths from EBs were first observed on d 2 in both ouabain-treated EBs and controls (12.5%), and progressively increased until reaching a plateau at d 9. The administration of ouabain resulted in a higher percentage of spontaneous beating outgrowths of EBs compared with controls from d 6 to d 9 (Figure 1B). Standard, counting of beating outgrowths from EBs is nevertheless a very crude measurement of the efficiency of cardiac differentiation<sup>[4]</sup>. The percentage of cardiomyocytes was consistently significantly higher in the ouabain group (9.50%±1.82% vs 2.90%±0.20%; *n*=3, *P*<0.05).

Figure 2 shows the immunocytochemical pattern of cardiac-specific cytoskeletal proteins including troponin-T in mESC-derived cardiomyocytes. Cardiomyocytes derived from controls showed a homogeneous distribution of troponin-T protein resembling early stage cardiomyocytes (Figure 2)<sup>[23]</sup>; cardiomyocytes from the ouabain group demonstrated striations of the myofilament specific protein indicating sarcomere development, a marker for late-stage cardiomyocytes.

##### Ouabain-induced expression of cardiac specific genes

The effect of ouabain on the expression of cardiac marker genes in EBs was examined by quantitative RT-PCR. The expression of cardiac transcription factors, including Nkx2.5, significantly increased (almost double) in the ouabain group on d 7 (*n*=3, *P*<0.05), but not GATA-4 and GATA-6, the transcription factors common for mesoderm and endoderm (Figure 3A). In addition, gene expression of sarcomeric muscle proteins (α-MHC and β-MHC) was increased in the ouabain group compared with the control (*n*=3; *P*<0.05 and



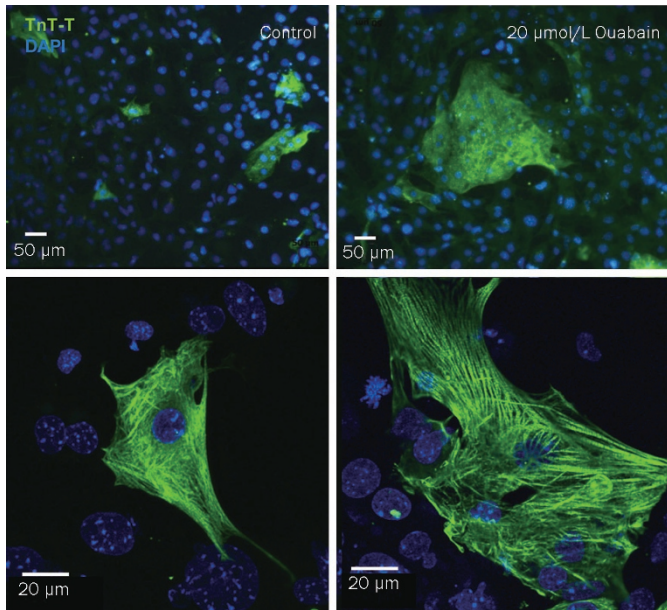
**Figure 1.** Enhanced *in vitro* cardiac differentiation with ouabain. (A) The effect of ouabain on the viability of differentiating D3 mESCs. The EBs were plated and exposed to various dosages of ouabain for 72 h and the viability of cells determined by MTT assay. Data are expressed as mean±SEM ( $n=6$ ). (B) Dot plots of the percentage of mESC-derived cardiomyocytes (troponin-T positive cells) as determined by flow cytometry at various concentrations of Ouabain. (C) Bar chart of the Troponin-T positive cell counts. (D) Effect of ouabain on percentage of beating clusters of the differentiated ESCs. Number of day is defined as time beating cluster shown after plating of suspended EBs. Data are expressed as mean±SEM ( $n=3$ ), whereas the significant difference was tested between ouabain-treated and control for each time point by unpaired *t*-test, <sup>b</sup> $P<0.05$ .

$P<0.01$  respectively). Of note, the expression of  $\alpha$ -MHC, the adult isoform of myosin heavy chain, was markedly up-regulated with ouabain treatment by about 3.5-fold (Figure 3A). The authenticity of increase  $\alpha$ -MHC gene expression was confirmed with Western blot experiment (Figure 3B). As a potential target of ouabain, the expression kinetics of  $\alpha$  subunit isoforms of  $\text{Na}^+/\text{K}^+$  ATPase in mESCs during differentiation was also investigated using RT-PCR. The mRNA of all three isoforms:  $\alpha 1$ ,  $\alpha 2$ , and  $\alpha 3$  subunits was detected in both undifferentiated mESCs and mESC-derived cardiomyocytes at 7-day differentiation; nonetheless  $\alpha 1$  and 2 isoforms were the most responsive to ouabain treatment (Figure 3C). In addition, mRNA expression of a panel of calcium handling proteins, including RyR2 and SERCA2a, (Figure 3D) was up-

regulated in the ouabain group.

#### Ouabain-enhanced calcium handling of cardiomyocytes derived from mESCs

To investigate whether up-regulation of calcium handling proteins in cardiomyocytes from the ouabain group was associated with more mature calcium handling properties, spontaneous calcium oscillations in single cardiomyocytes isolated from ouabain-treated EBs were characterized on d 7 using confocal laser microscopy and compared with that of the control. Consistent with the up-regulated calcium handling proteins, the ouabain group exhibited more mature calcium handling properties (Figure 4A). Specifically, cardiomyocytes from the ouabain group generated larger calcium transients



**Figure 2.** (A) Detection of typical cardiomyocyte proteins in mESC-CM of control (left) and ouabain-treated group (right). The cells were stained with anti-troponin T antibody (green). Nuclei are stained with DAPI (blue).

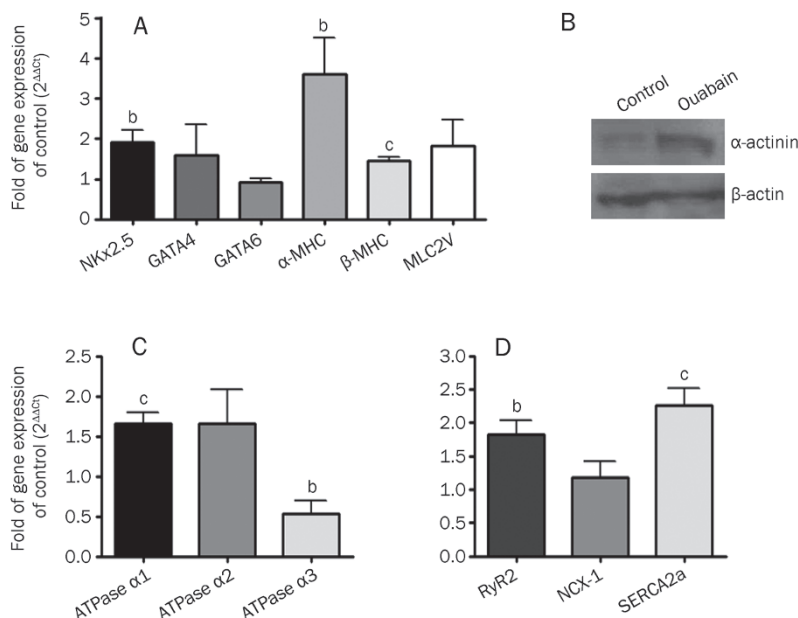
[14.52±4.45 (*n*=7) vs 6.86±0.87 (*n*=7)], as well as a significantly higher maximal and decay velocity (*n*=7; *P*<0.01 and *P*<0.05 respectively), suggestive of a more mature sarcoplasmic reticular (SR) function (Figure 4B–4D). Administration of caffeine (10 mmol/L) also elicited a significant larger surge in cytosolic Ca<sup>2+</sup> in the ouabain group (1163±213.9 vs 612.4±99.7; *P*<0.05), indicating a substantial increase in SR calcium content conferred by ouabain treatment (Figure 5).

### Ouabain increases cardiac differentiation of mESCs by Erk1/2 activation

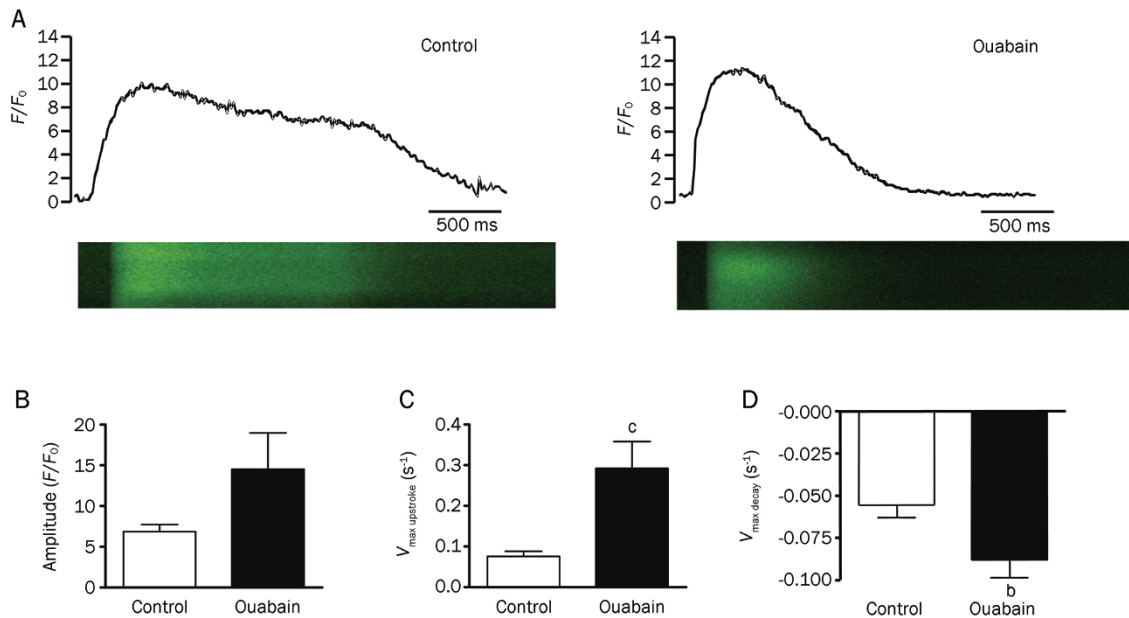
Various MAPK-signaling cascades including ERK, c-JNK and p38 MAPK play important roles in cardiac hypertrophy<sup>[17, 24]</sup> and cardiac remodeling following myocardial infarction. In order to determine whether the enhanced cardiac differentiation on administration of ouabain is related to MAPK-signaling cascades involved in the hypertrophy pathway, phosphorylation of ERK, c-JNK, and p38 MAPK in ouabain-treated EBs was determined and compared with controls. On d 7, ouabain treatment resulted in a significant surge in phosphorylation of ERK1/2 (Figure 6A); no significant difference between the ouabain group and control was found in phosphorylation of either JNK1/2 or p38 MAPK (Figure 6B). The MEK1/2 inhibitor U0126 suppressed ouabain-activated tyrosine phosphorylation of ERK1/2 (Figure 7A), indicating that ERK1/2 was likely activated by ouabain stimulation of MEK1/2 activity. To further study the effect of Erk1/2 activation on cardiac differentiation of mESCs, FACS experiments were performed by blocking the Erk1/2 pathway. Addition of the MEK1 inhibitor, U0126, which subsequently blocks MEK1/2, upstream of ERK1/2, suppressed the percentage of troponin-T positive cells to 0.53%±0.28% (*n*=3, *P*<0.01 compared with control) (Figure 7) while ouabain rescued the effect to control levels (*n*=3, *P*<0.05 compared with U0126).

### Discussion

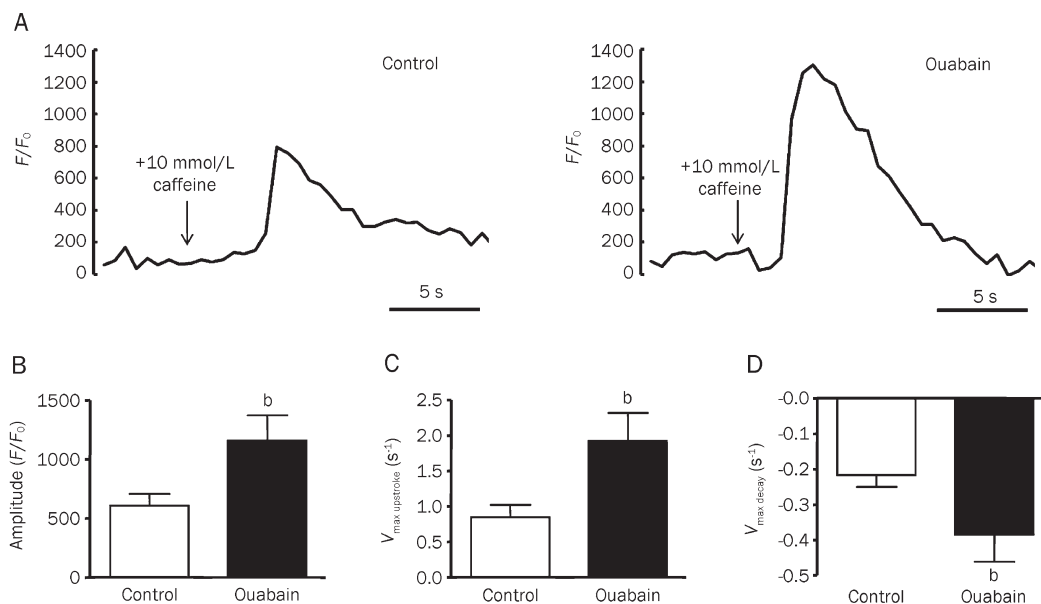
We evaluated the effects of ouabain on cardiac differentiation of mESCs. Our results demonstrate that ouabain promotes cardiogenesis and myofibrillogenesis of ESCs, and matures the calcium handling properties of cardiomyocytes derived there-



**Figure 3.** (A) Cardiac maker gene expression of mESC with ouabain-induced cardiac differentiation as revealed by quantitative RT-PCR. Nkx2.5, NK2 transcription factor related locus 5; GATA 4 and 6, GATA-binding protein 4 and 6; MLC2V, myosin light chain 2 ventricular transcripts; α- and β-MHC, α- and β-myosin heavy chain. GAPDH was used as internal control. (B) Western blot of cardiac cytoskeletal protein, alpha-MHC in differentiated mESCs. (C) Gene expression of three isoforms of sodium-potassium ATPase (Na<sup>+</sup>/K<sup>+</sup> ATPase), α1, α2, and α3. (D) Gene expression of calcium handling proteins on sarcoplasmic reticulum (SR). Three different experiments were repeated with similar results. RyR2, ryanodine receptor 2; SERCA2a, sarcoplasmic reticulum Ca<sup>2+</sup> ATPase; NCX-1: Na/Ca exchanger. GAPDH was used as internal control. Data shown as mean±SEM from 3 independent experiments, significance difference was tested by unpaired *t*-test with <sup>b</sup>*P*<0.05 and <sup>c</sup>*P*<0.01.



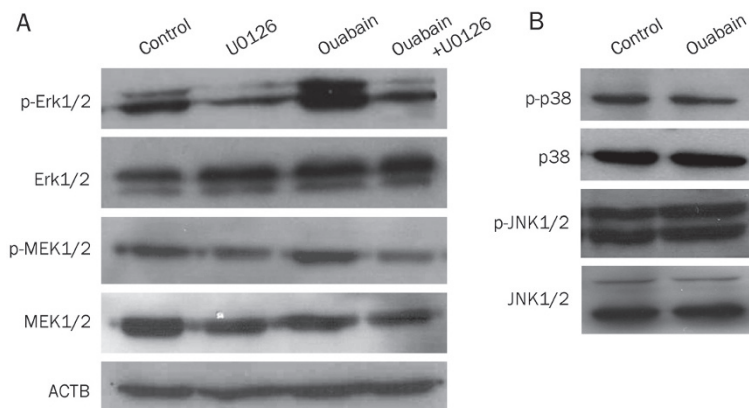
**Figure 4.** (A) Representative tracings of rhythmic spontaneous  $Ca^{2+}$  transients showing in ouabain treated ESC-CM (right) and control (left). (B) Amplitude, (C) Maximal upstroke velocity ( $V_{\max \text{ upstroke}}$ ), (D) Maximal decay velocity ( $V_{\max \text{ decay}}$ ) of  $Ca^{2+}$  transients in the mESC-derived cardiomyocytes; Data shown as mean $\pm$ SEM ( $n=7$ ), seven cells of interest were selected from in three independent experiments. Unpaired  $t$ -test was performed between ouabain treated and control group; <sup>b</sup> $P<0.05$ , <sup>c</sup> $P<0.01$ .



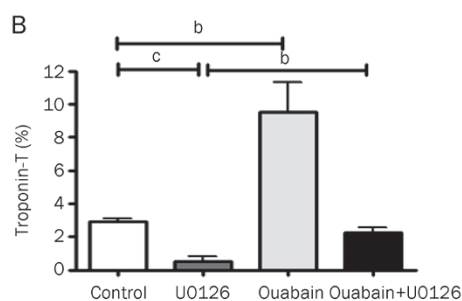
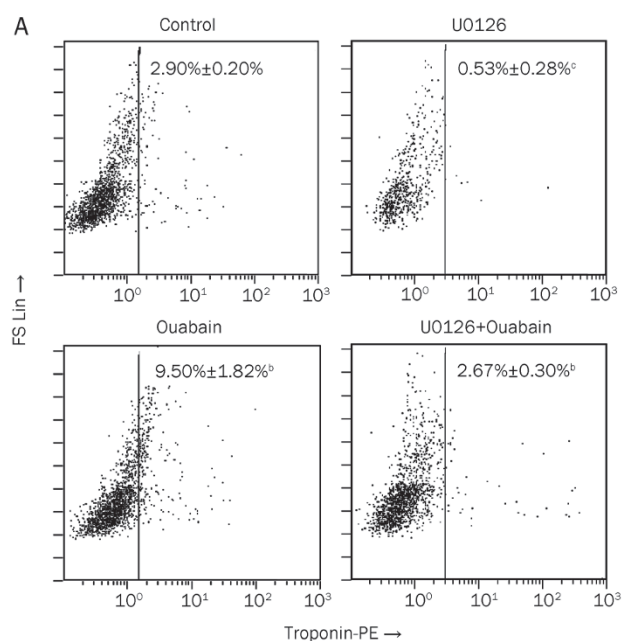
**Figure 5.** (A) Representative tracings of caffeine-induced SR  $Ca^{2+}$  release in control (left) and ouabain-treated (right) mESC-CM, demonstrating caffeine-sensitive  $Ca^{2+}$  stores and fractional release of total SR  $Ca^{2+}$  load during spontaneous activation. (B) Amplitude, (C) Maximal upstroke velocity ( $V_{\max \text{ upstroke}}$ ), (D) Maximal decay velocity ( $V_{\max \text{ decay}}$ ) of  $Ca^{2+}$  transients in the mESC-derived cardiomyocytes; Data shown as mean $\pm$ SEM ( $n=5$ ), five cells were analyzed in three independent experiments. Unpaired  $t$ -test was performed between ouabain treated and control group; <sup>b</sup> $P<0.05$ .

from. The main findings are as follows: 1) Ouabain-treated EBs showed an increased differentiation into cardiomyocytes (in terms of the percentages of beating outgrowths and troponin-positive cardiomyocytes), probably via Erk1/2 activation; 2) Ouabain increased gene expression of cardiogenesis (Nkx2.5)

and myofibrillogenesis ( $\alpha$ -MHC and  $\beta$ -MHC); 3) Ouabain increased the mRNA level of calcium handling protein (RyR2 and SERCA2a) with corresponding maturation of calcium handling properties as determined by confocal microscopy; 4) Ouabain significantly enhanced expression of the  $\alpha 1$  isoform



**Figure 6.** Expression of major MAPK, ERK1/2, p38 and JNK, involved in hypertrophy pathway of mESCs. (A) Ouabain increased Erk1/2 and MEK1/2 phosphorylation and rescued the suppression by U0126; (B) Unchanged phosphorylation of p38 and JNK upon ouabain treatment; at least three independent experiments were repeated with a similar result for each of the MAPK examined.



**Figure 7.** Mechanistic study on the relative number of ESC-derived cardiomyocytes calculated as the percentage of troponin-T positive cells on d 7 as determined by flow cytometry. (A) Dot plots and (B) bar chart of the troponin-T positive cell counts. Data of independent experiments were expressed as mean $\pm$ SEM ( $n=3$ ,  $^bP<0.05$ ,  $^cP<0.01$ ). Significant difference was analyzed by unpaired *t*-test.

of Na<sup>+</sup>/K<sup>+</sup>-ATPase, the predominant form in cardiac tissue.

ESC-derived cardiomyocytes hold great promise for cardiac regeneration. Nonetheless current protocols for cardiac differentiation of ESCs are in general inefficient, making it very difficult to obtain adequate numbers of cardiomyocytes for clinical therapy. Despite the well-known hypertrophic effects of ouabain on cardiomyocytes<sup>[17, 24]</sup> and the documented functional expression of Na<sup>+</sup>/K<sup>+</sup>-ATPase in undifferentiated mESCs<sup>[5]</sup>, the potential procardiogenic effects of ouabain have not been explored. Binding of ouabain to Na<sup>+</sup>/K<sup>+</sup>-ATPase, in addition to the positive inotropic effect, also activates multiple MAPK pathways in a cell-type specific manner. Previous studies have suggested that MAPK activation may play a crucial role in mesoderm induction, which leads subsequently to cardiogenesis during embryonic development<sup>[25, 26]</sup>. Coordinated activation of the three major MAPKs involved in cardiac hypertrophy namely ERK1/2, JNK, and p38 MAPK, are essential to induce cardiac differentiation of P19 embryonic carcinoma cell line<sup>[27, 28]</sup>. In the present study, application

of ouabain to undifferentiated mESCs resulted in a modest increase in the number of troponin-positive cells differentiated from mESCs. This was associated with increased expression of early cardiac specific transcription factors (Nkx2.5) and cardiac specific markers ( $\alpha$ -MHC and  $\beta$ -MHC). Consistent with a previous study<sup>[29]</sup>, ERK1/2, JNK and p38 MAPK were endogenously activated in mESCs during differentiation. Only ERK1/2 though was significantly activated upon ouabain treatment. Since specific blocker for ERK1/2 is not available, U0126, an upstream MEK1/2 blocker, were used to study the pathway, of which ouabain induced cardiogenesis. Due to the non-specificity of MEK1/2 blocker, it is possible that other mechanisms independent of ERK1/2 activation may be involved in ouabain induced cardiac differentiation in mESCs. Nonetheless, the specific role of ouabain in Erk1/2 activation was well defined by the inhibitor experiment. Interestingly, various cytokines or growth factors, including cardiotrophin-1<sup>[30]</sup>, VEGF<sup>[31]</sup>, and heregulin- $\beta$ 1<sup>[8]</sup>, which promote cardiac differentiation of mESCs, also mediate via the



activation of ERK1/2 pathway. It has recently been shown that icariin, the active ingredient of the plant herb *Epimedium*, significantly enhances cardiac differentiation of mESCs via activation of p38 MAPK<sup>[29]</sup>. Taken together, this evidence suggests that adult cardiac hypertrophic signals, particularly the MAPK pathway, may play a role in the cardiac differentiation in ESCs, and may be exploited to improve the efficiency of cardiac differentiation.

Another important hurdle for ESC-based cardiac therapies is the relative immature calcium handling properties of ESC-derived cardiomyocytes<sup>[2, 32-34]</sup>. In adult cardiomyocytes, calcium enters the cell through L-type calcium channels during phase 2 of action potentials. This relative small calcium influx in turn triggers a large calcium release from the internal calcium store, SR through ryanodine receptors<sup>[35]</sup>. This process is known as calcium-induced calcium release (CICR), the primary mechanism that links electrical excitation and mechanical contraction in cardiomyocytes. During diastole, calcium is actively removed from cytosol, mainly through sarco/endoplasmic reticulum Ca<sup>2+</sup>-ATPase pump (SERCA), back into the SR and via Na<sup>+</sup>-Ca<sup>2+</sup> exchanger (NCX) out of cell<sup>[36]</sup>. mESC-derived cardiomyocytes are known to exhibit immature calcium dynamics: small cytosolic calcium transient amplitudes, slow rise and decay kinetics, and reduced calcium content of SR. This adversely affects excitation-contraction coupling<sup>[37]</sup> and is partly related to the relatively underdeveloped SR and partly to the developmental expression profiles of calcium handling proteins in mESC-derived cardiomyocytes. In the present study, ouabain treatment favorably altered the calcium handling properties of mESC-derived cardiomyocytes including larger calcium transients, a faster rate of rise and decay of calcium transients, and thus resulted in a stronger contractile force. In addition, cardiomyocytes isolated from ouabain-treated EBs also appeared to have a larger internal store of calcium as evidenced by larger amplitude of caffeine-mediated calcium release. These changes could be related to the corresponding upregulation of key calcium handling proteins in cardiomyocytes isolated from ouabain-treated EBs. Specifically, the upregulated ryanodine receptor could result in the faster rate of calcium release, while the rate of calcium transient decay corresponds to the higher expression of SERCA in ouabain-treated cardiomyocytes. The enhanced intracellular calcium concentration due to Na<sup>+</sup>/K<sup>+</sup>-ATP inhibition nonetheless remains another plausible mechanism for such improvement.

Our results shed new light on the potential use of a hypertrophic stimulus on adult cardiomyocytes to enhance cardiac differentiation and maturation of ESC-CMs *in vitro*. Ouabain-driven cardiac differentiation of mESC-CMs is mediated by activation of Erk1/2 in the hypertrophy pathway. The relationship of the pathway with calcium handling in the cells was not identified. Nonetheless, our findings broaden knowledge of the differentiation processes of cultured ESCs, and may also contribute to the future development of step-cell based therapy for heart disease.

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## Author contribution

This research was designed by Chung-wah SIU, Hung-fat TSE, Chu-pak LAU, Deborah K LIEU, Cornilia MAN. The experiments were performed by Yee-ki LEE, Kwong-man NG, Wing-hon LAI. The new analytical tools and reagents were provided by Chung-wah SIU and Hung-fat TSE. Yee-ki LEE and Kwong-man NG were responsible for analyzing data. The manuscript was written by Yee-ki LEE, Kwong-man NG, and Chung-wah SIU.

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