



Fibroblast Growth Factors and Vascular Endothelial Growth Factor Promote Cardiac Reprogramming under Defined Conditions

Hiroyuki Yamakawa,¹ Naoto Muraoka,¹ Kazutaka Miyamoto,¹ Taketaro Sadahiro,¹ Mari Isomi,¹ Sho Haginiwa,¹ Hidenori Kojima,¹ Tomohiko Umei,¹ Mizuha Akiyama,¹ Yuki Kuishi,¹ Junko Kurokawa,² Tetsushi Furukawa,² Keiichi Fukuda,¹ and Masaki Ieda^{1,3,*}

¹Department of Cardiology, Keio University School of Medicine, Tokyo 160-8582, Japan

²Department of Bio-Informational Pharmacology, Medical Research Institute, Tokyo Medical Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8510, Japan

³JST, CREST, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan

*Correspondence: mieda@z8.keio.jp

<http://dx.doi.org/10.1016/j.stemcr.2015.10.019>

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SUMMARY

Fibroblasts can be directly reprogrammed into cardiomyocyte-like cells (iCMs) by overexpression of cardiac transcription factors, including *Gata4*, *Mef2c*, and *Tbx5*; however, this process is inefficient under serum-based culture conditions, in which conversion of partially reprogrammed cells into fully reprogrammed functional iCMs has been a major hurdle. Here, we report that a combination of fibroblast growth factor (FGF) 2, FGF10, and vascular endothelial growth factor (VEGF), termed FFV, promoted cardiac reprogramming under defined serum-free conditions, increasing spontaneously beating iCMs by 100-fold compared with those under conventional serum-based conditions. Mechanistically, FFV activated multiple cardiac transcriptional regulators and converted partially reprogrammed cells into functional iCMs through the p38 mitogen-activated protein kinase and phosphoinositol 3-kinase/AKT pathways. Moreover, FFV enabled cardiac reprogramming with only *Mef2c* and *Tbx5* through the induction of cardiac reprogramming factors, including *Gata4*. Thus, defined culture conditions promoted the quality of cardiac reprogramming, and this finding provides new insight into the mechanism of cardiac reprogramming.

INTRODUCTION

Direct reprogramming generates the desired cell types from fibroblasts without passing through a stem cell state by overexpression of tissue-specific transcription factors. Transduction with reprogramming factors rapidly suppresses the fibroblast signature and concomitantly activates the target cell program to generate partially reprogrammed cells, which become more fully reprogrammed functional cells after prolonged cultivation (Sadahiro et al., 2015; Vierbuchen and Wernig, 2012). We and others reported the generation of cardiomyocyte-like cells (iCMs) from fibroblasts using combinations of cardiac-specific factors, including *Gata4*, *Mef2c*, and *Tbx5* (GMT) (Addis et al., 2013; Chen et al., 2012; Ieda et al., 2010; Jayawardena et al., 2012; Protze et al., 2012; Song et al., 2012). Transduction with GMT suppresses the expression of fibroblast-related genes and activates a cardiac reporter, α MHC promoter-driven GFP (α MHC-GFP), and cardiac protein expression in ~20% of fibroblasts after 1 week; however, few cells (~0.1% of the starting fibroblasts) are fully reprogrammed into functional iCMs after 4 weeks under conventional serum-based culture conditions, suggesting that most α MHC-GFP⁺ cells remain partially reprogrammed or immature iCMs by the original method (Ieda et al., 2010; Sadahiro et al., 2015). We and others have reported that *miR-133* and inhibition of pro-fibrotic

signaling promote cardiac reprogramming by silencing fibroblast signatures at the early stage of reprogramming; however, the molecular mechanisms underlying the conversion of partially reprogrammed cells into functional iCMs at the later stage remain undetermined (Muraoka et al., 2014; Zhao et al., 2015). Given that endogenous cardiac fibroblasts can be converted into more fully reprogrammed functional iCMs by in vivo reprogramming, undefined extrinsic factors may promote the quality of cardiac reprogramming (Inagawa et al., 2012; Qian et al., 2012; Song et al., 2012).

Recently, multiple groups showed that modification of the reprogramming factors could promote cardiac reprogramming (Sadahiro et al., 2015). Cahan et al. (2014) analyzed the gene regulatory network of the α MHC-GFP⁺ population, in which a vast majority of the cells were partially reprogrammed iCMs, using a network biology platform, CellNet, that assesses the fidelity of engineered cells (Morris et al., 2014). The α MHC-GFP⁺ cells were found to be exclusively classified as cardiomyocytes; however, multiple cardiac transcriptional regulators, including *Gata6*, *Hand2*, and *Nkx2.5*, were incompletely activated in the α MHC-GFP⁺ population, and supplementation of these factors could improve cardiac reprogramming. Indeed, addition of *Hand2* to GMT (GHMT) increases generation of cells expressing cardiac proteins and spontaneously beating iCMs, and addition of *Nkx2.5* to GMT or

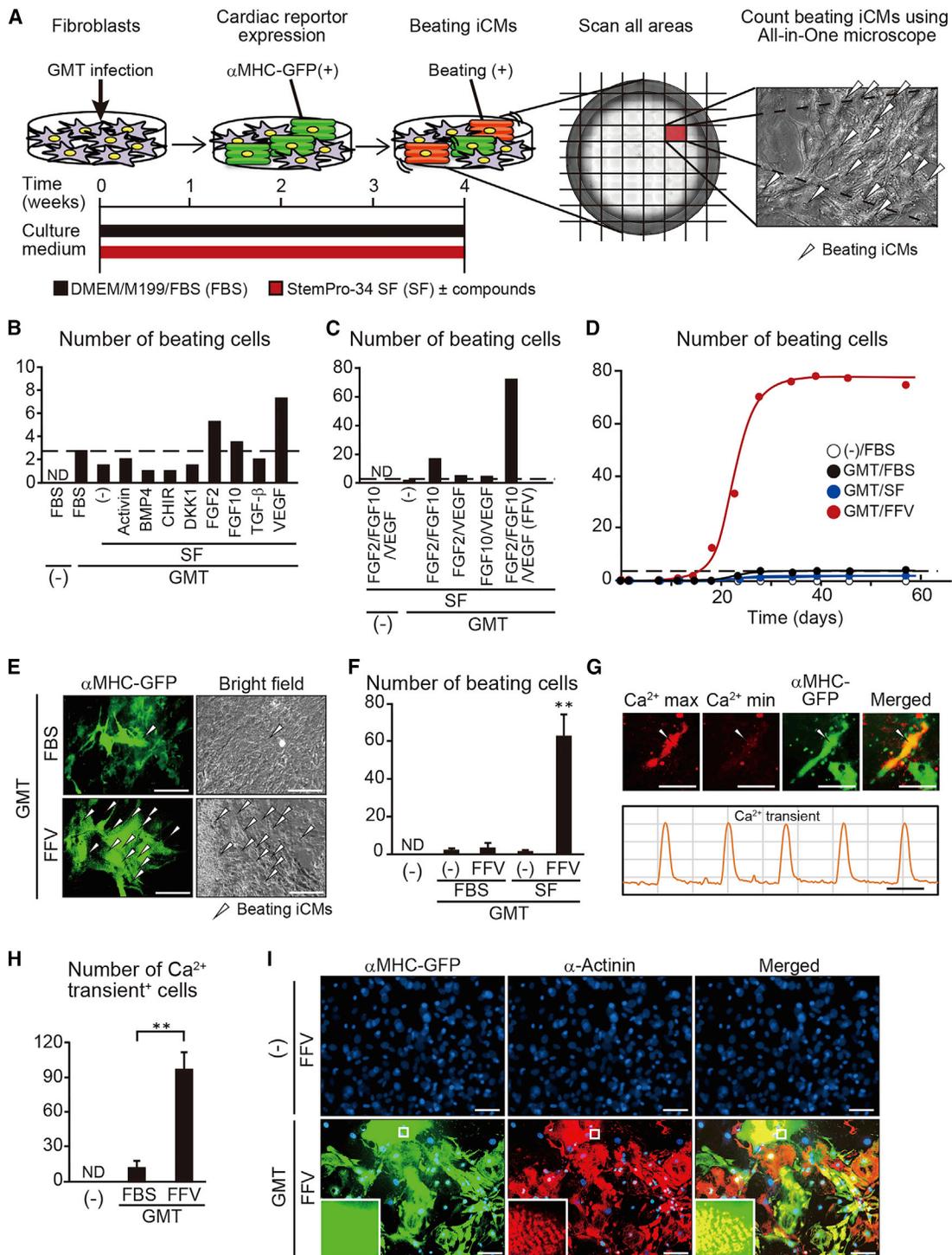


Figure 1. FFV Promoted GMT-Induced Cardiac Functional Reprogramming

(A) Schematic representation of the strategy used to test candidate compounds in SF (red bar) compared with FBS (black bar). The number of beating cells in each well was counted after 4 weeks.

(B and C) The number of spontaneously beating cells in each well after transduction of mock or GMT in MEFs. GMT-transduced cells were cultured under the indicated conditions.

(D) The number of spontaneously beating cells in each well after transduction of mock or GMT in FBS, SF, or FFV.

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GHMT increases the number of iCMs exhibiting calcium activity (Addis et al., 2013; Song et al., 2012). However, to date, extrinsic factors that regulate expression of multiple reprogramming factors have not been identified.

The use of undefined serum-containing medium has been associated with considerable batch-to-batch variation in cardiac reprogramming, leading to variable and low reprogramming efficiency in previous studies (Chen et al., 2012; Qian et al., 2013). Standardization of efficient, reproducible, and defined culture conditions for cardiac reprogramming is needed. In contrast, cardiomyocytes can be efficiently differentiated from pluripotent stem cells (PSCs) under defined serum-free media containing compounds that mimic the developmental signals in embryos (BurrIDGE et al., 2014; Kattman et al., 2011). It remains unknown whether these cardiogenic compounds can promote cardiac reprogramming under defined culture conditions and reduce the genetic manipulations required for cardiac reprogramming.

Here, we screened eight cardiogenic compounds and found that a combination of fibroblast growth factor (FGF) 2, FGF10, and vascular endothelial growth factor (VEGF), termed FFV, greatly improved the quality of cardiac reprogramming in mouse fibroblasts under serum-free conditions. FFV treatment activated multiple cardiac transcriptional regulators and converted partially reprogrammed iCMs into functional iCMs through the p38 mitogen-activated protein kinase (MAPK) and phosphoinositol 3-kinase (PI3K)/AKT pathways. It also eliminated the need of *Gata4* in cardiac reprogramming.

RESULTS

FFV Promoted the Direct Reprogramming of Fibroblasts into Functional iCMs under Defined Conditions

We previously screened cardiac reprogramming factors by analyzing the induction of cardiac reporter and protein expression after 1 week; however, this method may not

allow for the identification of factors that induce functional iCMs. Thus, in this study, we analyzed cardiac reprogramming efficiency by counting the number of spontaneously contracting iCMs after 4 weeks. We tested eight small molecules and growth factors involved in cardiac differentiation from PSCs in serum-free StemPro-34 SF medium (SF) (BurrIDGE et al., 2014; Kattman et al., 2011). We used mouse embryonic fibroblasts (MEFs) from α MHC-GFP transgenic mice to determine cardiac gene expression in the beating cells. GMT-transduced MEFs were cultured with DMEM/M199/10% fetal bovine serum (FBS; the culture condition in the previous studies) or SF with or without individual reagents for 4 weeks (Ieda et al., 2010; Muraoka et al., 2014), and the number of contracting iCMs was counted using an all-in-one fluorescence microscope system (Figure 1A). Addition of FGF2, FGF10, or VEGF to SF resulted in a higher number of contracting iCMs than that observed with SF alone or FBS, while activin A, bone morphogenic protein (BMP) 4, CHIR99021, Dickkopf-related protein 1 (DKK1), and transforming growth factor (TGF)- β had no significant effects (Figure 1B). Intriguingly, a combination of FGF2, FGF10, and VEGF resulted in markedly improved cardiac reprogramming compared to that observed with the addition of only one or two growth factors in SF (Figures 1C, S1A, and S1B). The uninfected MEFs did not contract, suggesting that no starting MEFs contaminated the CM population. GMT-transduced MEFs cultured with FFV started to contract earlier, and a greater population of α MHC-GFP⁺ cells contracted in FFV-containing medium than in FBS (Figures 1D, 1E, and S1C; Movies S1 and S2). FFV treatment resulted in a markedly higher number of beating iCMs and spontaneous Ca²⁺ oscillations compared to FBS, suggesting that FFV increased the generation of functional iCMs (Figures 1F–1H and S1D; Movie S3). Addition of FFV to FBS did not increase the reprogramming efficiency, suggesting that the serum may contain inhibitory factors (Figure 1F). Immunostaining confirmed that the beating iCMs in FFV expressed multiple cardiac markers, including α MHC-GFP, α -actinin, cardiac troponin T (cTNT), and atrial

(E) Spontaneously beating GMT-iCMs in FBS or FFV after 4 weeks (arrowheads), corresponding to Movie S1. See also Figure S1C and Movie S2.

(F) The number of spontaneously beating cells in three independent triplicate experiments is shown.

(G and H) Spontaneous Ca²⁺ oscillations in GMT-iCMs (arrowheads) after 4 weeks of FFV treatment, corresponding to Movie S3. Maximum and minimum concentrations of Ca²⁺ are shown in the upper panels, and the Rhod-3 intensity trace is shown in the lower panel (G). The total number of Ca²⁺ oscillation⁺ cells in ten randomly selected fields per well is shown (n = 3 independent triplicate experiments) (H). See also Figure S1D.

(I) Immunocytochemistry for α MHC-GFP, α -actinin, and DAPI. GMT-iCMs expressed cardiac proteins, and high-magnification views in insets show sarcomeric organization. See also Figure S1E.

All data are presented as mean \pm SD. **p < 0.01 versus the relevant control. ns, not significant. Scale bars represent 100 μ m (E, G upper panels, and I) or 500 ms (G, lower panel).

See also Figure S1 and Movies S1, S2, and S3.

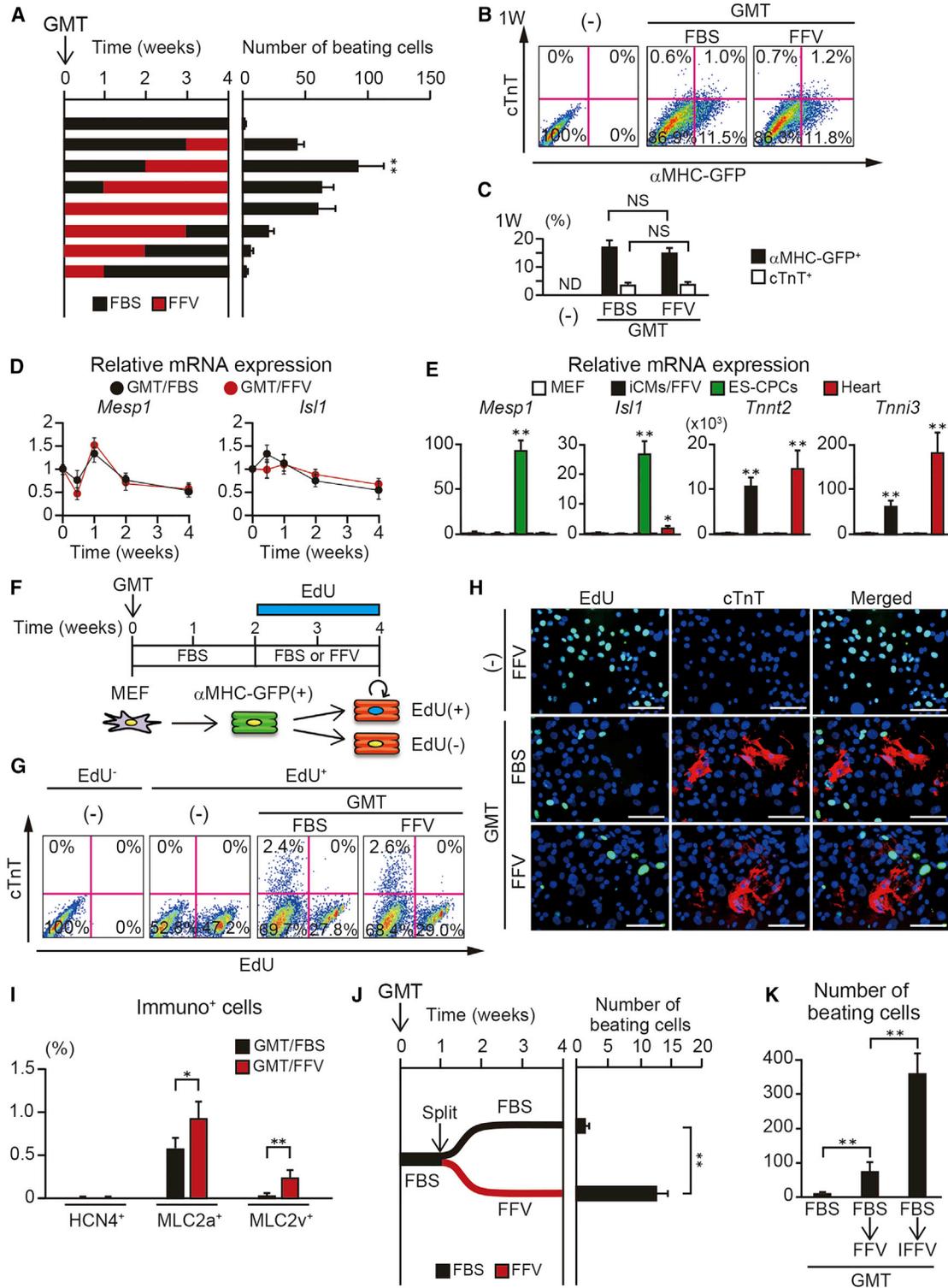


Figure 2. FFV Promoted Cardiac Reprogramming by Functioning during the Late Phase

(A) GMT-transduced MEFs were cultured with either FBS (black bars, left) or FFV (red bars) as indicated for 4 weeks. The number of beating cells in three independent triplicate experiments is shown (right).

(B and C) FACS analyses for αMHC-GFP and cTnT expression in GMT-transduced MEFs cultured with FBS or FFV for 1 week. Quantitative data are shown in (C) (n = 3 independent triplicate experiments).

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natriuretic peptide (ANP), and had well-defined sarcomeric structures (Figures 1I and S1E).

FFV Promoted Cardiac Reprogramming by Converting Partially Reprogrammed Cells into Functional iCMs

Next, we analyzed the time during which FFV plays a critical role in the generation of functional iCMs by changing the culture medium sequentially after GMT infection. Treatment with FFV for the last 2 weeks was sufficient to enhance cardiac reprogramming, while FFV treatment for the first 1 week did not affect reprogramming, suggesting that FFV was critical for the late but not early stage of reprogramming (Figure 2A). Consistent with this, fluorescence-activated cell sorting (FACS) analyses demonstrated that induction of α MHC-GFP and cTNT expression was not altered in the FFV-containing medium after 1 week, indicating FFV did not increase the generation of partially reprogrammed iCMs (Figures 2B and 2C). There are several possibilities for the increase in the numbers of spontaneously beating iCMs, without the generation of partially reprogrammed iCMs, caused by the addition of FFV—induction of mitotic cardiac progenitor cells (CPCs), stimulation of iCM proliferation, and generation of pacemaker type cardiomyocytes. The lineage-tracing experiments performed using *Mesp1-Cre/GFP* mice demonstrated that iCM generation was not mediated through *Mesp1*-expressing CPCs in either FFV or FBS (Figures S2A and S2B). Moreover, *Mesp1* and the later CPC marker, *Isl1*, were not expressed in the starting MEFs or the GMT-transduced cells, suggesting that the CPCs were not contaminated or induced during the reprogramming process (Figures 2D and 2E). Next, we performed EdU incorporation assays to determine the cell proliferation status. A 2-week pulse labeling of EdU at the late stage of reprogramming, during which FFV increased the number of functional iCMs, demonstrated that a vast majority of iCMs did not express EdU (Figures 2F–2H).

Consistent with this, the numbers of total cells, α MHC-GFP⁺ cells, and cTNT⁺ cells did not increase with FFV treatment after 4 weeks, suggesting that FFV did not stimulate cell proliferation (Figures S2C–S2E). qRT-PCR and immunostaining for sinoatrial nodal, atrial, and ventricular myocytes revealed that the proportion of HCN4⁺ pacemaker cells did not increase with FFV (Figures 2I and S2F–S2I). These results suggested that FFV increased the number of beating iCMs by functioning at the late stage of reprogramming; however, this effect was not due to the generation of partially reprogrammed iCMs or mitotic CPCs, proliferation of iCMs, or induction of pacemaker cells.

Our observation that FFV increased the ratio of beating iCMs/ α MHC-GFP⁺ cells suggested that FFV may promote the conversion of partially reprogrammed iCMs into functional iCMs. To test this, after culturing GMT-transduced MEFs with FBS for 1 week, at which point the cells remained in a partially reprogrammed state, we split them into FBS or FFV groups and cultured the cells for an additional 3 weeks. The number of beating iCMs was significantly increased with FFV, and the cardiac reprogramming efficiency was further increased with the addition of IWP4, an inhibitor of Wnt signaling, to the FFV-containing medium; the reprogramming efficiency increased by approximately 100-fold compared to that observed in the conventional FBS condition (Figures 2J and 2K). Therefore, FFV improved the quality of cardiac reprogramming by enhancing the conversion of partially reprogrammed cells into functional iCMs.

FFV Increased the Expression of Multiple Cardiac Transcriptional Regulators through p38MAPK and PI3K/AKT Pathways

Next, we studied the gene expression profiles of the iCMs cultured with FBS, SF alone, or FFV by microarray analyses. We isolated the α MHC-GFP⁺ cells using FACS

(D) qRT-PCR for *Mesp1* and *Isl1* expression in GMT-transduced cells cultured with FBS or FFV (n = 3 independent triplicate experiments). (E) qRT-PCR for *Mesp1*, *Isl1*, *Tnnt2*, and *Tnni3* expression in MEFs, iCMs, mouse ES-derived CPCs, and hearts (n = 3 independent triplicate experiments). The iCMs expressed cardiac genes, but not CPC genes.

(F) Schematic representation of EdU treatment during cardiac reprogramming.

(G) FACS analysis of EdU incorporation in mock-transduced cells and GMT-transduced MEFs cultured with FBS or FFV after 4 weeks of transduction.

(H) Immunocytochemistry for cTNT in mock-transduced and GMT-transduced MEFs cultured with FBS or FFV for 4 weeks, coupled with 2 weeks of EdU incorporation. The majority of immunopositive cells were negative for EdU in the iCMs.

(I) Quantitative data of immunopositive cells for HCN4, MLC2a, and MLC2v in iCMs cultured with FBS or FFV for 4 weeks (n = 7 independent triplicate experiments).

(J) GMT-transduced cells were cultured with FBS for 1 week and then split into either FBS or FFV groups (left). The number of beating cells per well on 24-well plates is shown (right, n = 3 independent triplicate experiments).

(K) GMT-transduced cells were cultured with FBS for 2 weeks, and the media were changed to FFV or IFFV (FFV + IWP4). The number of beating cells after 4 weeks is shown (n = 3 independent triplicate experiments).

All data are presented as mean \pm SD. **p < 0.01; *p < 0.05 versus the relevant control. ns, not significant. Scale bars represent 100 μ m. See also Figure S2.

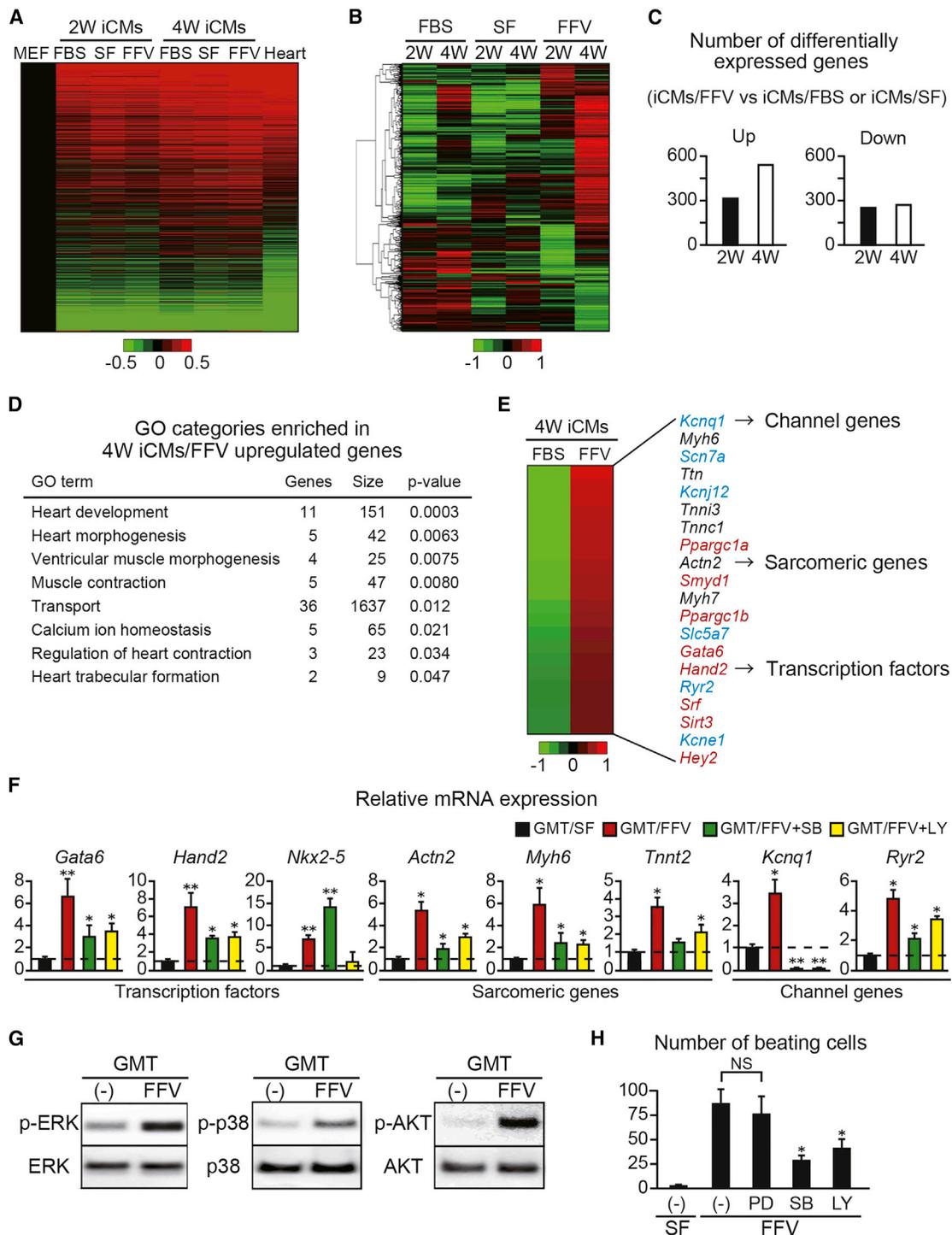


Figure 3. FFV Promoted Cardiac Reprogramming via the Activation of Cardiac Transcriptional Regulators

(A) Heatmap images of microarray data illustrating the global gene expression patterns of MEFs, iCMs cultured under the indicated conditions, and the hearts. The iCMs were sorted as α MHC-GFP⁺ cells at 2 and 4 weeks after GMT transduction. Differentially expressed genes between MEFs and the hearts are shown.

(B and C) Differentially expressed genes between iCMs cultured with FFV and those cultured with FBS or SF are shown (B). From this analysis, it was found that 306 and 548 genes were upregulated in iCMs cultured with FFV for 2 and 4 weeks, respectively, while 245 and 271 genes were downregulated after 2 and 4 weeks, respectively (C).

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at 2 and 4 weeks after GMT transduction, before and after the cells started contraction, and identified the differentially expressed genes between MEFs and the hearts. The heatmap image revealed a shift in the global gene expression patterns of iCMs from a MEF state toward a cardiac state after 2 and 4 weeks for all culture conditions (Figure 3A). Next, to determine the mechanisms responsible for the effect of FFV on cardiac reprogramming, we examined the differentially expressed genes in iCMs cultured with FFV compared with those in cells cultured with FBS or SF. FFV upregulated 306 and 548 genes after 2 and 4 weeks, respectively, but did not alter the number of downregulated genes (Figures 3B and 3C). Consistent with the occurrence of functional reprogramming after 4 weeks, genes that were upregulated following treatment with FFV at 4 weeks were enriched for gene ontology (GO) terms associated with heart development and cardiac functional properties (Figure 3D). Heatmap images and qRT-PCR analyses revealed that FFV upregulated a panel of cardiac genes related to different functions, including transcription factors (*Gata6*, *Hand2*, *Nkx2.5*, *Hey2*, *Ppargc1a*, *Smyd1*, *Sirt3*, and *Srf*), sarcomeric structures, and ion channels (Figures 3E, 3F, and S3A). The upregulated transcription factors were cardiac reprogramming factors or candidate factors that have been found to enhance cardiac reprogramming in previous studies and CellNet analyses (Figures 3E and S3A) (Addis et al., 2013; Cahan et al., 2014; Christoforou et al., 2013; Song et al., 2012). In contrast, fibroblast gene expression (*Col1a1* and *Snail*) was not significantly altered by FFV treatment (Figure S3B), suggesting that FFV promoted cardiac reprogramming by activating cardiac programming rather than suppressing fibroblast signatures, which is consistent with the observation that FFV functioned at the late stages of reprogramming.

FGF and VEGF activate MAPK and PI3K/AKT pathways. To investigate the signaling pathways involved in FFV-mediated cardiac reprogramming by GMT, we analyzed the activation of MAPK and AKT. Western blot analyses

revealed that the expression of phospho-ERK1/2, phospho-p38MAPK, and phospho-AKT was strongly induced by FFV treatment (Figure 3G). To identify the pathways involved in cardiac functional reprogramming, the GMT-transduced cells were cultured with FFV with or without specific inhibitors of these pathways during the late stage of reprogramming. We found that SB203580 (p38MAPK inhibitor) and LY294002 (PI3K inhibitor), but not PD98059 (MEK inhibitor), reduced the generation of beating iCMs, suggesting that FFV improved cardiac reprogramming through p38MAPK and PI3K/AKT, but not through MEK/ERK1/2 (Figure 3H). qRT-PCR analyses revealed that the key cardiac transcriptional regulators and a panel of cardiac genes were also suppressed by SB203580 and LY294002, suggesting that FFV activated the cardiac transcriptional profile via p38MAPK and PI3K/AKT pathways (Figure 3F).

FFV Enabled the Direct Reprogramming of Fibroblasts into Functional iCMs with *Mef2c* and *Tbx5*

Next, we investigated whether FFV could generate functional iCMs with fewer reprogramming factors. A combination of two factors, *Mef2c* and *Tbx5* (MT), induced functional iCMs under FFV culture (Figures 4A and 4B). In contrast, different two-factor combinations or the application of one factor alone did not induce the generation of beating iCMs. The efficiency of induction of functional iCMs with MT and FFV was similar to that with GMT and FBS, suggesting that FFV replaced the need for *Gata4*. qRT-PCR revealed that FFV upregulated the expression of cardiac reprogramming factors, such as *Gata4*, *Hand2*, and *Nkx2.5* (Figure 4C). Morphologically, the MT-iCMs in FFV appeared thicker and more refractive than MEFs, with a polygonal or rod-like shape (Figure 4D; Movie S4). Moreover, the MT-iCMs demonstrated spontaneous Ca^{2+} oscillations, expressed several cardiac markers, including α MHC-GFP, α -actinin, and cTNT, and had well-defined sarcomeric structures, suggesting that they are cardiac myocytes (Figures 4E–4I). However, the efficiency of cardiac reprogramming by MT with FFV was significantly lower than

(D) GO analyses of the upregulated genes in GMT-iCMs treated with FFV for 4 weeks. Cardiac-related GO terms are shown.

(E) Heatmap image of the expression of cardiomyocyte-enriched genes, related to transcription factors (red letters), sarcomeric structures (black letters), and ion channels (blue letters), in the iCMs cultured with FBS and FFV.

(F) GMT-transduced cells were cultured with SF (black bars), FFV without inhibitors (red bars), and FFV with SB203580 (green bars) or LY294002 (yellow bars). Relative mRNA expression was determined by qRT-PCR after 4 weeks ($n = 3$ independent triplicate experiments). Data were normalized to the values of GMT-transduced cells in SF. See also Figures S3A and S3B.

(G) Western blot analyses for p-ERK, p-p38MAPK, p-AKT, total ERK, total p38MAPK, and total AKT expression in GMT-transduced MEFs cultured with or without FFV.

(H) GMT-transduced cells were cultured with FBS for 2 weeks, and the media were changed to SF or FFV with or without inhibitors. The number of beating cells after 4 weeks is shown ($n = 3$ independent triplicate experiments).

All data are presented as mean \pm SD. ** $p < 0.01$; * $p < 0.05$ versus the relevant control. ns, not significant; PD, PD98059; SB, SB203580; LY, LY294002. The scales are -0.5 to $+0.5$ in log₁₀ (A) and -1 to $+1$ in log₁₀ (B and E).

See also Figure S3.

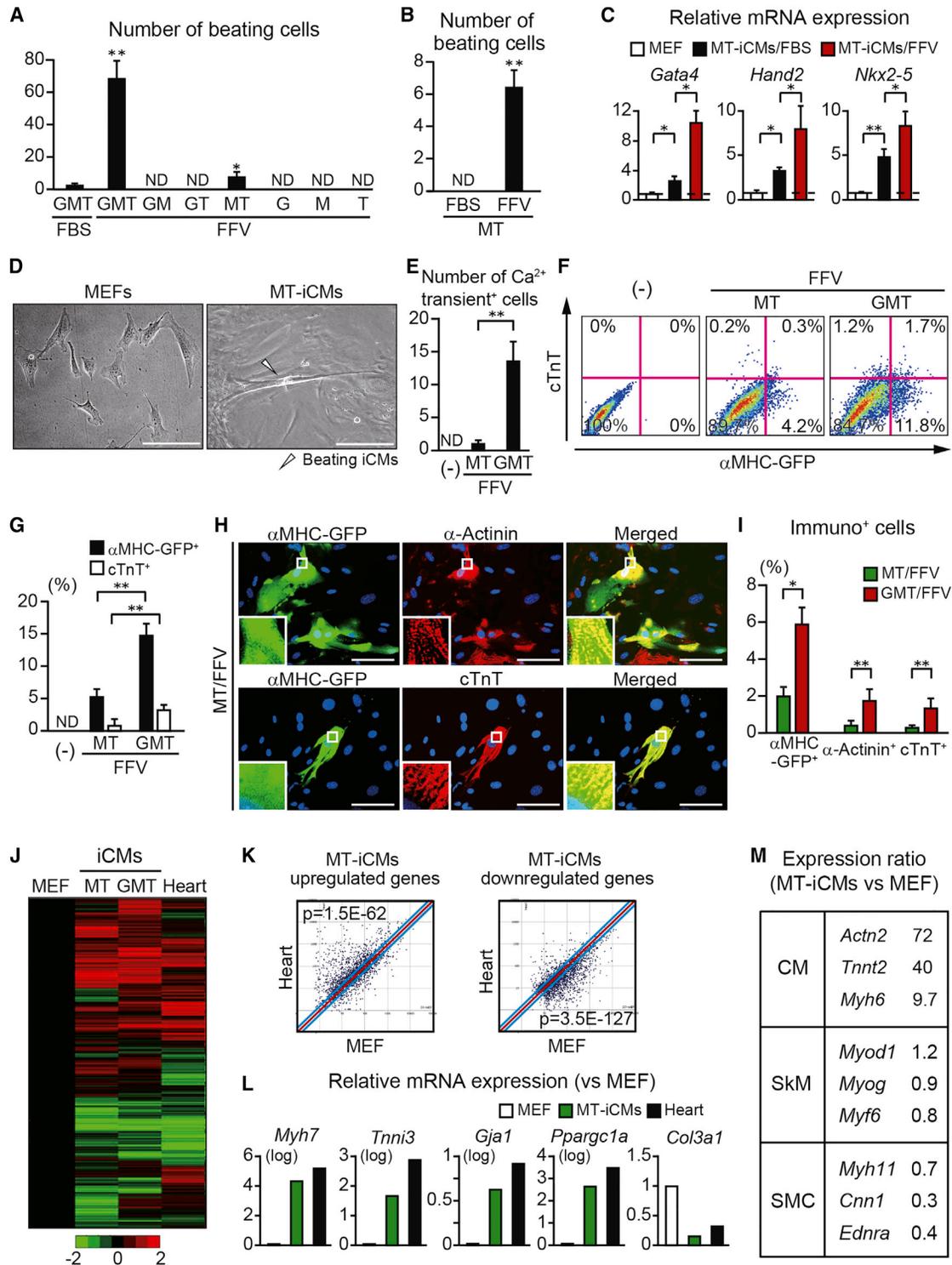


Figure 4. *Mef2c* and *Tbx5* Converted Fibroblasts into Functional iCMs under Defined Conditions

(A) The number of beating cells after transduction with GMT, one single factor, or combinations of two factors in FFV or FBS medium (n = 3 independent triplicate experiments). G, *Gata4*; M, *Mef2c*; T, *Tbx5*.

(B) The number of beating cells after transduction of MT in FBS or FFV (n = 3 independent triplicate experiments).

(C) Relative mRNA expression of *Gata4*, *Hand2*, and *Nkx2.5* in MEFs and MT-iCMs cultured with FBS or FFV were determined by qRT-PCR (n = 3 independent triplicate experiments). Data were normalized against the values in MEFs.

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that of GMT with FFV, suggesting that *Gata4*, while dispensable, played an important role in cardiac reprogramming (Figures 1I, 4A, 4H, 4I, and S1E). The heatmap image and scatter-plot analyses demonstrated that the global gene expression profile of MT-iCMs changed from that similar to the gene expression profile of fibroblasts toward that of cardiac cells (Figures 4J and 4K). qRT-PCR and array data confirmed that a panel of cardiac genes was higher, while fibroblast gene expression was lower in MT-iCMs than in MEFs (Figures 4L and 4M). Importantly, the MT-iCMs did not express genes of other muscle cells, such as the skeletal muscle or smooth muscle cells, suggesting that they were cardiac-specific myocytes (Figure 4M). These results indicated that FFV upregulated the expression of multiple cardiac reprogramming factors, including *Gata4*, and enabled cardiac reprogramming with only *Mef2c* and *Tbx5*.

FFV Promoted Cardiac Reprogramming in Postnatal Tail-Tip Fibroblasts

Next, to test the universality of the effect of FFV on cardiac induction by other reprogramming factors, we investigated whether FFV could promote cardiac reprogramming by GHMT. GHMT generated more functional iCMs than GMT alone in FBS, and this effect was enhanced following treatment with FFV (Figure 5A). Interestingly, GHMT-iCMs sometimes beat synchronously, similar to contractile cell sheets, when treated with FFV (Figure S4A; Movie S5). FACS analyses demonstrated that GHMT generated more cTNT⁺ cells than GMT after 1 week, suggesting that *Hand2* promoted cardiac reprogramming, at least in part, by functioning at the early stage of reprogramming. Immunocytochemistry analysis confirmed that GHMT-iCMs expressed cardiac proteins and had sarcomeric structures (Figures S4B–S4D). To determine whether FFV treatment played a role at the late stage of reprogramming by

GHMT, we replaced FBS with FFV after 2 weeks of GHMT transduction (Figure 5B). The cardiac reprogramming efficiency was further increased up to 9% (4628 beating iCMs out of 50,000 starting fibroblasts), suggesting that FFV promoted GHMT-mediated cardiac reprogramming at the late stage of reprogramming, which is consistent with the results of GMT-mediated reprogramming.

Next, to determine the cardiac reprogramming from other type of fibroblasts and to exclude the possibility of contamination of rare CPCs in the starting MEFs, we transduced mouse postnatal tail-tip fibroblasts (TTFs) with GMT, GHMT, or GMT plus *Mesp1* and *Myocd* (GMTMM), and cultured the cells with FBS or FFV for 8 weeks (Figure 5C) (Wada et al., 2013). Although GMT transduction did not generate beating iCMs in FBS, FFV treatment induced the generation of a small number of functional iCMs. GHMT transduction with FFV increased the number of beating cells, which was further increased with GMTMM. The numbers of beating iCMs and spontaneous Ca²⁺ oscillations in the GMTMM-transduced TTFs were significantly higher with FFV than with FBS (Figures 5C–5E; Movie S6). The GMTMM-iCMs expressed several cardiac markers and had well-defined sarcomeric structures under FFV (Figure 5F). FFV did not increase the generation of partially reprogrammed iCMs, as shown by FACS (Figures 5G). Microarray analyses revealed that FFV resulted in higher expression of 1,461 genes and lower expression of 801 genes compared to the gene expression observed with FBS treatment. GO terms related to cardiac function and heart development were highly associated with the upregulated genes, but not with the downregulated genes, suggesting that FFV globally activated cardiac programming in TTFs (Figures 5H and S4E). qRT-PCR confirmed that FFV upregulated a panel of cardiac genes, including *Gata6*, *Hand2*, and *Nkx2.5*, without changing fibroblast gene expression (Figure 5I).

(D) MEFs and MT-induced beating iCMs (arrowhead), corresponding to Movie S4.

(E) The number of Ca²⁺ oscillation⁺ cells in ten randomly selected fields per well (n = 3 independent triplicate experiments).

(F and G) FACS analyses of α MHC-GFP⁺ and cTNT⁺ cells 1 week after MT or GMT transduction with FFV. Quantitative data are shown in (G) (n = 3 independent triplicate experiments).

(H and I) Immunocytochemistry analysis of α MHC-GFP, α -actinin, cTNT, and DAPI staining. MT induced α MHC-GFP and cardiac protein expression. The high-magnification views in the insets show sarcomeric organization. Quantitative data are shown in (I) (n = 5 independent triplicate experiments). See also Figures 1I and S1E.

(J) Heatmap images of microarray data for MEFs, MT- and GMT-iCMs, and the hearts. The iCMs were sorted as α MHC-GFP⁺ cells after 4 weeks. Differentially expressed genes between MEFs and hearts are shown.

(K) Scatter plots of gene expression changes in MT-iCMs compared with that in MEFs.

(L) Relative mRNA expression levels of target genes in MEFs, MT-iCMs, and the heart were determined by qRT-PCR (n = 3 independent triplicate experiments). Data were normalized against the values in MEFs. All data are presented as means, and the scales are log₁₀ for *Myh7*, *Tnni3*, *Gja1*, and *Ppargc1a* expression.

(M) The relative expression of cardiomyocyte (CM), skeletal muscle (SkM), and smooth muscle cell (SMC) genes in MT-iCMs compared to those in MEFs, as determined by microarray analysis.

Data are presented as mean \pm SD (D, F, and H). **p < 0.01; *p < 0.05 versus the relevant control.

See also Movie S4.

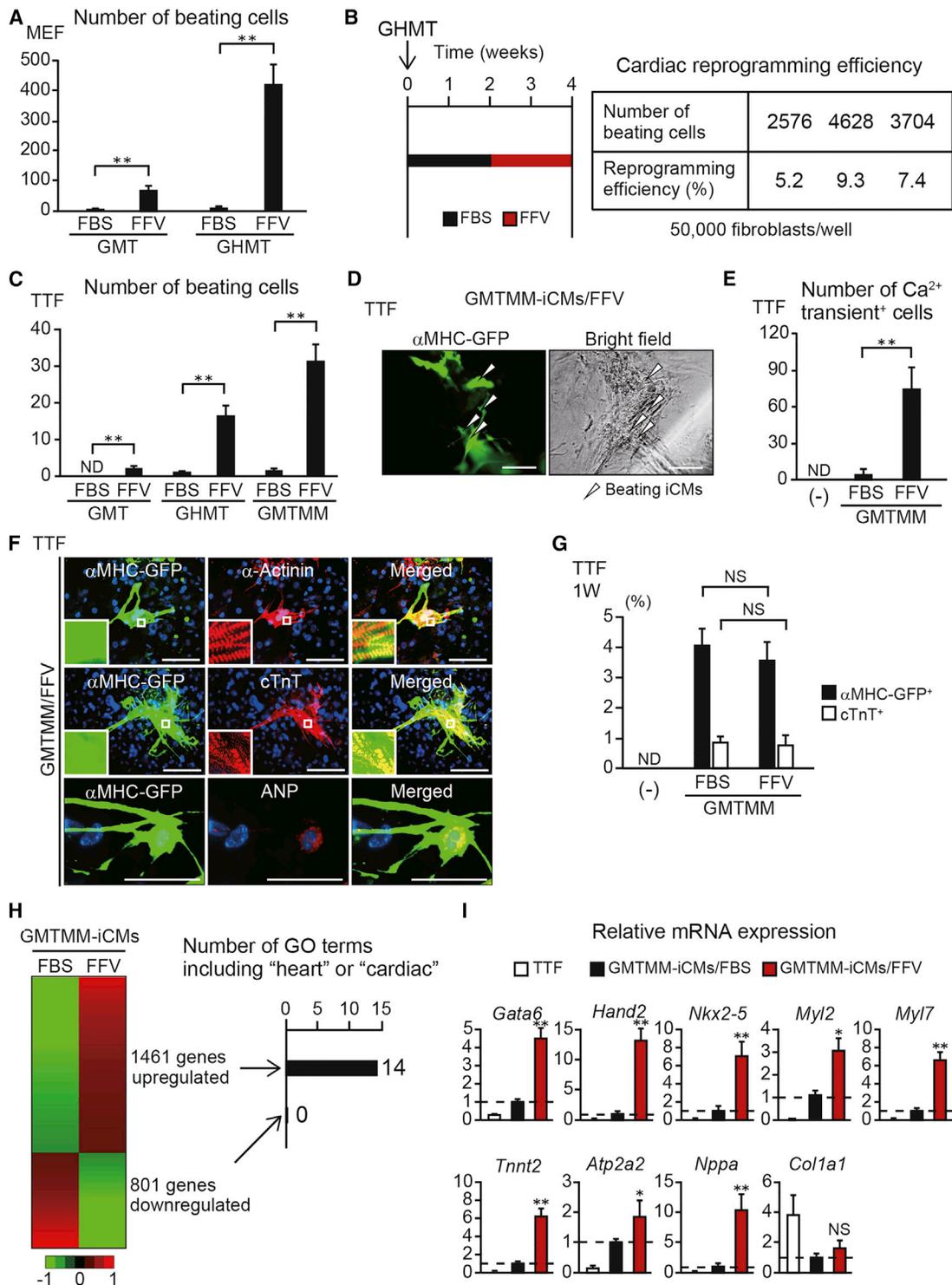


Figure 5. FFV Promoted Cardiac Reprogramming by GMTMM in Postnatal TTFs

(A) The number of beating cells after transduction with GMT or GHMT in MEFs after culture with FBS or FFV for 4 weeks ($n = 3$ independent triplicate experiments). See also [Figure S4A](#) and [Movie S5](#).

(B) GHMT-transduced cells were cultured with FBS for 2 weeks and FFV for an additional 2 weeks (left). Cardiac reprogramming efficiency was calculated as the number of beating cells divided by the number of starting fibroblasts (right).

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DISCUSSION

Here, we identified the defined culture conditions that greatly promoted the quality of cardiac reprogramming. We also determined the molecules and signaling pathways critical in the late stage of cardiac reprogramming, which has been a major hurdle for successful reprogramming. Use of the defined condition could allow for the standardization of the cardiac reprogramming protocol and provide new insights into the mechanisms of cardiac reprogramming. Our work showed that cardiac reprogramming is not merely a nuclear event, but rather that it is determined by the fine cross talk between intrinsic and extrinsic factors.

We revealed that the cardiac reprogramming process can be divided into early and late stages with distinct molecular features, and that the factors enhancing cardiac reprogramming may function at different stages. Consistent with previous reports, we found that the addition of *Hand2* to GMT increased the generation of spontaneously contracting iCMs, at least in part, by promoting the generation of cells expressing sarcomeric genes during early reprogramming (Addis et al., 2013; Song et al., 2012). In contrast, FFV promoted cardiac reprogramming by converting partially reprogrammed iCMs into functional iCMs at the late stage of reprogramming, without increasing the numbers of partially reprogrammed cells or cell proliferation. Thus, the addition of *Hand2* and FFV to GMT functioned at both the early and late stages of reprogramming and synergistically increased the cardiac reprogramming efficiency in this study.

For directed cardiac differentiation from PSCs, Activin-Nodal, BMP, and Wnt activation cause PSCs to commit to the CPC fate, and subsequent inhibition of Wnt signaling induces cardiac differentiation (BurrIDGE et al., 2014). Kattman et al. (2011) used FGF and VEGF for cardiac differentiation, but the effects of these growth factors on cardiac

differentiation and the underlying mechanisms remain undetermined. We found that Activin, BMP, and Wnt signaling activators did not promote cardiac reprogramming, while FFV activated multiple cardiac transcriptional regulators and increased the generation of functional iCMs through the p38MAPK and PI3K/AKT pathways. FFV-induced *Gata6*, *Hand2*, and *Ppargc1a* expression was inhibited by pretreatment with either p38MAPK or PI3K inhibitor, while the expression of *Nkx2.5* was largely mediated through the PI3K/AKT pathway. In agreement with this, the expression of *Nkx2.5* was regulated by PI3K/AKT activities in the cardiac differentiation of PSCs, suggesting a link between the mechanisms of iCM maturation and cardiac differentiation of PSCs (Naito et al., 2003; Roggia et al., 2007). Consistent with our results, Zhou et al. (2015) recently reported that addition of AKT1 to GHMT greatly enhanced cardiac reprogramming through pathways involving the mitochondrial target of rapamycin complex1 (*mTORC1*) and forkhead box o3 (*Foxo3*). While we found that the p38MAPK and PI3K/AKT pathways were critical for cardiac reprogramming, there are many other pathways involved in FFV signaling. Identification of such pathways could enhance our understanding of the molecular mechanism underlying cardiac reprogramming and promote reprogramming efficiency.

We also found that FFV enabled cardiac reprogramming with only *Mef2c* and *Tbx5* through the induction of *Gata4* and other reprogramming factors. Recently, Baeyens et al. (2014) reported that the transient administration of two growth factors, epidermal growth factor and ciliary neurotrophic factor, into adult diabetic mice efficiently converted pancreatic exocrine cells into functional β -cells, and ameliorated hyperglycemia without genetic manipulation in vivo. This effect was mediated through the induction of the expression of Neurogenin 3, which is one of the reprogramming factors for β -cells. Given that the in vivo environment might be more permissive than

(C) The number of beating cells in each well after transduction with GMT, GHMT, or GMTMM in TTFs cultured with FBS or FFV for 8 weeks (n = 3 independent triplicate experiments).

(D) Spontaneously beating GMTMM-iCMs cultured with FFV for 8 weeks (arrowheads), corresponding to Movie S6. The iCMs originated from postnatal TTFs.

(E) The total number of Ca^{2+} oscillation⁺ cells in ten randomly selected fields per well (n = 3 independent triplicate experiments).

(F) Immunocytochemistry analysis for α MHC-GFP, α -actinin, cTNT, ANP, and DAPI. GMTMM transduction induced α MHC-GFP and cardiac protein expression in TTFs after 8 weeks. Insets are high-magnification views.

(G) FACS analysis of α MHC-GFP and cTNT expression in GMTMM-TTFs cultured with FBS or FFV for 1 week (n = 3 independent triplicate experiments).

(H) Heatmap image of microarray data for differentially expressed genes between GMTMM-iCMs cultured with FBS and FFV. From this analysis, 1,461 genes were upregulated and 801 genes were downregulated in GMTMM-iCMs treated with FFV (left). The number of GO terms, including "heart" or "cardiac," in the differentially expressed genes is shown (right). See also Figure S4E.

(I) mRNA expression patterns of TTFs and GMTMM-iCMs cultured in medium containing FBS or FFV were determined by qRT-PCR (n = 3 independent triplicate experiments). Data were normalized to the values of GMTMM-iCMs cultured in FBS.

All data are presented as mean \pm SD. **p < 0.01; *p < 0.05 versus the relevant control. ns, not significant.

See also Figure S4 and Movies S5 and S6.

culture dishes for reprogramming, FFV with fewer reprogramming factors might be sufficient to repair damaged hearts by *in vivo* reprogramming. In agreement with this, *in vivo* cardiac reprogramming by GMT with VEGF administration enhanced the efficacy of GMT-mediated functional recovery in mice after myocardial infarction (Mathison et al., 2012). Further *in vitro* studies in human cells and *in vivo* experiments in larger animals are needed to apply this strategy to regenerative therapies. Nevertheless, we believe that our defined culture conditions could serve as a powerful platform that can be used to identify new compounds that enhance cardiac reprogramming and reduce genetic manipulation, which would be highly desirable for the development of pharmacological cardiac regeneration.

EXPERIMENTAL PROCEDURES

Generation of α MHC-GFP and *Mesp1*-GFP Mice

The Keio University Ethics Committee for Animal Experiments approved all experiments in this study. Transgenic mice overexpressing GFP under the α MHC promoter were generated as described previously (Ieda et al., 2010). *Mesp1*-GFP mice were obtained by crossing *Mesp1-Cre* mice and *CAG-CAT-EGFP* reporter mice (Ieda et al., 2010; Kawamoto et al., 2000; Saga et al., 1999).

qRT-PCR

Total RNA was isolated from the cells, and qRT-PCR was performed using the StepOnePlus Real-Time PCR system with the following TaqMan probes (Applied Biosystems): *Actn2* (Mm00473657_m1), *Atp2a2* (Mm01201431_m1), *Col1a1* (Mm00801666_g1), *Col3a1* (Mm01254476_m1), *Gja1* (Mm00439105_m1), *Hcn4* (Mm01176086_m1), *Isl1* (Mm00517585_m1), *Kcnq1* (Mm00434640_m1), *Mesp1* (Mm00801883_g1), *Myh6* (Mm00440354_m1), *Myh7* (Mm01319006_g1), *Myl2* (Mm00440384_m1), *Myl7* (Mm01183005_g1), *Nppa* (Mm01255747_g1), *Ppargc1* (Mm01208835_m1), *Ryr2* (Mm00465877_m1), *Slc8a1* (Mm01232254_m1), *Snai1* (Mm00441533_g1), *Smyd1* (Mm00477663_m1), *Tnnt2* (Mm00441922_m1), and *Tnmi3* (Mm00437164_m1). mRNA levels were normalized to those of *Gapdh* (Mm99999915_g1).

Fibroblast Isolation

For MEF isolation, embryos isolated from 12.5-day pregnant mice were washed with PBS, followed by the careful removal of head and visceral tissues (Muraoka et al., 2014). The remaining parts of the embryos were washed in fresh PBS, minced using a pair of scissors, transferred to a 0.125% trypsin/EDTA solution (GIBCO, 25200-072; 3 ml per embryo), and incubated at 37°C for 20 min. An additional 3 ml of trypsin/EDTA solution was then added, and the mixture was further incubated at 37°C for 20 min. After trypsinization, an equal amount of medium (6 ml of DMEM containing 10% FBS per embryo) was added and pipetted several times to allow tissue dissociation. After incubation of the tissue/medium

mixture for 5 min at room temperature, the supernatant was transferred to a fresh tube, and cells were collected by centrifugation and resuspended in DMEM/10% FBS (Thermo Scientific, SV30014.03) for culturing at 37°C in 5% CO₂. For TTF isolation, tails of α MHC-GFP TG mice were minced into pieces smaller than 1 mm³ in size (Muraoka et al., 2014). The explants were plated on gelatin-coated dishes and cultured for 10–14 days in explant medium (IMDM with L-glutamate and 25 mM HEPES [GIBCO, 12440-053]/20% FBS). Fibroblasts that migrated were harvested and filtered with 40- μ m cell strainers (BD Biosciences) to avoid contamination with tissue fragments. The cells were plated at a density of 1 \times 10⁴ cells/cm² for retrovirus transduction. For all experiments, fibroblasts at early passage numbers (P1–P3) were used.

Retroviral Infection and Cell Culture

To construct the pMXs retroviral vectors, we amplified the coding regions of *GFP*, *Gata4*, *Mef2c*, *Tbx5*, *Hand2*, *Myocd*, and *Mesp1* by PCR and subcloned them into respective pMXs vectors for transfection into Plat-E cells using Fugene 6 (Promega, E2691) to generate retroviruses (Ieda et al., 2010). Fibroblasts were transduced with the retrovirus mixture as indicated. The medium was replaced with FBS or FFV after 24 hr of infection and at the indicated time points in some experiments. FBS medium contained DMEM (high glucose) with L-glutamate and phenol red (Wako, 044-29765), Medium199 with Earle's salts, L-glutamate, and 22 g/l sodium bicarbonate (GIBCO, 11150-059), and 10% HyClone Characterized FBS (Thermo Scientific, SV30014.03). FFV medium contained StemPro-34 SF medium (GIBCO, 10639-011), GlutaMAX (10 μ l/ml, GIBCO, 35050-061), ascorbic acid (50 μ g/ml, Sigma Aldrich, A-4544), recombinant human VEGF165 (5 ng/ml, R&D Systems, 293-VE-050), recombinant human FGF basic146 aa (10 ng/ml, R&D Systems, 233-FB-025), and recombinant human FGF10 (50 ng/ml, R&D Systems, 345-FG-025). Unless otherwise stated, the cells were cultured in SF (StemPro-34 SF medium/GlutaMAX/ascorbic acid) supplemented with or without recombinant human/mouse/rat activin A (5 ng/ml, R&D Systems, 338-AC), recombinant human BMP4 (20 ng/ml, R&D Systems, 314-BP), CHIR99021 (3 μ M, Selleck, S2924), recombinant human DKK1 (50 ng/ml, R&D Systems, 5439-DK/CF), FGF2, FGF10, recombinant human TGF- β (2 ng/ml, PeproTech, 100-21C), VEGF, and IWP4 (5 μ M, STEMGENT, 04-0036). PD98059 (5 μ M), SB203580 (5 μ M), or LY294002 (10 μ M, Calbiochem) was added after 2 weeks of culture in some experiments (Ieda et al., 2009). The medium was changed every 3 days.

FACS Analyses and Sorting

For α MHC-GFP/cTNT expression, cells were fixed with 4% paraformaldehyde (PFA; MUTO PURE CHEMICALS, 3311-1) for 15 min, permeabilized with saponin (Sigma Aldrich, 47036-250G-F), stained with anti-cTNT (Thermo Scientific, MS-295-P1) and anti-GFP (MBL, 598) antibodies, followed by secondary antibodies conjugated with Alexa Fluor 488 (Invitrogen, A11008) and 647 (Invitrogen, A21240), respectively. Cells were then analyzed using a FACS Calibur instrument (BD Biosciences) with the FlowJo software (Tomy Digital Biology). For iCM sorting, cells were sorted



as α MHC-GFP⁺ cells, and for Mesp1-GFP⁻/Thy1⁺ cell sorting, cells were incubated with APC-conjugated anti-THY1 antibodies (eBioscience, 17-0909-41) and sorted using a FACS Aria III instrument (BD Biosciences). Negative controls used for FACS gating were based on the cells stained with isotype control antibodies.

EdU Labeling Assay

For assessing cell proliferation, 10 μ M EdU was added to the culture medium after 2 weeks of transduction and maintained throughout the culture for an additional 2 weeks. Cells were fixed with 4% PFA for 15 min, permeabilized, and incubated with anti-cTNT antibodies. Cells were then incubated with secondary antibodies conjugated with Alexa Fluor 546 (for immunocytochemistry) or 647 (for FACS) and then incubated with the EdU reaction cocktail following the manufacturer's instructions (Click-iT EdU Alexa Fluor 488 HCS Assay; Invitrogen, C10350).

Immunocytochemistry

Cells were fixed in 4% PFA for 15 min at room temperature, blocked with 5% Normal Goat Serum blocking solution (Vector Laboratories, S-1000), and incubated with primary antibodies against atrial natriuretic peptide (ANP; Millipore, AB5490), cTNT (Thermo Scientific, MS-295-P1), cTNI (Abcam, ab47003), GFP (MBL, 598), MLC-2A (Synaptic Systems, 311-011), MLC-2V (Synaptic Systems, 310-111), or sarcomeric α -actinin (Sigma Aldrich, 111M4845). Cells were then incubated with secondary antibodies conjugated with Alexa Fluor 488 or 546, followed by DAPI (Invitrogen, D1306) counterstaining. The percentage of cells immunopositive for GFP, α -actinin, cTNT, and ANP were counted in 10–15 randomly selected fields per well in at least three independent experiments, and 2,000–4,000 cells were counted in total. The measurements and calculations were conducted in a blinded manner.

Western Blot Analysis

Lysates were prepared by homogenization of cells in RIPA buffer and run on SDS-PAGE to separate proteins prior to the immunoblot analyses as described previously (Muraoka et al., 2014). After transfer to nitrocellulose membranes, immunodetection was performed using antibodies specific to p-ERK(Cell Signaling Technology, 9101S), p-p38MAPK(Cell Signaling Technology, 9211S), p-AKT(Cell Signaling Technology, 9271S), ERK(Cell Signaling Technology, 9102S), p38MAPK(Cell Signaling Technology, 9212S), and AKT(Cell Signaling Technology, 9272S), followed by the HRP-conjugated anti-rabbit immunoglobulin G secondary antibody (Cell Signaling Technology, 7074S). The antibody-bound proteins were visualized by chemiluminescence detection (ECL, Amersham).

Ca²⁺ Imaging and Counting Beating Cells

Ca²⁺ imaging was performed following standard protocols. Briefly, cells were labeled with Rhod-3 from a Calcium Imaging Kit (Invitrogen, R10145) for 1 hr at room temperature, washed, and incubated for an additional hour, to allow de-esterification of the dye. Rhod-3-labeled cells were analyzed at 37°C using an LSM 510 META confocal microscope (Carl Zeiss). Ca²⁺ oscillations could

only be imaged for a short period, owing to the increasing background fluorescence from the medium; thus, the measurements were taken within 30 min after changing to Tyrode's buffer. Ca²⁺ oscillation⁺ cells were counted in ten randomly selected fields per well in at least three independent experiments, and a minimum of 1,000 cells were counted.

To count the number of beating cells, we seeded 50,000 fibroblasts per well on 12-well plates, performed cell transductions, cultured the cells with the indicated media, and then monitored cell contraction. For accurate analyses of the cell count, we used an all-in-one fluorescence microscope (BZ-9000; Keyence). The cells were maintained at 37°C and 5% CO₂ using the controlled chamber within the microscope. We first acquired images of the cells in all the areas in a well with a 20 \times phase contrast lens by moving the motorized stage sequentially. Next, we moved the field to cover all the areas in a well and counted the number of spontaneously contracting cells in each field with the 20 \times phase contrast lens in at least three independent experiments. We identified the individual beating iCMs based on differences in beating frequency, cell-membrane boundary, and nuclei identified by phase contrast and GFP fluorescence filter. The cardiac reprogramming efficiency was determined as the number of beating cells per 50,000 starting fibroblasts. The measurements and calculations were conducted in a blinded manner.

Gene Microarray Analyses

Genome-wide gene expression analyses were performed using the 3D-Gene Mouse Oligo Chip 25k (Toray Industries). For efficient hybridization, this microarray was 3D and constructed with a well as the space between the probes and cylinder-stems with 70-mer oligonucleotide probes on the top. RNA was extracted from neonatal mouse heart tissue, MEFs, TTFs, and MT-, GMT-, or GMTMM-induced α MHC-GFP⁺ cells using the RNeasy RNA Cell Miniprep System (Promega, Z6012). Total RNA was labeled with Cy5 by using an Amino Alkyl MessageAMP II aRNA Amplification Kit (Applied Biosystems), and hybridization was performed using the supplier's protocols (www.3d-gene.com). Hybridization signals were scanned using a 3D-Gene Scanner (Toray Industries) and processed by extraction (Toray Industries). The raw data for each spot were normalized by substitution with the mean intensity of the background signal determined by the combined signal intensities of all blank spots at 95% confidence intervals. Raw data intensities greater than 2 SD of the background signal intensity were considered valid. Signals detected for each gene were normalized by the global normalization method. Heatmap images for differentially expressed genes (more than 2- or 1.5-fold differences) were processed using Cluster (v.2.0) software, and the results were displayed with the TreeView program (http://www.eisenlab.org/eisen/?page_id=42). Scatter plot analyses were processed using Microsoft Excel. Gene ontology (GO) analysis was performed using GeneCodis (Carmona-Saez et al., 2007; Nogales-Cadenas et al., 2009). This method computes hypergeometric p values for the over- or underrepresentation of each GO term in the specified ontology for the gene set of interest. Moderated t-statistics and the associated p values were calculated by the Welch t test using Microsoft Excel. Differential gene expression was defined using the statistics/threshold combination.



Statistical Analyses

Differences between groups were examined for statistical significance using the Student's *t* tests or ANOVA. Differences with *p* values of less than 0.05 were regarded as significant.

ACCESSION NUMBERS

The accession number for the microarray data reported in this paper has been uploaded to GEO:GSE73839.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and six movies and can be found with this article online at <http://dx.doi.org/10.1016/j.stemcr.2015.10.019>.

AUTHOR CONTRIBUTIONS

H.Y. and M. Ieda designed the experiments. H.Y., N.M., K.M., T.S., M. Isomi, S.H., H.K., T.U., M.A., and Y.K. carried out the experiments. H.Y., N.M., J.K., T.F., and K.F. analyzed the data. H.Y. and M. Ieda wrote the paper.

ACKNOWLEDGMENTS

M. Ieda was supported by research grants from JST CREST, JSPS, Keio University Program for the Advancement of Next Generation Research Projects, Banyu Life Science, Senshin Medical Research Foundation, and Takeda Science Foundation, and H.Y. was supported by research grants from Ichiro Kanehara Foundation and Keio University Medical Science Fund.

Received: September 29, 2015

Revised: October 29, 2015

Accepted: October 29, 2015

Published: November 25, 2015

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