Stem Cell Reports, Volume *7*

Supplemental Information

Autonomous and Non-autonomous Defects Underlie Hypertrophic Cardiomyopathy in BRAF-Mutant hiPSC-Derived Cardiomyocytes

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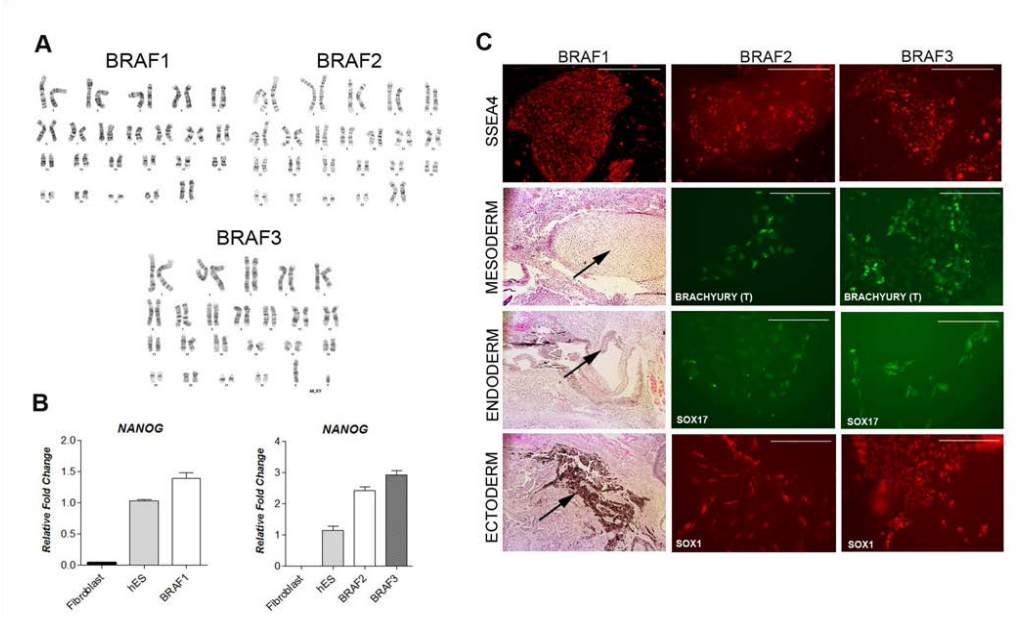
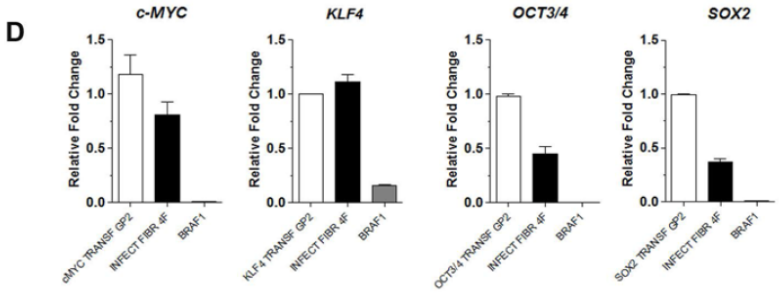
Supplemental Information

Supplemental Figures





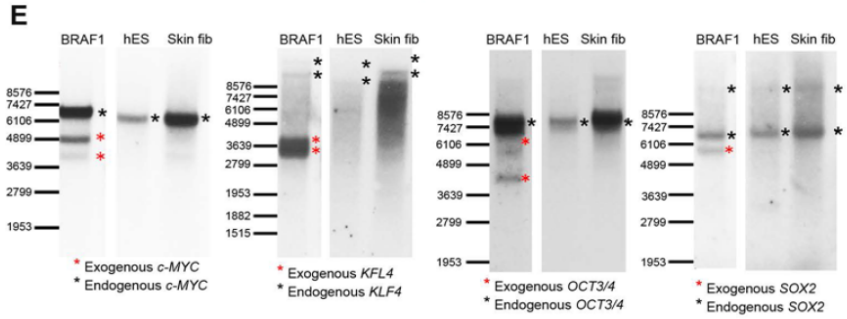


Figure S1 (refers to Figure 1). Pluripotency analysis of BRAF-mutant hiPSCs. (A) Karyotype G-banding analysis of all BRAF-mutant hiPSC lines demonstrated normal diploid chromosomes. (B) Gene expression for the pluripotency marker *NANOG* demonstrated similar expression levels in BRAF-mutant hiPSCs to hESCs. Barr graphs show mean ± standard error of the mean. (C) BRAF-mutant hiPSCs were positive for the stem cell surface marker SSEA4 by immunocytochemistry (magnification 10x). BRAF-mutant hiPSCs could be differentiated into three germ layers, as assessed by BRACHYURY (mesoderm), SOX17 (endoderm) and SOX1 (ectoderm) immunocytochemistry in BRAF2 and BRAF3 samples (magnification 10x). Haematoxylin and eosin staining demonstrated the presence of the three germ layers in teratomas generated from BRAF1 hiPSCs (magnification 4x). (D) Exogenous transgenes were silenced in BRAF1 hiPSCs generated with retrovirus. Positive controls introduced GP2 cells transfected with individual factors (TRANS GP2) and original skin fibroblast lines transfected with all four factors (INFECT FIBR 4F). Bar graphs show mean ± standard error of the mean. (E) Southern blot demonstrating integrations of exogenous transgenes in BRAF1 hiPSCs generated with retrovirus compared to hESCs and original un-infected skin fibroblast samples. For (A-E), n=3 technical replicates for each condition.

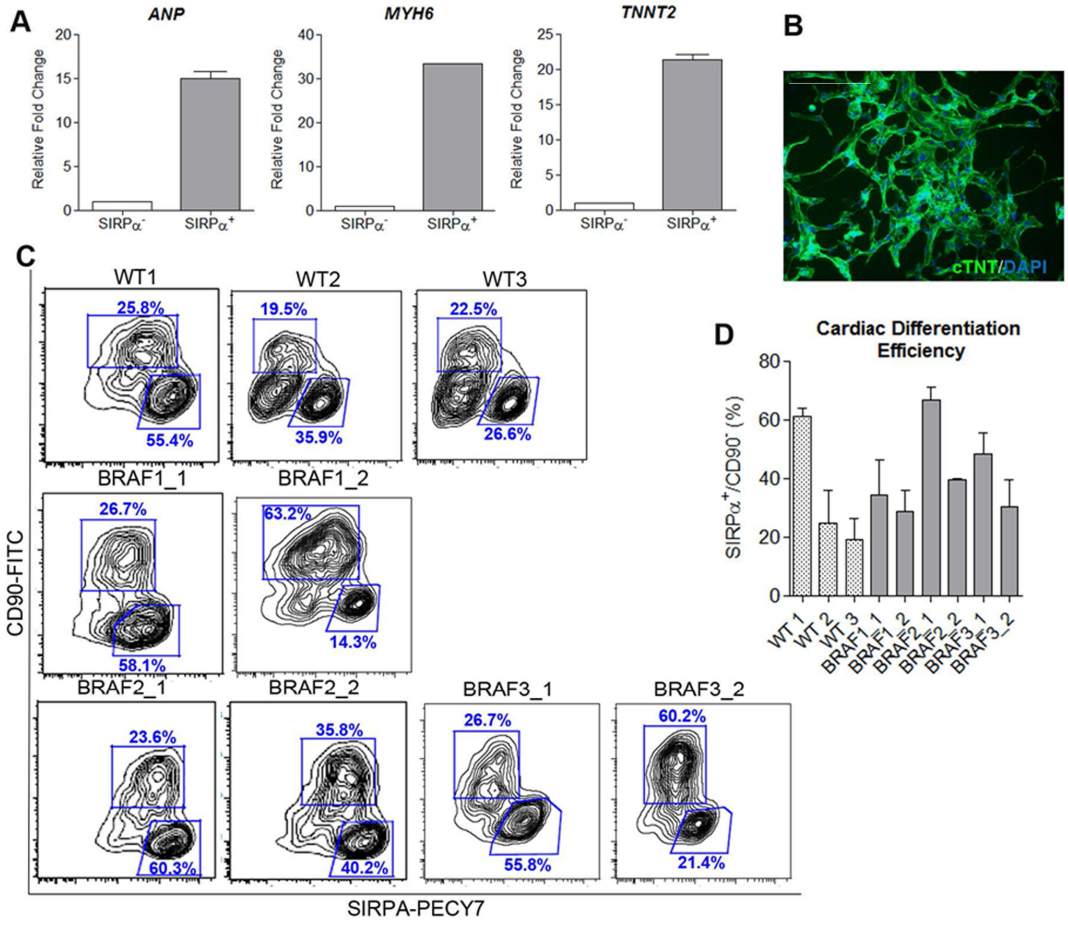


Figure S2 (refers to Figure 1). Purification of hiPSC-derived CM. (A) Expression of cardiac-specific genes *ANP*, *MYH6*, and *TNNT2* were up-regulated in the SIRPα^{+}/CD90^{-} population. (B) Immunostaining for cTNT in monolayers generated from sorted and re-cultured SIRPα^{+}/CD90^{-} cells (scale bars 200 μm). (C) Representative FACS plots demonstrating the percentage of SIRPα^{+}/CD90^{-} cells across all hiPSCs. (D) Quantification of the SIRPα^{+}/CD90^{-} population across all hiPSCs lines (n=3 independent experiments per group). Bar graphs show mean± standard error of the mean.

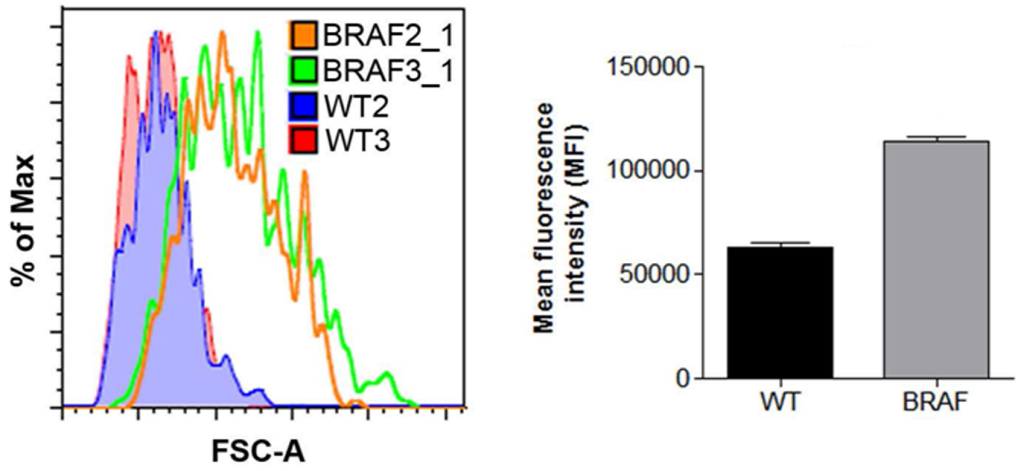


Figure S3 (refers to Figure 2). Three-dimensional cellular area of hiPSCs-derived CM. Three-dimensional cellular area was quantified by comparing the mean fluorescence intensity (MFI) of the FSC property for WT (n=2) and BRAF-mutant (n=2) SIRPα^{+}/CD90^{-} populations. BRAF-mutant CMs displayed increased three-dimensional area compared to WT. Data represents 2 biological replicates (WT2, WT3, BRAF2, and BRAF3) and 2 technical replicates. Bar graphs show mean± standard error of the mean.

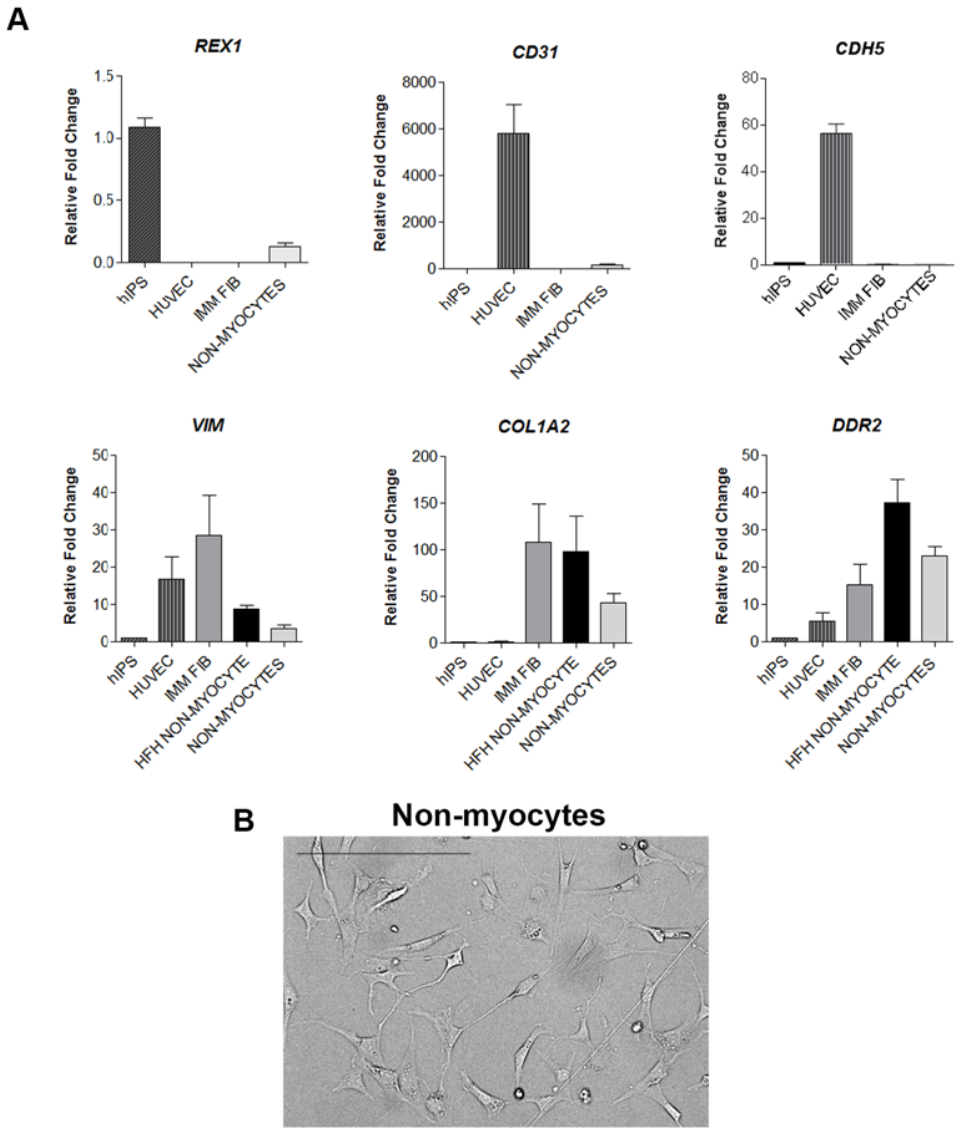


Figure S4 (refers to Figure 5). Purified SIRPα^{+}/CD90^{-} cells are fibroblast-like. (A) Expression of pluripotency (*REX1)*, endothelial (*CD31, CDH5)*, and fibroblast-associated (*Vimentin; VIM, Collagen Type I; COL1A2,* and *Discoidin Domain Receptor Tyrosine Kinase 2; DDR2*) genes in purified SIRPα^{-}/CD90^{+} non-myocytes. hiPS cells, human umbilical vein endothelial cells (HUVECs), immortalized primary fibroblasts (IMM FIB), and SIRPα^{-}/CD90^{+} cells derived from human fetal hearts (HFH NON-MYOCYTES) were used for comparison. All samples relative to gene expression in hiPS cells. Non-myocytes exclusively expressed fibroblast-associated genes, which were also highly expressed by SIRPα^{-}/CD90^{+} cells purified from human fetal hearts. Non-myocyte cell lines used were BRAF1 and BRAF2. Data represents 3 independent experiments. Bar graphs show mean± standard error of the mean. (B) Spindle-shaped morphology of purified SIRPα^{-}/CD90^{+} non-myocytes (scale bars 400 μm).

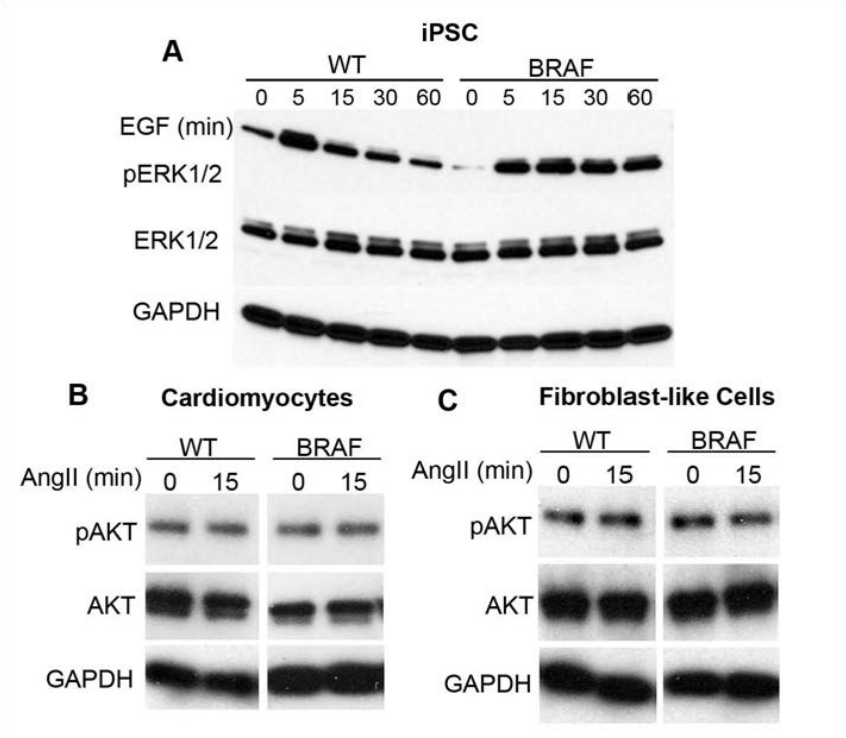


Figure S5 (refers to Figure 4). Signaling pathway activation in hiPSCs and derived cells. (A) BRAF hiPSCs stimulated with EGF over time displayed prolonged activation of pERK compared to WT by western blot. (B) Levels of pAKT were not different from WT in purified BRAF-mutant cardiomyocytes or fibroblast-like cells (C) with or without AngII stimulation, indicating a lack of AKT pathway activation. Cell lines shown are WT1 and BRAF1.

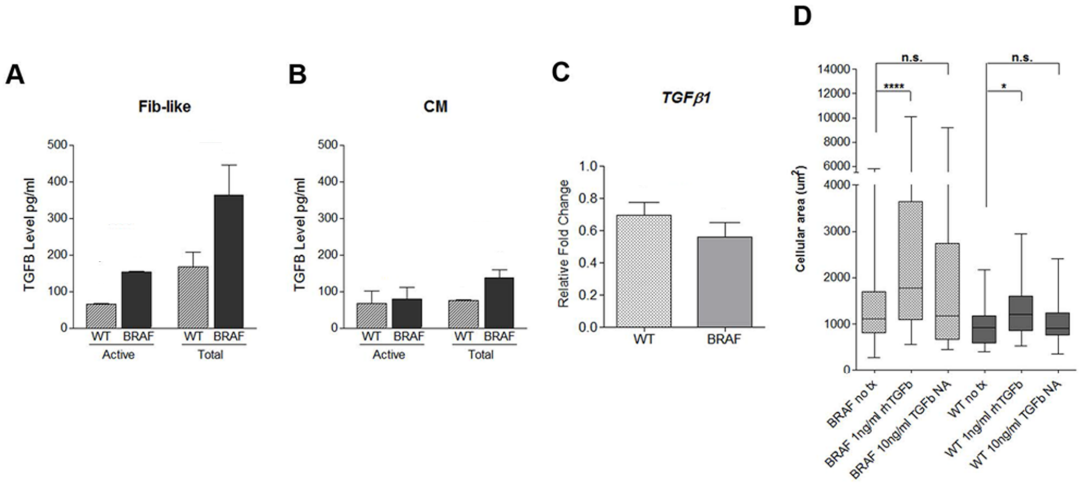


Figure S6 (refers to Figure 6). BRAF-mutant FLCs secrete increased levels of TGFβ protein and TGFβ is sufficient to induce cellular hypertrophy in hiPSC-derived CM. (A-B) Levels of active and total TGFβ protein in conditioned media from purified BRAF-mutant and WT FLCs (Fib-like) and CMs were measured by ELISA. (A) Purified BRAF-mutant Fib-like cells secreted increased levels of active and total TGFβ protein compared to WT (BRAF n=3, WT n=3). (B) Purified BRAF-mutant CMs secreted similar levels of active and total TGFβ protein to WT CMs (BRAF n=3, WT n=3). (C) Purified BRAF-mutant CMs expressed similar levels of TGFβ1 gene compared to WT CMs (BRAF n=12, WT n=12). Bar graphs show mean ± standard error of the mean. Data in (A-C) represent 2 biological replicates (WT1, WT3, BRAF1, and BRAF3) and 3 technical replicates. (D) Purified BRAF-mutant and WT CMs were incubated with recombinant human TGFβ (rhTGFβ). BRAF-mutant CMs exposed to rhTGFβ (n=67) were significantly larger than BRAF-mutant CMs without treatment (n=70) (p=0.0001). WT CMs exposed to rhTGFβ (n=30) were also significantly larger than WT CMs without treatment (n=29) (p=0.03). Treatment of BRAF-mutant (n=25) or WT (n=36) CMs with TGF NA did not alter their cellular size (p=n.s). Data in (D) represents 2 biological replicates (WT2, WT3, BRAF 1, and BRAF3) and 2 technical replicates. Box-and-whisker plots show the median to the first and third quartiles and the minimum and maximum values.

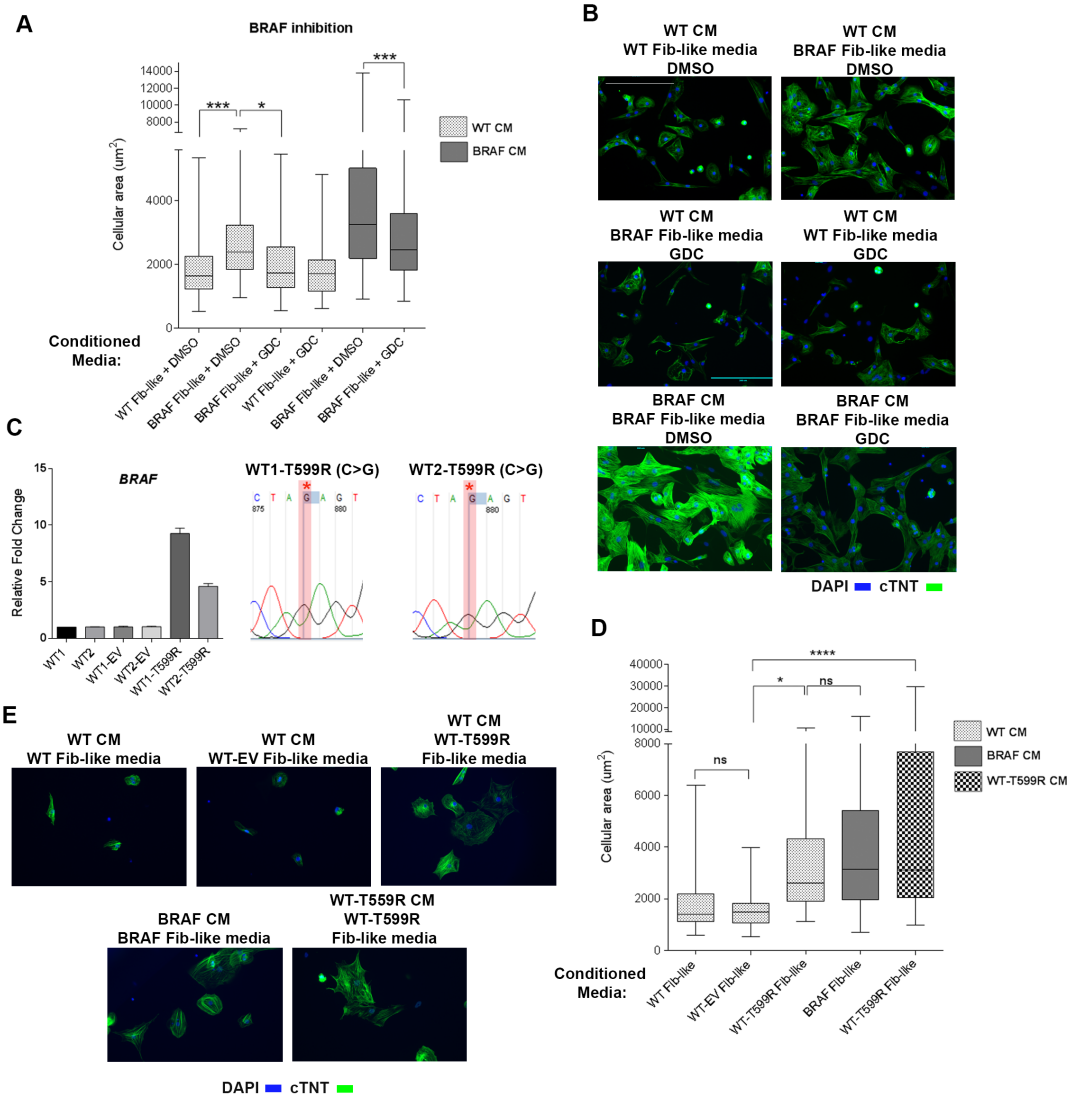

A
BRAF inhibition
Cellular area (um2)
WT CM
BRAF CM
Conditioned Media:
WT Fib-like + DMSO
BRAF Fib-like + DMSO
BRAF Fib-like + GDC
WT Fib-like + GDC
BRAF Fib-like + DMSO
BRAF Fib-like + GDC
B
WT CM
WT Fib-like media
DMSO
WT CM
BRAF Fib-like media
DMSO
WT CM
BRAF Fib-like media
GDC
WT CM
WT Fib-like media
GDC
BRAF CM
BRAF Fib-like media
DMSO
BRAF CM
BRAF Fib-like media
GDC
DAPI
cTNT
C
BRAF
Relative Fold Change
WT1
WT2
WT1-EV
WT2-EV
WT1-T599R
WT2-T599R
WT1-T599R (C>G)
WT2-T599R (C>G)
D
Cellular area (um2)
WT CM
BRAF CM
WT-T599R CM
Conditioned Media:
WT Fib-like
WT-EV Fib-like
WT-T599R Fib-like
BRAF Fib-like
WT-T599R Fib-like
E
WT CM
WT Fib-like media
WT CM
WT-EV Fib-like media
WT CM
WT-T599R
Fib-like media
BRAF CM
BRAF Fib-like media
WT-T559R CM
WT-T599R
Fib-like media
DAPI
cTNT
F
ANP
BNP
Relative Fold Change
WT Fib-like + WT CM
WT-EV Fib-like + WT CM
WT-T599R Fib-like + WT CM
BRAF Fib-like + BRAF CM

Figure S7 (refers to Figure 7). Activation of BRAF induces the hypertrophic phenotype. (A) Purified WT CMs exposed to BRAF-mutant FLC (Fib-like) conditioned media (n=99) significantly increased in cellular area compared to exposure to WT Fib-like conditioned media (n=84) (p=0.0005), but cell size was normalized upon addition of BRAF inhibitor GDC-0879 (GDC) (n=86) (p=0.01). WT CM cell size was unchanged when GDC was added to WT Fib-like conditioned media (n=56). Cellular area of BRAF-mutant CMs was significantly reduced upon exposure to BRAF-mutant Fib-like conditioned media with GDC (n=105) compared to BRAF-mutant Fib-like conditioned media alone (n=80) (p=0.0006). (B) Representative images of conditions depicted in (A). (C) Integration of *BRAF* T599R lentivirus in WT1 and WT2 hiPSC clones was confirmed by Sanger sequencing for presence of the mutation in *BRAF* cDNA, as well as increased expression of *BRAF* by qPCR. (D) WT CMs exposed to conditioned media from WT Fib-like cells over-expressing an empty vector (WT-EV) (n=45) displayed no change in cellular area compared to un-transduced WT Fib-like cells (n=67). Cellular area of WT CMs significantly increased upon exposure to conditioned media from WT Fib-like cells over-expressing the *BRAF* T599R mutation (WT-T599R) (n=75) (p=0.02), similar in size to BRAF-mutant CMs exposed to BRAF-mutant Fib-like conditioned media (n=44). The largest increase in cellular area was observed in WT-T599R CMs exposed to conditioned media from WT-T599R Fib-like cells (n=50) ($p<0.0001$). (E) Representative images of conditions depicted in (D). (F) WT CMs expressed increased levels of *ANP* and *BNP* when exposed to WT-T599R Fib-like conditioned media compared to WT-EV Fib-like conditioned media or media from un-transduced WT Fib-like cells (n=3 for all conditions). Scale bars 200μm. For each graph data represents 2 (WT1, WT2) or 3 (BRAF1, BRAF2, and BRAF3) biological replicates and 2 technical replicates. Bar graphs show mean ± standard error of the mean. Box-and-whisker plots show the median to the first and third quartiles and the minimum and maximum values.

Supplemental Tables

Experiment	WT1	WT2	WT3	BRAF1_1	BRAF1_2	BRAF2_1	BRAF2_2	BRAF3_1	BRAF3_2
Cellular size	✔	✔	✔	✔	✔	✔	✔	✔	
Sarcomeric organization	✔	✔	✔	✔	✔	✔		✔	
Cardiomyocyte gene expression	✔	✔	✔	✔	✔	✔		✔	
Calcium studies	✔	✔	✔	✔	✔	✔	✔	✔	✔
Western blots	✔	✔	✔	✔	✔	✔		✔	
Co-culture		✔	✔	✔		✔			
Fibroblast-like cell gene expression	✔	✔	✔	✔		✔	✔	✔	
Fibroblast-like cell proliferation		✔	✔	✔		✔		✔	
Fibroblast-like cell TGFβ ELISA	✔		✔	✔				✔	
Conditioned media cross-culture	✔	✔	✔	✔	✔	✔		✔	
TGFβ-NA studies		✔	✔	✔		✔			
MEK-inhibitor studies		✔	✔	✔	✔	✔	✔	✔	✔
BRAF inhibitor studies	✔	✔		✔		✔		✔	
BRAF T599R over-expression studies	✔	✔		✔		✔		✔	

Table S1: hiPSC lines used in experimental procedures.

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')
QPCR		
NANOG	CCTGAAGACGTGTGAAGATGAG	GCTGATTAGGCTCCAACCATAC
pMX (Transgene)	CCCTCAAAGTAGACGGCATC	
c-MYC (Transgene)		AGGCTGCTGGTTTTCCACTA
KLF4 (Transgene)		GTGGAGAAAGATGGGAGCAG
OCT3/4 (Transgene)		GCGAGAAGGCAAAATCTGAA
SOX2 (Transgene)		TTCAGCTCCGTCTCCATCAT
cTNT	AGCATCTATAACTTGGAGGCAGAG	TGGAGACTTTCTGGTTATCGTTG
ANP	ACGCAGACCTGATGGATTTC	GCTTCTTCATTCGGCTCACT
BNP	CGGGTTACAGGAGCAGCG	CTCCAGGGATGTCTGCTCCA
MYH6	CCGTCTTCCTCACGCTCTT	TGAACTCATCCTTGTTCACCAC
ATP2A2	GGTGCTGAAAATCTCCTTGC	ATCAGTCATGCACAGGGTTG
PLN	CTGCCAAGGCTACCTAAAAG	AGCTGAGCGAGTGAGGTATT
BRAF	ACCACCCAATACCACAGGAAGAGGCGT	GGATCGGTCTCGTTGCCCAAATTGATTTC
REX1	AAAGCATCTCCTCATTCATGGT	AAAGCATCTCCTCATTCATGGT
CD31	ATGCCGTGGAAAGCAGATAC	CTGTTCTTCTCGGAACATGGA
DDR2	CCAGTCAGTGGTCAGAGTCCA	GGGTCCCCACCAGAGTGATAA
COL1A2	GGAGCTCCAAGGACAAGAAA	ATGAAGGCAAGTTGGGTAGC
CDH5	ATGAGATCGTGGTGGAAGCG	TGTGTACTTGGTCTGGGTGA
VIM	GACAGGATGTTGACAATGCG	GTTCCTGAATCTGAGCCTGC
POSTN	TGCCCAGCAGTTTTGCCCAT	CGTTGCTCTCCAAACCTCTA
TGFβ1	CCCTGGACACCAACTATTGC	CTTCCAGCCGAGGTCCTT
CTGF	CAGGCTAGAGAAGCAGAGCC	TGGAGATTTTGGGAGTACGG
ET-1	TCTCTGCTGTTTGTGGCTTG	GACTGGGAGTGGGTTTCTCC
GAPDH	GAGTCAACGGATTTGGTCGT	TTGATTTTGGAGGGATCTCG
SEQUENCING		
BRAF EXON 6 (Q257R)	TCCATCTATTATCTTGAACCCC	CCCTCACAGACTTTTAGACATCG
BRAF EXON 15 (T599R)	TAGGAAAGCATGTCACCTCATC	AACTCAGCAGCATCTCAGGG

Table S2: Primers used in PCR and qRT-PCR experiments.

Supplemental Experimental Procedures

hiPSC generation and maintenance. To generate BRAF1 hiPSCs, $8x10^5$ fibroblasts were plated onto 10 cm gelatin-coated dishes. After 24 and 48h (Day 0,1), equal amounts of retroviruses were filtered and added to fibroblasts with 6 μg/ml polybrene. On Day 3, media was changed to DMEM (Life Technologies). On Day 4, fibroblasts were trypsinized and re-plated at $5x10^4$ cells on 10 cm dishes coated with $1.3x10^6$ mitotically inactivated mouse embryonic fibroblasts (MEFs). Media was changed every two days using hiPSC maintenance media composed of DMEM/F12 (Cellgro, Mediatech) with 20% (vol/vol) knockout serum replacement (Life Technologies), 5% (vol/vol) MEF-conditioned medium, penicillin/streptomycin, L-glutamine (L-Gln), non-essential amino acids (Life Technologies), β-mercaptoethanol (β-ME, Sigma) and 10 ng/ml bFGF (R&D Systems). From Day 6-13 valproic acid was added to hiPSC medium at a final concentration of 2 mM. After 30 days, hESC-like colonies were mechanically isolated and re-plated onto 48-well plates coated with MEFs. Colonies were passaged and maintained in hiPSC media on MEF-coated plates. Rho Kinase inhibitor (10 μM) (Y-27632; Tocris, Bristol, UK) was added to hiPSC media for 24h after passaging.

To generate BRAF 2 and 3 hiPSCs, fibroblasts at passage 5 were trypsinized, centrifuged at 500 x g for 5 min, and resuspended in DMEM High-Glucose media (Life Technologies). $3.6x10^5$ cells were used per reprogramming. 1 μg of each plasmid pCXLE-hOCT3/4-shp53-F, pCXLE-hSK, pCXLE-hUL, and pCXLE-EGFP (Addgene) was mixed with 100 μl Resuspension Buffer R (Life Technologies), and added to fibroblasts. 100 μL of the cell suspension was dispensed into the Neon Transfection System (Life Technologies) and electroporated with three pulses of 1,650 V for 10 ms, and transfered into gelatin-coated dishes containing pre-warmed fibroblast medium and incubated in a 37 ºC, 5% CO_2 incubator. After Day 5, transfected fibroblasts were detached and plated onto mitomycin C-treated MEFs (Millipore) at $1x10^5$ cells/cm^2 and maintained in hiPSC medium.

***In vitro* three germ layer differentiation.** $2.5x10^4$ hiPSCs were plated on matrix-coated 96-well plates in 100 μl hiPSC medium. After 24 h, Day 1 media for the three lineages was added. Cells were maintained for two additional days (mesoderm) or five additional days (endoderm, ectoderm) at 37 °C in 5% CO_2, 5% O_2, and 90% N_2 before fixation. hiPSCs were blocked for 1 h at room temperature in 3% milk, 1% BSA, and 0.1% Triton-X100 in PBS and stained with provided primary antibodies Brachyury (mesoderm), Sox17 (endoderm), or Sox1 (ectoderm) for 2 h at room temperature, followed by corresponding secondary antibody – Alexa Fluor® 488 goat-anti-rabbit IgG (mesoderm), Alexa Fluor® 488 goat-anti-mouse IgG (endoderm), Alexa Fluor® 594 donkey-anti-goat IgG (ectoderm) (Life Technologies) at 1:400 dilution for 1 h at room temperature.

Flow cytometry. Cardiomyocytes were stained with 1:500 anti-human SIRPα-PE/Cy7 (BioLegend) and 1:250 anti-human CD90-FITC (BD Pharmingen) for 1 h at 4 °C in PBS+10% FBS staining buffer. Cells were filtered and resuspended at 10^6 cells/mL in staining buffer for cell sorting. Sorting was performed on an AriaII cell sorter (BD Biosciences). Flow cytometric gates were set using control cells stained with the appropriate isotype control antibody. Gates were determined for each differentiation independently to account for variability in differentiation and staining efficiencies. To determine cardiomyocyte purity, dissociated single cells were fixed with 4% PFA for 15 min at room temperature. The primary antibody mouse-anti-human cTNT (ThermoScientific, clone 13-11) was conjugated to AlexaFluor 488 *in vitro* using the Zenon Mouse IgG Labeling Kit (Life Technologies), according to manufacturer's instructions. Cells were incubated with the conjugated primary antibody at 1:100 final dilution of cTNT antibody for 2 h at room temperature. Cells were analyzed on an LSR-II flow cytometer (BD Biosciences). Data were analyzed using FlowJo software, Version 9.3.2.

Calcium transient analysis. Calcium (Ca^{2+}) transients were recorded from cardiomyocytes 7 days post sort on matrigel-coated coverslips. Cells were loaded with the acetomethoxy (AM) form of the Ca^{2+} indicator fluo-3 at 10 μM for 30 min at room temperature (Biotium, Hayward, CA), washed, and allowed 30 min for dye de-esterification. During experiments, cells were superfused with Tyrode's solution containing (in mM): NaCl 140, KCl 5.4, HEPES 10, NaH_2PO_4 1, $MgCl_2$ 1, $CaCl_2$ 2, glucose 5 (pH 7.4). Fluo-3 was excited at 488 nm, and fluorescence above 505 nm was recorded by a confocal microscope (LSM 5 Exciter Carl Zeiss AG, Jena, Germany) at 40x magnification. Cardiomyocytes were paced to steady-state at 1Hz, and transients recorded at 37 °C using the line-scan mode of the microscope. To quantify irregularity, the percentage of transients with extra peaks was calculated (10 transients per recording). For SR Ca^{2+} content, a solution of 0 Na^+ 0 Ca^{2+} Tyrode with 20 mM caffeine was rapidly washed on to the cells (NaCl replaced with equimolar LiCl). Analysis of data from line scan recordings

consisted of: (1) averaging across the cell length, and (2) converting to relative units (F/F_0) by normalizing to fluorescence prior to stimulation. Ca^{2+} transient decay time constants were calculated by fitting exponential functions to the declining phase of the transient. Recordings were processed and analyzed using custom MATLAB scripts.

Lentiviral over-expression. The pSIN-EF2-LIN28-PURO transfer plasmid (Addgene #16580) was used for lentiviral production. *LIN28* coding region was removed via restriction enzyme digestion, and *BRAF* T599R cDNA synthesized from patient iPSCs, or nothing (empty vector) was subcloned into the transfer plasmid. Packaging plasmid pCMV-dR8.2-dvpr (Addgene #8455) and envelope plasmid pCMV-VSV-G (Addgene #8454) were used for subsequent viral production. HEK 293T cells were transfected with 8 μg transfer plasmid, 6 μg packaging plasmid, and 2 μg envelope plasmid at 90% confluence using the Superfect Transfection Reagent (Qiagen), and maintained at 37 °C. 48 h and 72 h post-transfection, media containing viral particles was collected and pelleted via ultra-centrifugation at 16000rpm for 4 h at 4 °C. Viral pellets were re-suspended in iPSC media supplemented with 6μg/mL polybrene (Sigma), and used for two rounds of infection of 70-80% confluent WT1 and WT2 iPSC lines. 48 h after the second infection, media was replaced with fresh iPSC media supplemented with 1 ug/mL puromycin. Puromycin-resistant clones were isolated and verified for viral integration via PCR. Presence of *BRAF* T599R mutant cDNA was verified via Sanger sequencing, and over-expression of *BRAF* verified via qPCR according to previously described methods.