

SUPPLEMENTARY DATA

Increased cardiogenesis in P19-GFP teratocarcinoma cells expressing the propeptide IGF-1Ea

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Supplementary Materials and Methods

P19-GFP cell culture condition and differentiation

P19-GFP cells have been previously characterized for their efficiency to differentiate into cardiac myocytes and to be maintained easily in culture without feeder layer [1]. The expression of green fluorescent protein (GFP) under the myosin light chain 2v promoter favours the identification of differentiated cardiomyocytes [1]. Cells were incubated in a humidified chamber at 37°C and 5% CO₂. Cells were grown on 0.1% gelatin-coated dishes in media consisting of 78% DMEM/F-12 (Ham) (1:1), 10% Fetal Bovine Serum, 1% Pen/Strep, 1% MEM Non Essential Amino Acid and 1% L-glutamine. Cells for differentiation were trypsinized and re-suspended in density of 400,000 cells/ml in growth medium in presence of 1%DMSO. Cell suspension was transferred to bacteriological Petri dishes without gelatin for 4 days to facilitate Embryoid Bodies (EBs) formation. Media was replenished after 2 days. After 4 days in suspension, the EBs were transferred to tissue culture dishes with normal growth medium to allow cell attachment. Media was changed every two days and cells were carefully monitored throughout. Images of embryoid bodies and GFP positive cells were acquired by a Zeiss Axiovert 25 microscope connected to a Nikon Pix 5400 camera.

IGF-1Ea cloning and lentiviral production

Rat IGF-1Ea cDNA was directionally cloned into the Gateway entry vector backbone pENTR Directional TOPO Cloning Kit (Invitrogen # K2400-20), using a blunt-end PCR product. IGF-1Ea was further cloned into a destination lentiviral backbone, p*Lenti4/TO/V5-DEST*, ViraPower T-Rex Lentiviral Expression System (Invitrogen # K4965-00) by site-specific recombination of attL and attR recombinant sites present on the entry and destination vectors respectively. This lentiviral system ensures tetracycline regulation of the gene of interest. In this study, we used clones that express IGF-1Ea in a constitutive manner.

Viral production was performed as instructed by the manufacturer (Invitrogen, *ViraPower T-Rex Lentiviral Expression System User Manual*, 2004). In brief, 293 cells grown in adherent conditions at 90% confluency were transfected with IGF-1Ea lentiviral backbone by lipofectamine 2000 (Invitrogen). 9 μ g of packaging mix and 3 μ g of IGF-1Ea expression vector were used in Opti-MEM I Medium without serum. An optimized mixture of pLP1, pLP2, and pLP/VSVG plasmids formed the Packaging/Envelope Mix (Invitrogen #K4975-00). Virus containing supernatant was harvested 48 hours post-transfection and used for titering. Titering was performed with a mammalian HT1080 fibrocarcinoma cell line (ATCC # CCL-121). The cells were transduced with lentiviral supernatant in a 10-fold serial dilution (10^0 to 10^5). A plate was left untransduced as a mock control. Forty-eight hours post-transduction, cells were treated with 10 μ g/ml Zeocin. After 10-14 days of selection, the transduced cells were stained with 5ml/10cm-dish crystal violet for 10 minutes at room temperature and the titer of the virus was determined by counting Crystal violet-stained colonies as described in the instruction manual (Invitrogen, *ViraPower T-Rex Lentiviral Expression System User Manual*, 2004).

RNA isolation and quantitative Real time PCR (qRT-PCR)

Total RNA was isolated from frozen tissue and from cells in culture following the standard TRIzol protocol (Invitrogen). To ensure a pure preparation of RNA to be used for quantitative Real Time

PCR analyses (qRT-PCR), Trizol isolated RNAs were further purified using RNeasy mini-columns (Qiagen). Gene expression was analyzed using real-time PCR. cDNA was first synthesized using the Taqman reverse transcription kit supplied by Applied Biosystems (Applied Biosystems). 100 ng/ μ l RNA samples were prepared for cDNA production. 6 ng/ μ l of cDNA was used for real-time PCR analysis. The reagents for real-time PCR, the 20X 18S assay and the 20X off-the-shelf probes were purchased from Applied Biosystem. cDNA was used in duplicate for the analysis. Real-time PCR was performed in an ABI Prism 7700 Sequence Detection System (Applied Biosystem). PCR conditions are performed as recommended by the Manufacture (Applied Biosystem). All probes used in this study for qRT-PCR have been purchased from Applied Biosystem. Data were collected and analyzed using Sequence Detection Software (Applied Biosystem, SDS). Data from SDS were exported into Excel and analyzed using the comparative Ct method [2].

Immunofluorescence

Immunofluorescence was performed on differentiated cells to analyse alpha actinin expression. DMSO-differentiated cells were incubated with 4% PFA for 10 minutes at room temperature. Following fixation, they were washed with 1x PBS and permeabilize with 0.1% Triton X-100. Cells were blocked with 3% BSA (bovine serum albumin) for an hour at room temperature. Alpha actinin monoclonal antibody (Sigma) was added at 1:200 dilution for 2 hours at room temperature. Cells were treated with Cy3-conjugated secondary antibody (Jackson laboratoty) at 1:400 and incubated for an hour at room temperature. Nuclei were stained with 1:1000 Hoechst. Mounting was performed with Mountant, PermaFlour (Thermo Scientific) and analysed by confocal (Zeiss, LSM 510).

Hypoxia Induction and LDH Assay

Hypoxia was induced by exposing undifferentiated P19-GFP/IGF-1Ea and control P19-GFP cells to 3% oxygen in a hypoxic incubator (Sanyo #MCO-5M) for 24 hours. The activity of lactate

Dehydrogenase (LDH) released into the media was measured using CytoTox96 Non-Radioactive Cytotoxicity Assay as described by the Manufacturer (Promega). The percentage of LDH activity, as an indicator of cell death, was measured relative to control cells and to normoxia conditions. The analysis was performed in three independent experiments.

Western blot analysis

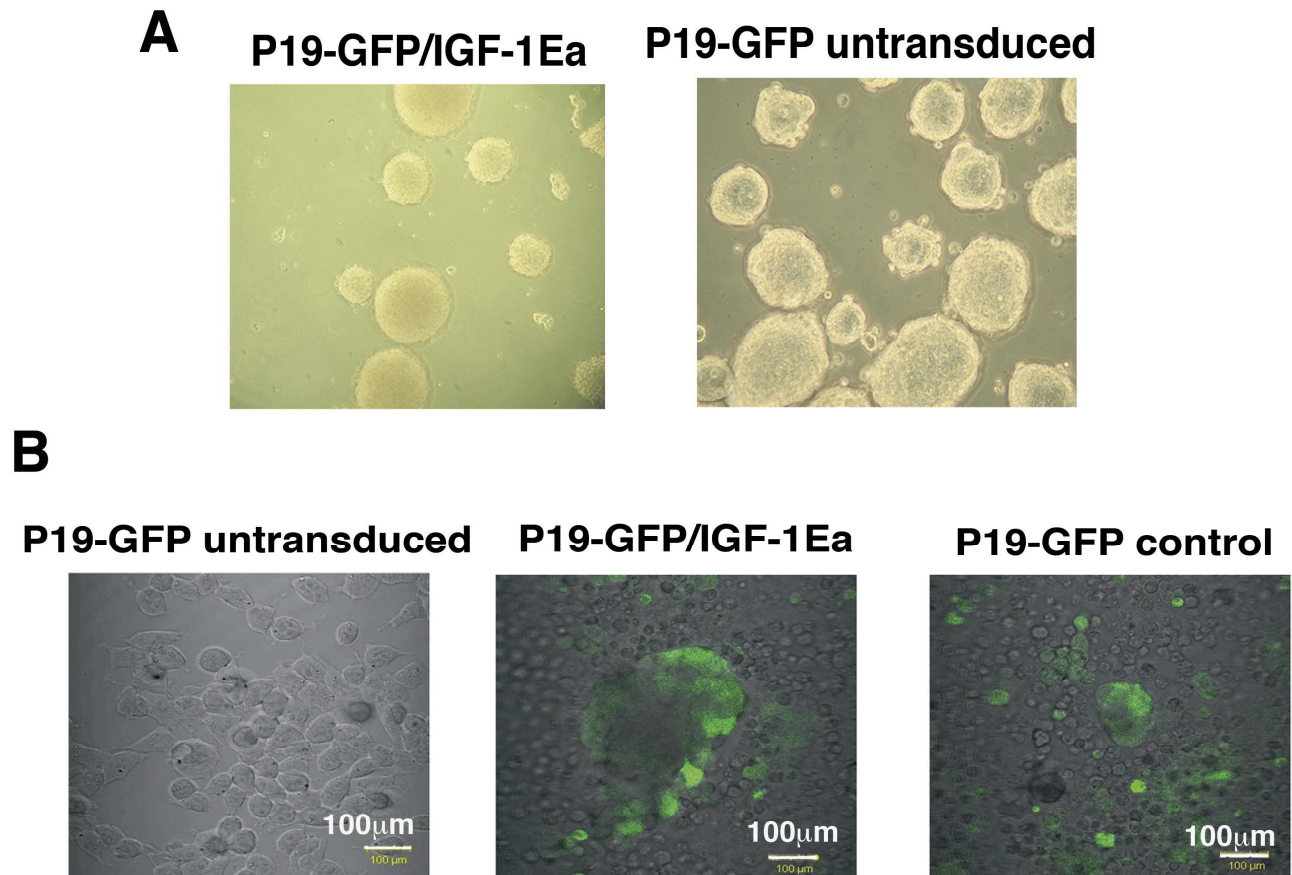
Cultured cells and tissue from infarcted and cell-transplanted hearts were lysed in lysis buffer (20mM Tris pH 7.5, 5mM MgCl₂, 150mM NaCl, 1% Triton, supplemented with 1mM protease and phosphatase inhibitors' cocktail (Roche)) and centrifuged at 13000 rpm for 20 min at 4°C. 50 µg of proteins were loaded onto SDS-PAGE gel and blotted on PVDF membrane. Polyclonal antibody recognizing phosphorylated AKT1 and PDK1, as well AKT1, PDK1 and the VEGF-A receptor KDR were purchased from Cell signalling and used at 1:500 dilution in 5% milk. Alpha-tubulin (Sigma) was used at 1:1000 dilution.

PI Staining for DNA Analysis using FACS

Control P19-GFP cells and P19-GFP/IGF-1Ea cells were harvested from adherent cultures and 10⁶ cells were resuspended in 1ml of the chilled 70% Ethanol solution and left at 4°C to fix overnight. After centrifugation, the cell pellet was gently vortexed in the remaining ethanol and added with 1 mL of PI staining solution (0.5ml 20X PI stock solution) at 50µg/ml final concentration. RNase A was added at a final concentration of 100U/ml to the PI solution. The samples were incubated at room temperature for at least 30 minutes, protected from light before FACS analysis. FACS analysis was performed for PI staining using PE-A channel, PI fluorescence at ≥ 600 nm wavelength.

Supplementary Figures

Supplementary Figure 1

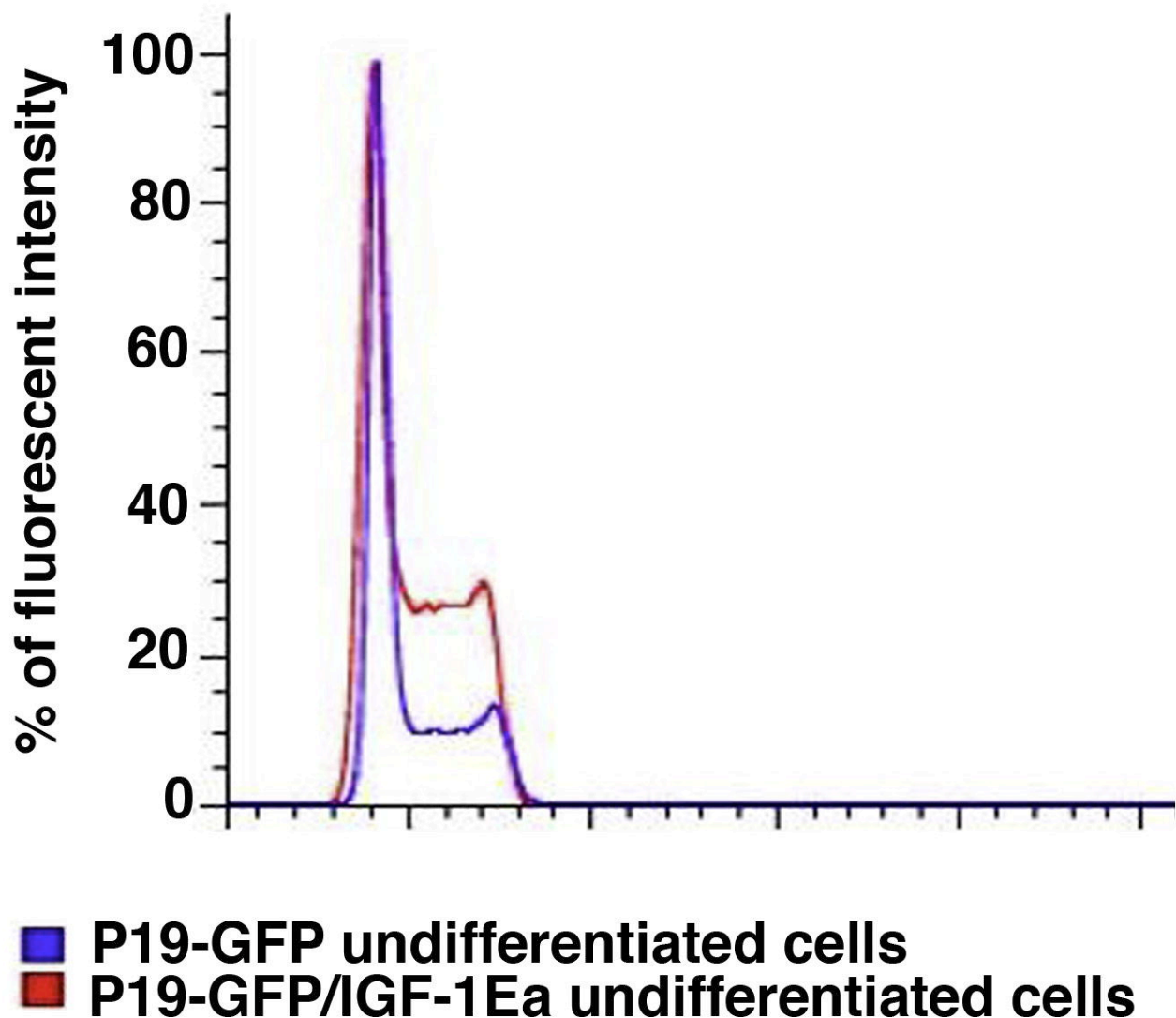


Supplementary Figure 1: Normal embryoid body formation and cardiomyocytes differentiation in IGF-1Ea overexpressing P19-GFP cells.

A) Embryoid bodies were formed as described in the Supplementary Materials and Methods. IGF-1Ea overexpression did not alter embryoid body formation in the selected Clone 6. B) IGF-1Ea transduced cells (P19-GFP/IGF-1Ea), control cells transduced with lentivirus not expressing IGF-1Ea (P19-GFP) and untransduced cells were cultured after embryoid bodies' formation on 0.1%

gelatin. Differentiated ventricular cardiomyocytes expressed green fluorescent protein. Images were acquired by a Zeiss Axiovert 25 microscope connected to a Nikon Pix 5400 camera.

Supplementary Figure 2

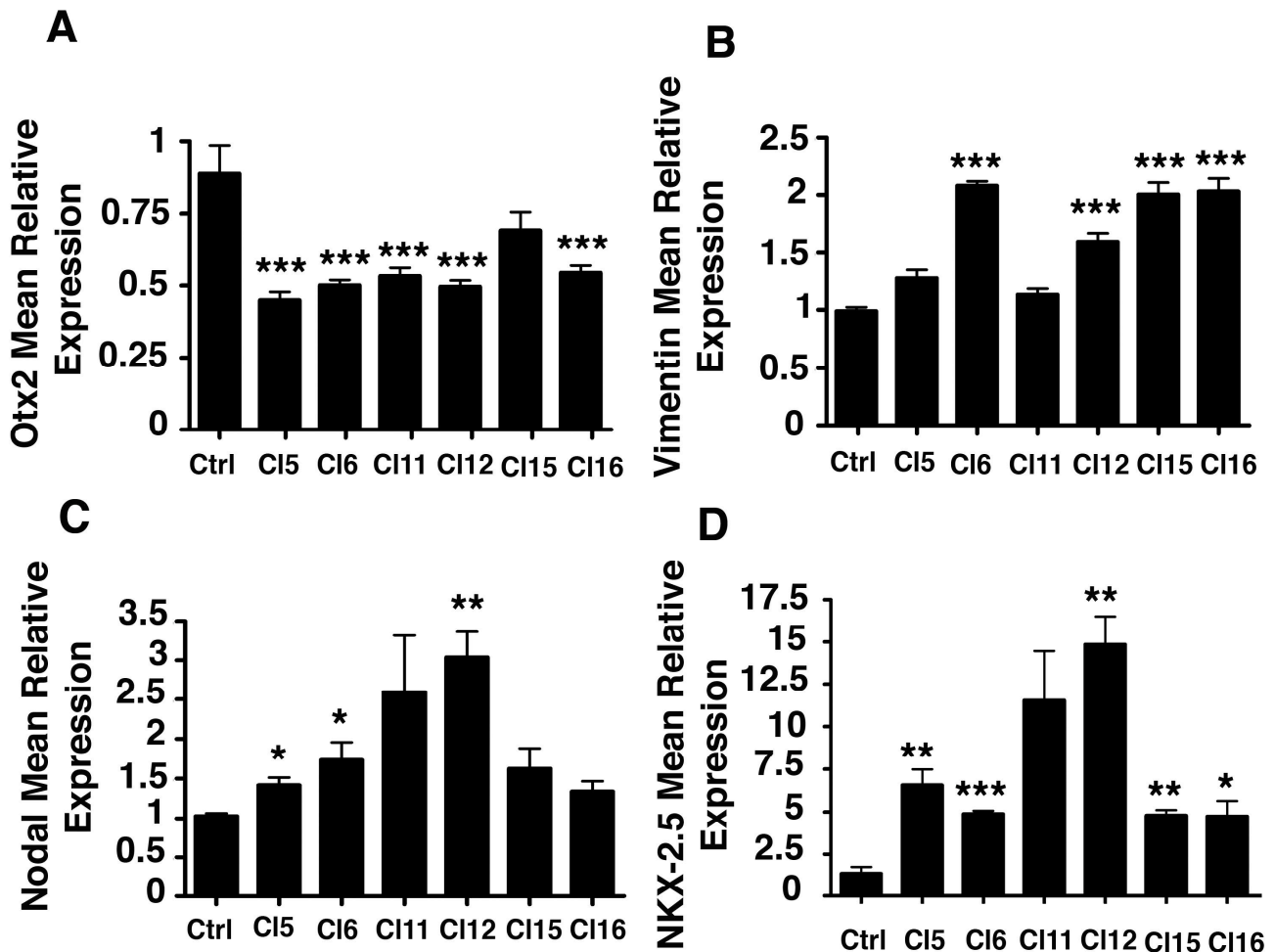


Supplementary Figure 2: Proliferation profile of overexpressing IGF-1Ea and control clone 5.

Cell cycle analysis of undifferentiated P19-GFP cells, transduced with empty (blue) or IGF-1Ea (red) viral vectors. Percentage of cells in S phase increased in IGF-1Ea expressing cells. Results

were obtained by PI staining as described in Materials and Methods, using Flow Jo analyses. X-axis represents percentage of cells and y-axis fluorescent intensity.

Supplementary Figure 3



Supplementary Figure 3: Lineage commitment in undifferentiated clones expressing IGF-1Ea.

Real time PCR was performed with applied Biosystem probes for the ectodermal marker Otx2 (A), the mesodermal markers Vimentin (B) and Nodal (C) and the cardiac marker NKX-2.5 (D). 18S rRNA was used as internal reference gene. Results are expressed as Mean \pm SEM. Asterisk (*) indicates significant values ($p < 0.05$); ** indicates $p < 0.01$, whereas *** indicates $p < 0.001$.

References

- [1] J.C. Moore, R. Spijker, A.C. Martens, T. de Boer, M.B. Rook, M.A. van der Heyden, L.G. Tertoolen, C.L. Mummery, A P19Cl6 GFP reporter line to quantify cardiomyocyte differentiation of stem cells, *Int J Dev Biol* 48 (2004) 47-55.
- [2] T.D. Schmittgen, K.J. Livak, Analyzing real-time PCR data by the comparative C(T) method, *Nat Protoc* 3 (2008) 1101-1108.