

## Cultured Neonatal Rat Cardiomyocytes Continue Beating Through Upregulation of CTGF Gene Expression

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**Background:** Some cultured neonatal rat cardiomyocytes continue spontaneous beating even in serum-free medium. The present study explored the cause and genes responsible for this phenomenon.

**Methods:** Ingenuity Pathway Analysis (IPA) software was used to analyze fold changes in gene expression in beating neonatal rat cardiomyocytes, as compared with non-beating cardiomyocytes, which were obtained from DNA microarray data of total RNA extracts of cardiomyocytes. To confirm the involvement of the 8 genes selected by IPA prediction, cellular protein abundances were determined by Western blot. The gene expression of connective tissue growth factor (CTGF) was substantially higher in beating cardiomyocytes than in non-beating cardiomyocytes; thus, CTGF protein content released from cardiomyocytes into the culture medium was examined.

**Results:** IPA showed that the “Apelin Cardiac Fibroblast Signaling Pathway” was significantly inhibited and that microtubule dynamics and cytoskeleton organization were significantly activated. Each fluctuation in the cellular abundances of the 8 proteins in beating cardiomyocytes, as compared with non-beating cardiomyocytes, was primarily in the same direction as that of gene expression. However, the cellular CTGF protein abundance as well as CTGF content released into the medium did not substantially differ between beating and non-beating cardiomyocytes.

**Conclusions:** The present results suggest that the large increase in CTGF gene expression in beating cardiomyocytes is not a cause but a result of beating, which may provide a putative pathway for controlling beating. Beating is sustained by developed cardiomyofibrils and directly upregulates CTGF gene expression, which is not followed by CTGF protein synthesis. (J Nippon Med Sch 2020; 87: 268–276)

**Key words:** cardiomyocyte, beating, DNA microarray, Ingenuity Pathway Analysis, Western blot

### Introduction

Continuous contraction to circulate blood is the primary function of the heart, and dysfunction of cardiac contraction is attributable to various causes<sup>1–3</sup>. Although cardiomyocytes cultivated from neonatal rats spontaneously beat in medium containing serum, beating sometimes stops in the absence of serum. No study, however, has investigated genes related to maintenance of heartbeat in cultured cardiomyocytes. This study used a DNA microarray to identify differences in mRNA expression between beating and non-beating neonatal rat cardiomyocytes. Analysis of mRNA expression suggested that connective tissue growth factor (CTGF) is involved in persis-

tence of cardiomyocyte beating.

Several studies have utilized Ingenuity Pathway Analysis (IPA) software to predict cardiac functions by using two-dimensional electrophoresis data of protein extracts from cultured neonatal rat cardiomyocytes<sup>4</sup> and to identify target mRNAs involved in mouse cardiac hypertrophy<sup>5</sup>. IPA uses a database of the scientific literature to predict pathways, upstream regulators, and biological functions relevant to the uploaded data set and to provide biological interpretations and their statistical probabilities. We used IPA software to analyze differences in mRNA expression between beating and non-beating neonatal rat cardiomyocytes and to identify regulator pro-

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teins that control heartbeat. In addition, we attempted to determine intracellular protein abundances of several molecules, which were selected on the basis of IPA-predicted pathways and regulators. Additionally, the level of CTGF protein released was measured in culture medium. Further studies based on IPA prediction from gene expression could identify a putative mechanism for continuous myocardial beating.

## Materials and Methods

### Culture of Cardiomyocytes

Ventricular cardiomyocytes were isolated from neonatal rats by using a modification of a previously described method<sup>6</sup>. Briefly, hearts removed from 1-day-old Sprague-Dawley rats were dispersed by repeated digestion with 0.06% collagenase (Worthington Biochemical Corp., Lakewood, NJ, USA) in ADS buffer (130 mM NaCl, 5.4 mM KCl, 0.8 mM NaH<sub>2</sub>PO<sub>4</sub>, 0.8 mM MgSO<sub>4</sub>, 5.5 mM glucose, 20 mM HEPES, pH 7.4). Excluding the first digestion, after every subsequent digestion, dispersed cells were suspended in newborn calf serum (NCS, Sigma-Aldrich Inc., Saint Louis, MO, USA), centrifuged, and resuspended in new NCS. The combined cell suspension was rinsed twice with culture medium (68% M199 [Sigma-Aldrich], 17% Dulbecco's minimum essential medium [DMEM; GIBCO Invitrogen Corp.], 10% heat-inactivated horse serum [GIBCO], 5% heat-inactivated fetal bovine serum [FBS, GIBCO], 100 U/ml penicillin/0.1 mg/ml streptomycin, 0.1% cytosine β-D-arabinofuranoside), resuspended in fresh culture medium, and preincubated in culture dishes (Nunclon, Nunc, Roskilde, Denmark) for 1 h in a CO<sub>2</sub> incubator. After preincubation, cardiomyocytes were plated in a gelatin-coated dish (diameter, 35 mm; Nunclon, Nunc) at a concentration of 1 × 10<sup>6</sup> cells/well. The rats were handled in accordance with "The regulations on Animal Experimentation of Nippon Medical School", which are based on "The Guidelines of the International Committee on Laboratory Animals, 1974".

After about 40 h of incubation, the culture medium was replaced with serum-free medium (DMEM:M199 = 4:1), incubated in a CO<sub>2</sub> incubator for 24 h, and replaced the serum-free medium with 1.5 mL of 21 mM HEPES-buffered serum-free medium (DMEM:M199 = 4:1). The plastic dishes were then put into a sealed glass container and incubated in a CO<sub>2</sub>-free environment at 37°C for 24 h. At the end of incubation each dish was observed with an inverted microscope to classify cardiomyocytes in the dish as beating or non-beating.

### Gene Expression Analysis—Total RNA Isolation

The techniques for sample preparation for gene expression analysis using the DNA microarray and subsequent analysis of digitized signal values were previously reported<sup>7</sup>. Briefly, the total RNA of cardiomyocytes was extracted with a NucleoSpin RNA XS kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany), Cy3-labeled, hybridized to SurePrint G3 Rat GE 8×60K Microarray (Agilent Technologies, Inc., Santa Clara, CA, USA), scanned using an Agilent G2565CA Microarray Scanner System (Agilent), and digitized with Feature Extraction 11.5 software (Agilent). Use of GeneSpring software (version 12.6.1, Agilent) allowed digitized signal values to be normalized, to correct differences between DNA microarrays and signal baselines, and transformed to fold changes (FCs) by calculating the ratio of gene expression values of beating cardiomyocytes (16 samples) to those of non-beating cardiomyocytes (4 samples). Differentially expressed genes (DEGs) were defined as those with an absolute FC of ≥2, and the dataset of DEGs was analyzed by using QIAGEN's Ingenuity Pathway Analysis (IPA, QIAGEN, Redwood City, CA, USA, [www.qiagen.com/ingenuity](http://www.qiagen.com/ingenuity)) to predict pathways and upstream regulators that control the beating of cardiomyocytes.

### Sample Preparation for Western Blot Analysis

#### —Protein Extraction

The techniques used for sample preparation for Western blot analysis were almost the same as those used in a previous study<sup>7</sup>. Briefly, cardiomyocytes were fixed with 1.5 ml of cold 20% (v/v) TCA in deionized water for 1 h on ice, scraped from the dish, transferred to an Eppendorf tube, and centrifuged at 3,000 g for 5 min. The pellet was washed 3 times by addition of cold acetone and centrifugation, completely dried, and stored at -80°C. To the dried pellet, 125 μl of MSS/Tris buffer (6 M urea, 2 M thiourea, 2.5% [w/v] 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate [CHAPS], Tris/HCl, pH 8.5) was added, homogenized with a plastic pestle in an Eppendorf tube, and solubilized by overnight incubation at room temperature. The homogenate was centrifuged for 30 min at 15,000 rpm at 4°C, and the obtained supernatant (ie, the protein extract of cardiomyocytes) was aliquoted and stored at -80°C. Protein concentration was determined by the Bradford method<sup>8</sup>.

#### Western Blot

Protein extract was added at an equal volume to the sample buffer (2×) (0.125 M Tris/HCl [pH 6.8], 4% [w/v] SDS, 20% [v/v] glycerol, 3.1% dithiothreitol, 0.002% bromophenol blue) and heated at 95°C for 10 min, except

for samples used in the detection of transforming growth factor-beta 2 (TGFB2). Next, the protein extract was loaded and separated on a stacking gel (TGX FastCast Acrylamide Kit, 12%; Bio-Rad Laboratories, Inc., Hercules, CA, USA; added with 0.05% ammonium persulfate, 0.1% TEMED) on top of 12% polyacrylamide gel (TGX FastCast Acrylamide Kit, 12%; Bio-Rad; added with 0.05% ammonium persulfate, 0.05% TEMED), and transferred to a polyvinylidene difluoride (PVDF) membrane (Trans-Blot Turbo Transfer system Transfer Pack, Bio-Rad). The membrane was blocked in 2% ECL Advance Blocking Reagent (GE Healthcare Life Sciences, Buckinghamshire, UK) in Tris (20 mM, pH 7.6)-buffered saline-Tween 20 (TBS-T) at gentle agitation for 1 h at room temperature, and washed with TBS-T. The membrane was incubated overnight at 4°C in the primary antibody of the target protein and the antibody of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) used as a loading control in TBS-T, and washed with TBS-T. The PVDF membrane incubated with anti-TGFB2 antibody not conjugated with horseradish peroxidase (HRP) was further incubated with Amersham ECL Anti-Rabbit IgG, HRP-linked whole antibody (from donkey; GE Healthcare, NA 934) for 1 h, and washed with TBS-T.

The membrane was then incubated with the detection reagent (Amersham ECL Prime Western Blotting Detection Reagent, GE Healthcare) for 5 min at room temperature. Chemiluminescence of protein bands was detected with a LAS-4000 Luminescent Image Analyzer (Fujifilm Corporation Life Science Division, Tokyo, Japan) and quantified with Image Quant TL Image Analysis Software (ver. 8.1, GE Healthcare). The chemiluminescence intensity of each protein band was normalized by using GAPDH on the same lane as a reference. NIH/3TC Whole Cell Lysate (Santa Cruz Biotechnology, Inc., Heidelberg, Germany, SC-2210) was used as a positive control.

All primary antibodies used were rabbit polyclonal antibodies. The dilution ratio of each antibody was described after each catalog number. The primary antibodies already conjugated with HRP were anti-CD105 (endoglin, ENG) antibody (Bioss; bs-4609R-HRP, 1:20,000), anti-Smad3 (Ser423) (SMAD family member 3) antibody (Bioss; bs-2225R-HRP, 1:10,000), anti-TGFB3 (transforming growth factor-beta 3) propeptide antibody (Bioss; bs-0099R-HRP, 1:800,000), and anti-ANKRD1 (ankyrin repeat domain 1) antibody (Bioss; bs-8074R-HRP, 1:400,000). The following antibodies were labeled with a labeling kit, after concentration with an Antibody Con-

centration/Clean Up Kit (Abnova Corp., Taipei, Taiwan; CB-KA1598), if necessary. Anti-GAPDH antibody (NOVUS; NB300-322, depending on the dilution of the target antibody) and anti-PAI-1 (plasminogen activator inhibitor type 1, = SERPINE1) antibody (Bioss; bs-1704R, 1:12,000) were conjugated with HRP by using a FastLink HRP Labeling Kit (Abnova). Anti-TGFB1 (transforming growth factor-beta 1) antibody (GeneTex; GTX110630, 1:150,000) was conjugated with HRP by using a Peroxidase Labeling Kit-SH (Dojindo Molecular Technologies, Inc.; Kumamoto, Japan, LK09). Additionally, anti-CTGF (GeneTex; GTX124232, 1:48,000) was conjugated with HRP by using an Ab-10 Rapid Peroxidase Labeling Kit (Dojindo, LK33). Anti-TGFB2 antibody (GeneTex; GTX 15539 1:1,500) was not labeled with HRP.

#### CTGF Assay

The culture medium was collected just before washing of cultured cardiomyocytes prior to fixation for protein extraction, and centrifuged for 1 h at 15,000 rpm and 4°C, to remove particulates. The supernatant was frozen at -80°C until measurement. CTGF content in the supernatant was assayed with a CTGF Elisa Kit (Rat): 96 Wells (Aviva Systems Biology, San Diego, CA, USA), in accordance with the manufacturer's instructions. Optical density at 450 nm was measured with a Multi-Mode Microplate Reader (FilterMax F5/SoftMax Pro, Molecular Devices, Inc., Sunnyvale, CA, USA).

#### Statistical Analysis

Student's *t*-test (IBM SPSS Statistics version 25, International Business Machines Corp., Armonk, NY, USA) was used to analyze the difference in protein expression and the CTGF content released in media of beating and non-beating cardiomyocytes. A *p* value of <0.05 was considered to indicate statistical significance.

## Results

### IPA-Predicted Pathways and Upstream Regulators and Functions

The 153 genes with an absolute FC value of  $\geq 2$  and significant differences for differential gene expression between beating and non-beating cardiomyocytes were uploaded to IPA. Western blot was used to determine protein abundances for 5 of these genes: CTGF, SERPINE1, TGFB3, TGFB2, and ANKRD1.

IPA predicted as top canonical pathways, "TGF- $\beta$  (transforming growth factor beta) Signaling", "HMGB1 (high mobility group-B1) Signaling" and "p38 MAPK (p38 mitogen activated protein family of kinases) Signaling", which were listed in every analysis and upregu-

Table 1 Canonical pathways related to heart function predicted by QIAGEN's Ingenuity Pathway Analysis (IPA) (Spring 2019 release) from differential gene expression with an absolute fold change greater than 2 and statistical significance of beating cardiomyocytes against non-beating cardiomyocytes

Canonical Pathways	Activation z-score	$-\log(p \text{ value})$	molecules*
Apelin Cardiac Fibroblast Signaling Pathway	-2	4.51	T3, T2, C, S
TGF- $\beta$ Signaling	1.342	3.9	T3, T2, S
HMGB1 Signaling	1.342	3.27	T3, T2, S
p38 MAPK Signaling	1	1.87	T3, T2

\* indicates molecules associated with the canonical pathway and analyzed by Western blot in this study. T3: TGFB3, T2: TGFB2, C: CTGF, S: SERPINE1

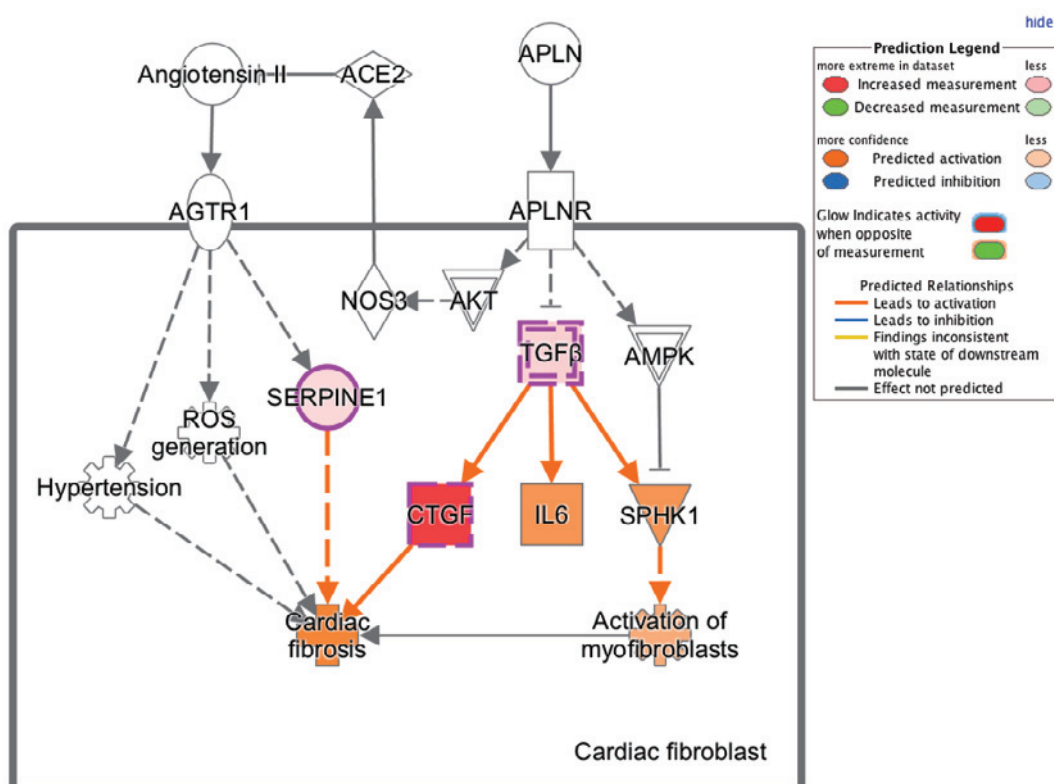


Fig. 1 Activation state of the “Apelin Cardiac Fibroblast Signaling Pathway” predicted by QIAGEN’s Ingenuity Pathway Analysis (IPA), released in Spring 2019. A differentially expressed gene set of beating neonatal rat cardiomyocytes against non-beating cardiomyocytes is shown; the genes have an absolute fold change value of  $\geq 2$  and are statistically significant. The pathway predicts upstream and downstream effects of activation or inhibition of molecules by specifying activation in silico, using the Overlay/Molecule Activity Predictor function of IPA. Molecules shown in red and pink in the pathway are genes with increased gene expression that were uploaded to IPA; molecules in green and pale green are those with decreased gene expression. Although molecules in orange or blue were not uploaded to IPA, IPA predicted that those in orange were activated and those in blue were inhibited. Orange lines indicate relationships that lead to activation; blue lines indicate relationships that lead to inhibition. Yellow lines indicate inconsistent relationships, and grey lines indicate unpredicted effects.

lated with a  $-\log(p \text{ value})$  of  $>1.3$ , even though the IPA system was upgraded quarterly (Table 1). Then, the “Apelin Cardiac Fibroblast Signaling Pathway” (Fig. 1), a pathway added to the canonical pathways of IPA in Fall

2018, was listed in Fall 2018 for the first time at the top of canonical pathways and was predicted to be significantly inhibited, with an “Activation z-score” of  $-2$ . In this pathway, TGF- $\beta$  was located inside cardiac fibro-

Table 2 “Diseases and Bio Functions” and “Tox Functions” relating heart function predicted by IPA (Spring 2019 release) from differential gene expression of beating cardiomyocytes against non-beating cardiomyocytes

Annotation of Diseases or Functions	Activation z-score	<i>p</i> value	molecules*
<b>Diseases and Bio Functions</b>			
Microtubule dynamics	3.281	0.00743	C, S, T3
Organization of cytoskeleton	3.061	0.00095	C, S, T2, T3
Cell movement	2.664	0.00968	C, S, T2, T3
Proliferation of connective tissue cells	2.417	3.44E-06	C, S, T2
Formation of cytoskeleton	2.09	0.00397	C, T3
Formation of actin filaments	1.782	0.00162	C, T3
Cell proliferation of fibroblasts	1.521	0.00485	C, S, T2
Activation of cells	1.363	0.00241	C, S, T2
Formation of actin stress fibers	1.029	0.0114	C, T3
Development of connective tissue cells	0.716	0.00562	S, T2
Contraction of heart	0.625	0.000334	C, T2
Fibrosis of heart	-0.896	0.0000554	C, S
Apoptosis of connective tissue cells	-1.258	0.00319	S, T2
<b>Tox Functions</b>			
Apoptosis of cardiomyocytes	-0.789	0.0324	-
Failure of heart	-1.000	0.0708	C, S
Hypertrophy of cardiomyocytes	None	2.65E-06	C, S
Dysfunction of heart	None	0.0458	A, S, T3

\* indicates molecules associated with the canonical pathway and analyzed by Western blot in this study.

A: ANKRD1, C: CTGF, S: SERPINE1, T2: TGFB2, T3: TGFB3

“None” means no “Activation z-score” was predicted.

blasts, directly activated CTGF, and induced “Cardiac fibrosis”.

Unlike the “Apelin Cardiac Fibroblast Signaling Pathway”, in “TGF- $\beta$  Signaling” and “p38 MAPK Signaling”, some molecules and routes overlapped, and TGF- $\beta$  locates extracellular space and acts through TGF- $\beta$  type II receptors in plasma membranes (the pathway figures are not shown). At the end of “TGF- $\beta$  Signaling”, both “Actin organization” and “Apoptosis” were predicted to be inhibited, but the former function was generally expected to be activated in beating cardiomyocytes. Thus, “TGF- $\beta$  Signaling” would not be sufficient to explain cardiomyocyte beating.

Conversely, in the “Diseases and Bio Functions”, “Microtubule dynamics”, “Organization of cytoskeleton”, and “Formation of cytoskeleton” were predicted to be significantly activated at an “Activation z-score” of  $\geq 2$  (Table 2). Additionally, “Formation of actin filaments” and “Formation of actin stress fibers” were predicted to be activated with an “Activation z-score” of  $< 2$ . Functions with “connective tissue cells” or “fibroblasts” in the title were also listed with a higher “Activation z-score”. However, “Fibrosis of heart” in “Diseases and Bio Func-

tions” as well as “Failure of heart” in “Tox Functions” were predicted to be somewhat inhibited, although the *p* value for “Failure of heart” was not significant (Table 2). The molecules CTGF, SERPINE1, TGFB2, and/or TGFB3 regulated most of these functions, and their proteins were analyzed by Western blot in this study.

IPA (Summer 2015 release) predicted 61 molecules as upregulated upstream regulators with an absolute “Activation z-score” of  $\geq 2$ , including the 4 molecules (TGFB3, TGFB1, TGFB2, SMAD3) analyzed by Western blot in this study. IPA predicted 12 “Networks”, among which “Network 2” was shown to regulate 3 top “Diseases and Functions”—“Cardiovascular System Development and Function”, “Cellular Movement”, and “Connective Tissue Development and Function”—, and had 4 molecules in the network: CTGF, SERPINE1, TGFB2 and TGFB3 (the network figure is not shown). Using the Build/Grow function of IPA, we attempted to add 10 molecules to “Network 2” on the upstream side of CTGF. Among the 10 added molecules, ENG was included and analyzed by Western blot in this study.

#### Western Blot

To confirm the predictions of IPA, 8 molecules

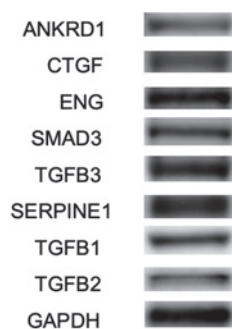


Fig. 2 Representative band images obtained by Western blot of protein extracts from cultured rat cardiomyocytes. Western blot was performed by standard SDS-PAGE. The blotted PVDF membrane was incubated with detection reagent (Amersham ECL Prime Western Blotting Detection Reagent). Chemiluminescence of each protein band was detected with an LAS-4000 Luminescent Image Analyzer, quantified with ImageQuant TL software, and normalized by using GAPDH in the same lane, as reference.

(ANKRD1, CTGF, ENG, SERPINE1, SMAD3, TGFB1, TGFB2, and TGFB3) were selected for Western blot analysis of protein extract from cardiomyocytes. Cardiomyocyte protein extracts were separated by SDS-PAGE, and chemiluminescence images of the PVDF membrane with the 8 types of antibodies were obtained, with GAPDH as loading control (Fig. 2). Table 3 shows the FC values for the 8 genes (ratio of mRNA expression values in beating versus non-beating cardiomyocytes) and FC values of chemiluminescence intensities of the 8 kinds of corresponding proteins obtained by Western blot. Most protein abundances showed the same direction of FC increase/decrease as the mRNA expression.

#### CTGF Assay

CTGF protein content in media for beating and non-beating cardiomyocytes was collected at the end of culture and measured, but there were no significant differences:  $77.05 \pm 3.41$  pg/mL and  $85.98 \pm 18.79$  pg/mL, respectively (mean  $\pm$  SD), 4 samples each,  $p = 0.47$ .

#### Discussion

Interestingly, CTGF gene expression was substantially higher than that of other genes in beating, as compared with non-beating, cardiomyocytes, but this was not reflected in cellular protein abundances (Table 3). An *in vitro* state that is peculiar to cell culture is a possible explanation for high gene expression of myocardial CTGF, because myocardial CTGF gene expression is believed to be upregulated during development of the fetal heart<sup>9,10</sup>.

Furthermore, in contrast to gene expression and cellular protein abundance (Table 3), the amount of CTGF protein released into culture media did not differ between beating cardiomyocytes ( $77.05 \pm 3.41$  pg/mL) and non-beating cardiomyocytes ( $85.98 \pm 18.79$  pg/mL), although the SD was higher for non-beating cardiomyocytes. The average of 2 samples with lower values was 69.72 pg/mL, which was lower than that of samples from beating cardiomyocytes, whereas the average of 2 samples with higher values was 102.25 pg/mL.

Research by Kireeva et al.<sup>11</sup> suggests that the markedly lower cellular abundance of CTGF protein, as compared with the high CTGF gene expression, could be attributable to its short half-life. Kireeva et al.<sup>11</sup> reported that the half-life of proteins in cells and extracellular matrix, as well as that released into medium, was 0.5 to 2 h in NIH 3T3 fibroblasts, ie, about half that of cysteine-rich angiogenic inducer 61 (CYR61) protein. CTGF protein is present not only in the extracellular space but also in the plasma membrane and Golgi apparatus. Therefore, CTGF protein is unlikely to be secreted outside of cells but is much more likely to be localized around the cell membrane, to act in cells anchoring to the substratum, namely, the culture dish. This discrepancy in gene expression, protein abundance, and amount of protein released in medium will be discussed below.

The ratios of protein abundances in beating and non-beating cardiomyocytes were determined by Western blot and were smaller than those for FCs of corresponding gene expression (Table 3). However, most protein abundances exhibited the same increasing/decreasing trend as that of corresponding gene expression and were statistically significant. A possible reason for these quantitative differences may be time lag between gene expression and protein synthesis. Second, densitometry obtained by Western blot might not have a linear proportional relation with actual protein abundance.

CTGF is a member of the CCN (CYR61, CTGF and NOV) family, which includes CYR61 and nephroblastoma overexpressed (NOV). CTGF and CYR61 are also referred to as CCN2 and CCN1, respectively. They are said to be inducible immediate-early genes and profibrotic and are believed to be involved in extracellular matrix production, cell adhesion, and cell survival, among other processes. CTGF was reported to be upregulated in various pathological states of the heart, in activating fibrosis<sup>12,13</sup>.

Apelin-related canonical pathways, including the “Apelin Cardiac Fibroblast Signaling Pathway”, first appeared in the IPA canonical pathways in the Fall 2018 re-

Table 3 Comparison of fold change values uploaded to IPA—ratios of mRNA expression values of beating cardiomyocytes against that of non-beating cardiomyocytes—and corresponding fold change values for chemiluminescence intensities obtained by Western blot analysis of 8 molecules

symbol	mRNA	Fold Change			
		protein		number	p value
		mean	S.D.		
ANKRD1	2.853	1.098	0.145	12	0.042
CTGF	23.370	1.175	0.284	13	0.047
ENG	1.027	1.161	0.140	13	0.001
SERPINE1	3.396	1.181	0.143	6	0.027
SMAD3	-1.355	1.078	0.141	12	0.081
TGFB1	-1.024	-1.053	0.091	11	0.096
TGFB2	4.213	-1.170	0.173	5	0.135
TGFB3	4.399	1.227	0.316	13	0.024
APLN	1.388	-	-	-	-

The fold change (FC) values of gene expression uploaded to IPA were obtained from 16 beating cardiomyocyte samples and 4 non-beating cardiomyocytes.

The chemiluminescence band intensity was normalized by using GAPDH on the same lane. The relative FC of protein abundance was calculated as the ratio of normalized band intensity of the 8 types of target proteins of beating cardiomyocytes against that of the non-beating cardiomyocytes; means and SD.

The average of relative FC values of protein abundance was calculated from the number indicated in the “number” column, which included pairs of samples from beating and non-beating cardiomyocytes blotted on the same PVDF membrane.

lease. Without this, it would have been difficult to interpret IPA prediction in this study. IPA predicted from the gene expression dataset that the “Apelin Cardiac Fibroblast Signaling Pathway” was significantly more inhibited in beating cardiomyocytes than in non-beating cardiomyocytes (**Table 1**). Inhibition originally should have resulted from downregulation of apelin to failure to stop CTGF activation, and thus lead to cardiac fibrosis (**Fig. 1**). In essence, experimental gene expression of apelin was slightly increased (FC = 1.388, **Table 3**), which indicates slight activation of this pathway, and is expected to lead to slight inhibition of CTGF gene expression, resulting in modest suppression of cardiac fibrosis.

In this study, however, a highly elevated gene expression of CTGF was predicted to directly activate cardiac fibrosis, which indicates why IPA predicted inhibition of this pathway. Likewise, this CTGF upregulation was predicted to be induced by TGF- $\beta$ , just upstream of CTGF. Previous studies have been inconsistent; TGF- $\beta$  did not induce CTGF expression in cultured smooth muscle

cells<sup>14</sup> but directly induced CTGF expression in fibroblastic cells<sup>15,16</sup> and cardiac myocytes<sup>15</sup>. IPA predicted slight inhibition of “Fibrosis of heart” in “Diseases and Bio Functions” (**Table 2**). Thus, there was a contradiction regarding cardiac fibrosis in the IPA prediction from the differential gene expression dataset between pathway (**Fig. 1**) and function (**Table 2**).

CTGF gene expression was much lower in non-beating cardiomyocytes than in beating cardiomyocytes, such that the “Apelin Cardiac Fibroblast Signaling Pathway” was predicted to be activated. Furthermore, “TGF- $\beta$  Signaling” was inhibited, which might result in suppression of cardiac fibrosis. From this perspective, this state seemed normal. However, the mean ( $\pm$ SD) of measured signal values of CTGF gene expression on the DNA microarray of beating and non-beating cardiomyocytes were  $21,735 \pm 7,543$  and  $1,052 \pm 603$ , respectively. We thus conclude that the gene expression level for non-beating cardiomyocytes was too low. Conversely, the CTGF gene expression level of beating cardiomyocytes appeared nor-

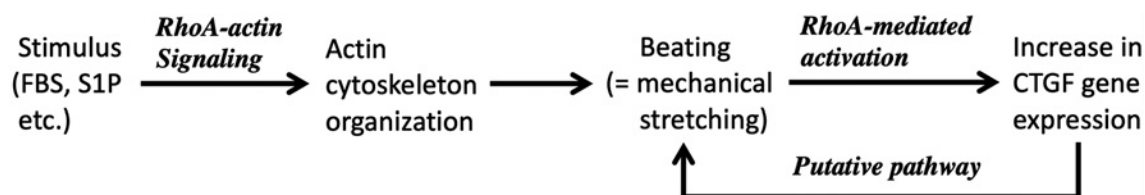


Fig. 3 Pathway model involved in continuation of cardiomyocyte beating, and a putative pathway. Most of the pathway is based on the work of Chowdhury and Chaqour (2004)<sup>14</sup>. RhoA: Ras homolog gene family, member a, FBS: fetal bovine serum, S1P: sphingosine 1-phosphate.

mal. The apelin level in left ventricles was reported to be elevated in patients with chronic heart failure<sup>17</sup>, probably because apelin might be compensatorily upregulated, to inhibit upregulated cardiac fibrosis. Thus, it is too late for apelin to inhibit cardiac fibrosis. It is also believed that the canonical pathway starting with apelin might not always proceed all the way to “fibrosis”. Although cardiac fibrosis is usually regarded as a pathological condition, some degree of inhibition of this apelin-related pathway and the ensuing activation of cardiac fibrosis appears necessary to keep a heart beating.

The model in **Figure 1** shows that this IPA-predicted pathway has only 7 colored factors, which means IPA cannot predict the activation state of other factors, because of the absence of FCs of gene expression. Non-colored molecules in the “Apelin Cardiac Fibroblast Signaling Pathway” had low FCs of differential gene expression (−1.5 to 1.4). Thus, CTGF and its upstream and downstream factors, TGF- $\beta$  and “Cardiac fibrosis”, were isolated from other factors. **Figure 1** suggests that these factors did not need to be included in this pathway. These views were interpreted in light of the following findings. In transgenic mice with cardiac-restricted overexpression of CTGF, cardiac fibrosis was not detected<sup>11,18</sup>. Indeed, ischemia-reperfusion injury was reduced<sup>11</sup> and hypertrophy due to chronic pressure-overload was inhibited<sup>12</sup>. In addition, induction of myocardial CTGF mRNA and protein in pigs with heart failure was not a decisive effector in fibrosis<sup>19</sup>. CTGF is a growth factor and has 4 modules, each of which interacts with different proteins and regulates many physiological processes<sup>4</sup>. Therefore, CTGF may also have an unknown function for maintaining cardiac physiological actions other than enhancement of fibrosis.

Using cultured smooth muscle cells from fetal bovine bladders, Chowdhury and Chaqour<sup>14</sup> found that RhoA (Ras homolog gene family, member a) GTPase activated organization of the actin cytoskeleton and induction of the CTGF gene (**Fig. 3**). The former RhoA-mediated or-

ganization of the actin cytoskeleton was induced through RhoA-actin signaling, in which various stimuli, including FBS and S1P (sphingosine 1-phosphate, a bioactive lysolipid), upregulated actin polymerization<sup>14</sup>. In the latter RhoA-mediated CTGF activation, RhoA translocated transcription factors into the nucleus to bind to the CTGF promoter and transcriptionally transactivated CTGF gene expression, while p38 activation enhanced the stability of CTGF mRNA<sup>14</sup>. RhoA-mediated upregulation of CTGF gene expression was also reported to be induced by mechanical stretching (**Fig. 3**), but disruption of the actin cytoskeleton architecture inhibited this upregulation<sup>20</sup>.

RhoA-actin signaling most likely corresponded to “Actin Cytoskeleton Signaling” in the IPA canonical pathways. Therefore, to predict the upstream and downstream effects of activation or inhibition *in silico*, we attempted to overlay on this signaling pathway the FC values of the differential gene expression set with an absolute FC value of  $\geq 2$  and statistically significant differences (153 genes), which was the same as that used for IPA prediction in this study. However, no prediction was obtained for any molecule of the pathway because no molecules on this pathway were included in this dataset. Following this, the differential gene expression dataset with an absolute FC value of  $\geq 2$ , but with no significant differences (526 genes), was overlaid on the pathway, and we obtained the predicted activation/inhibition state of the pathway, in which about 40% of molecules and functions were predicted to have activation or inhibition effects (pathway figure not shown). Likewise, most lines representing relationships between factors were indicated by solid lines, which shows that the relationships are confident. In the pathway, RhoA was activated, and 2 actin-related functions, “Actin polymerization” and “Cytoskeleton reorganization”, were predicted to be activated. Furthermore, IPA of gene expression had already predicted significant activation of “Microtubule dynamics” and “Organization of cytoskeleton” in “Diseases and Bio Functions” (**Table 2**). Importantly, under a phase-



contrast microscope, myofibrils were thick only in beating cardiomyocytes (data not shown). Activation of “Actin Cytoskeleton Signaling” seemed to have a role in maintaining contraction in beating cardiomyocytes.

Our findings suggest that beating cardiomyocytes have organized actin, which enables continuous beating and then induces elevation of CTGF gene expression in cardiomyocytes without cardiac fibrosis (Fig. 3). The extreme increase in CTGF gene expression is likely not a cause but a result of beating and may provide a putative pathway that controls beating. Beating is sustained by developed cardiomyofibrils and directly causes upregulation of CTGF gene expression, which may not be followed by CTGF protein synthesis. Furthermore, this marked CTGF gene expression, without protein synthesis, possibly ensures against unlikely events such as angina and cardiac overload<sup>12</sup> and possibly responds to emergencies by translationally synthesizing required proteins (Fig. 3). If anything interferes with this putative pathway, cardiomyocytes could not continue beating, which might lead to a decrease in CTGF gene expression.

**Conflict of Interest:** The authors declare no conflict of interest.

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