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ORIGINAL ARTICLE

Leukemia Inhibitory Factor Increases Survival of Pluripotent Stem Cell-Derived Cardiomyocytes

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Abstract Leukemia inhibitory factor (LIF) is a growth factor with pleiotropic biological functions. It has been reported that LIF acts at different stages during mesoderm development. Also, it has been shown that LIF has a cytoprotective effect on neonatal murine cardiomyocytes (CMs) in culture, but little is known about the role of LIF during human cardiogenesis. Thus, we analyzed the effects of LIF on human pluripotent stem cells (PSC) undergoing cardiac differentiation. We first showed that LIF is expressed in the human heart during early development. We found that the addition of LIF within a precise time window during the in vitro differentiation process significantly increased CMs viability. This finding was associated to a decrease in the expression of pro-apoptotic protein Bax, which coincides with a reduction of the apoptotic rate. Therefore, the addition of LIF may represent a promising strategy for increasing CMs survival derived from PSCs.

Keywords Pluripotent stem cells · Embryonic stem cells · Cardiac differentiation · Cardiomyocyte · Cardiac progenitor cell · LIF · Leukocyte inhibitory factor · Apoptosis

Introduction

The family of the interleukin-6 (IL-6) cytokines includes leukemia inhibitory factor (LIF), which has been shown to have relevant biological functions [1]. LIF exists in two isoforms: as a diffusible molecule with distant activities, or as an extracellular matrix-bound isoform with local functions [2]. LIF signals by binding to a heterodimeric membrane receptor complex formed by LIF-specific receptor (LIFR) and one signal transducer surface protein receptor, gp130, shared by a number of other cytokines of the IL-6 family, including Cardiotrophin-1 (CT-1) and IL-6 [3]. This receptor complex mediates LIF signaling via multiple pathways such as the JAK/STAT pathway, the MAPK pathway, and the PI3K/Akt pathway [4].

The heart is among the first organs to develop and function during embryonic development. Embryos with a homozygous null mutation for gp130 die *in uterus* between 12.5 days post-coitum and term [5]. Interestingly, the heart of gp130 knock out mice shows several defects, including a hypoplastic left ventricular myocardium without septal and trabecular abnormalities. The shape and structures of the heart are somehow conserved, although there is a lack of myocardial thickening due to a deficit of proliferating

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cardiomyocytes (CMs). Both gp130 ligands, LIF and CT-1, are expressed at high levels during the course of cardiogenesis in mice, and they promote the proliferation and survival of embryonic CMs. This implies that the LIF/LIFR/gp130 axis plays an essential role for the cardiac development, potentially by regulating early CMs survival and growth [6].

LIF has been involved at different stages during mesoderm development including the specification of the hematopoietic and endothelial cells and the development of the cardiovascular system. Notably, LIF appears to work in opposite ways during the specification of the mesoderm and mesoderm derivatives. Overexpression of LIF in mice identified it as an inhibitor of mesodermal differentiation during gastrulation [7]. In contrast, it has been shown the capability of LIF to have a hypertrophic and cytoprotective effect on neonatal murine CMs in culture [8, 9]. Moreover, the protective effects of LIF on CMs have been best studied in response to acute cardiac insults generated by ischemia/reperfusion (I/R). Signaling pathways that are activated by LIF are associated with short-term protection of adult CMs against acute I/R injury [10, 11]. Evidence suggests that these pathways are involved in the adaptive compensatory mechanism to maintain normal cardiac output in response to pressure overload. Then, LIF may have a significant role in preventing injury in the adult myocardium and facilitating repair of the injured heart in the setting of I/R.

Pluripotent stem cells (PSCs), which include human embryonic stem cells (hESCs) and induced pluripotent stem cells (iPSs) [5], can self-renew while maintaining their pluripotency to differentiate into all adult cell types, including CMs. PSCs rapidly has become a unique model to study human cardiogenesis allowing to closely recapitulate in vitro the developmental pattern of human cardiogenesis [12]. Moreover, PSCs are potentially a source of autologous CMs for cell-based cardiac treatments [13].

To this date, little is known about the presence and role of LIF during human cardiac development. Evidence suggests that LIF may have a beneficial role in cardiac differentiation from PSCs [14]. Therefore, our goal was to investigate the physiological relevance of LIF during human cardiac development using an in vitro model of cardiogenesis. In this study, we found that LIF is expressed in the human heart during early myocardium development and prevents cell death in hESC-derived CMs. We found that when LIF is added during the cardiac differentiation process, CMs viability is significantly increased and expression of the pro-apoptotic protein, Bax, is reduced concomitantly with apoptotic rates. Our results flag for a potential role of LIF in promoting anti-apoptotic signals during cardiac cells differentiation.

Methods

Cells and Cell Culture

The hESC line WA09 (H9) (46, XX karyotype) and H9-hTnTZ-PGZ-D2 (46, XX karyotype) [15] were purchased from WiCell Research Institute.. H9-hTnTZ-PGZ-D2 is a troponin-t reporter line expressing GFP and Zeocin-resistance genes under the promoter region of the troponin T gene. PSC were cultured as previously published [16–18]. Briefly, hESCs were maintained on inactivated mouse embryonic fibroblast feeder layers in DMEM/F12 medium supplemented with KSR 10%, 2 mM non-essential amino acids, 2 mM L-glutamine, 100 U/ml penicillin, 50 µg/ml streptomycin, 0.1 mM β-mercaptoethanol, and 4 ng/ml of bFGF. All reagents were purchased from Thermo Fischer Scientific (US). Inhibitors used for this paper were diluted in DMSO and stored at −20 °C. STAT3 inhibitor, STATTIC V (Millipore); PI3K inhibitors, wortmannin (Sigma, US) and LY294002 (Calbiochem, San Diego, CA, US), and the MEK inhibitor UO126 (Calbiochem, San Diego, CA, US). Blocking antibody anti-gp130 (5 µg/ml; R&D) was diluted in PBS and stored at −20 °C.

Immunohistochemistry

Human fetal samples were commercial obtained from Novogenix Laboratories (California, US). All samples were collected by the company following elective termination and informed consent. Developmental age was determined by ultrasound. All donated material was anonymous and carried no personal identifiers. Immunohistochemical analysis was done as previously described [19]. Primary antibodies used were sarcomeric myosin (MF-20, Developmental Studies Hybridoma Bank (DSHB), LIF, STAT3, gp130 (Santa Cruz). Primary isotype control antibodies were purchased from Santa Cruz. Detection following primary antibody incubation was done using chromogen-based HRP-conjugated secondary antibodies against rabbit or mouse IgG (Vector Laboratories, Burlingame, CA). Immunocomplexes were visualized with Peroxidase Substrate Kit DAB (Vector Laboratories). Nuclei were counterstained with hematoxylin.

Immunostaining and Fluorescent Microscopy

Embryonic bodies (EBs) were rinsed with PBS and fixed in 4% paraformaldehyde for 45 min. Cells were permeabilized with 0.1% Triton X-100 (Sigma) in PBSA (PBS with 0.1% BSA) with 10% normal goat serum for 30 min, washed and stained with the corresponding primary antibodies. Fluorescent secondary antibodies were used to locate the

antigen/primary antibody complexes. Cells were counterstained with DAPI and examined under a Nikon Eclipse TE2000-S inverted microscope equipped with a ×20 E-Plan objective. The following primary antibodies were used: anti-cTnT (1:100, Abcam ab8295), anti- Nkx 2.5 (1:100, Santa Cruz sc-14033).

Embryoid Bodies Differentiation

To induce differentiation, hESCs colonies were dispersed with 500 µl/well of Tryple Select (Invitrogen) for 5 min. Cells were passed through a 70 µm mesh (Corning) and centrifuged for 5 min at 1000 rpm. Cells were the resuspended in mTeSR (Stem Cell Technologies) supplemented with BMP4 (10 ng/ml) and Rho kinase inhibitor Y-27632 (10 µM). Cells were plated (25×10^4 cells/well) in 96-well v-shaped plates and centrifuged for 7 min at 1300 rpm to force aggregation. After 24 h, EBs were carefully removed and cultured in non-adherent 24-well plates until day 4 containing StemPro34 supplemented with BMP4 (10 ng/ml), bFGF (5 ng/ml), and Activin A (3 ng/ml). On day 4, EBs were plated onto 0.1% gelatin coated 24-well plates and cultured in StemPro34 supplemented with BMP4 (10 ng/ml), bFGF (5 ng/ml), Activin A (3 ng/ml), and IWR-1 (5 µM, Sigma) for 24 h. On day 5, medium was changed to StemPro34 supplemented with bFGF (10 ng/ml), VEGF (5 ng/ml), and IWR-1 (5 µM) and EBs were cultured until day 14. For antibiotic selection of H9-cTnT cells, Zeocin (100 µg/ml, Thermo-Fischer, US) was added at day 10 for further 72 h.

Cell Viability Assay

H9-cTnT EBs were plated in 24-well plates at day 4. On day 10, cells were treated with 100 µg/ml of Zeocin for 72 h. On day 14, 50 µg/well of activated 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5 [(phenylamino) carbonyl]-2-H-tetrazolium hydroxide (XTT) (Sigma) diluted in PBS containing 0.3 µg/well *N*-methyl dibenzopyrazine methyl sulfate (PMS) (Sigma, US) were added to a final volume of 250 µl and incubated for 2 h. Cellular metabolic activity was determined by measuring the absorbance of the samples with a spectrophotometer (Bio-Rad) at a wavelength of 450 nm and subtracting the background absorbance at 690 nm.

TUNEL Assay

An in situ cell death detection kit using TdT-mediated fluorescein-conjugated dUTP nick end labeling (TUNEL Kit, Roche) was used to detect apoptotic cells following manufacturer's instructions. EBs at day 14 were dissociated into a unicellular suspension using a trypsin-EDTA 0.25%/Collagenase type IV 1 mg/ml solution. After enzyme inactivation, cells were washed twice with PBS and fixed with 4% PFA, for 20 min on ice. Permeabilization was carried out O/N using ice-cold 70% ethanol. Cells were washed with PBS and resuspended in equilibration buffer supplemented with a nucleotide mix containing rTdT enzyme and incubated at 37 °C for 60 min protected from light. Incubation was inactivated by addition of EDTA 20 mM. Finally, cells were washed with a 0.1% Triton in PBS with 5 mg/ml BSA and the stained for sarcomeric myosin (MF-20, DSHB) following standard staining protocol for flow cytometry. Cardiac Fluorescein-labeled cells were analyzed by flow cytometry (FACSaria, BD, US) using BD FACSDiva software (BD Biosciences, US).

RNA Isolation and RT-qPCR

Total RNA was extracted as previously described [16]. For real-time PCR studies, cDNA samples were diluted fivefold and then PCR amplification and analysis were performed with StepOnePlus Real Time PCR System (Applied Biosystems). The SYBR GreenERTM qPCR SuperMix UDG (Thermo Fisher Scientific) was used for all reactions (For primers sequences see S1 Table).

Assessment of DNA Fragmentation

Apoptosis was quantified with Cell Death Detection ELISAPlus kit (Roche). Briefly, on day 4, equal number of EBs per treatment were plated on 24-well plates in 500 µl of culture medium. The mono and oligonucleosomes in the supernatants were determined at day 14 using an anti-histone-biotin antibody. Absorbance of the resulting color, was measured at 405 nm wavelength using a Benchmark microtiter plate reader (Bio-Rad Hercules). Results were expressed as DNA oligomer percentage, calculated from the ratio of absorbance of treated samples to that of the untreated ones.

Flow Cytometry

Single-cell suspensions were obtained by cell incubation with Tryple Select (Thermo Fisher Scientific). For detection of cardiac proteins, cells (1×10^6) were fixed with 300 µl of PFA 4%, washed with PBS and permeabilized using PermBuffer III (BD Biosciences). Staining was carried out using anti-sarcomeric myosin (1/400; AB-2147781, DSHB) or anti-cTnT (1/400; ab8295, Abcam) in PBS containing 0.5% BSA and 2 mM EDTA for 10 minutes at 4 °C and washed with 2 ml of PBS. Normal mouse IgG was used as isotype control. anti-mouse IgG conjugated with Alexa Fluor 488 (Thermo Fisher Scientific) was used as

secondary antibody. Finally, cells were centrifuged, resuspended in 0.5 ml PBS and analyzed by flow cytometry. Data were acquired on a FACSAria II flow cytometer and analyzed using BD FACSDiva software (BD Biosciences). Analysis of cardiac cells after Zeocin treatment of H9-cTnT EBs was carried out by digesting EBs with Tryple enzyme into a single cell suspension, and analyzed by flow cytometry to detect GFP expression of cardiac cells.

Western Blot

Protein sample preparation and Western blot assay were carried out as previously described [18]. Immunocomplexes were visualized using the Odyssey Infrared Imaging System (LI-COR). Membranes were scanned for protein bands detection using the 680- and 780-nm channels at a scanning intensity of 5. The following primary antibodies were used: anti-Bcl-XL/S (sc-634), anti-Bax (sc-493), anti-Actin (sc-1616), anti-AKT (sc- 1618) (all from Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-LIF (cat.ABD51; Millipore, Billerica, MA,USA), anti-STAT3 (cat. 9139), anti-p-AKT(S473) (cat. 9271S), anti-ERK (cat. 9102), anti-p-ERK (Thr202/Tyr204) (cat.9101) (all from Cell Signaling Technology, Beverly, MA, USA), and anti-p-STAT3(Y705) ([EP2147Y] ab76315) (Abcam Inc., Cambridge, MA, USA). Antigen/primary antibody complexes were detected with near infrared-fluorescence-labeled, IR-Dye 800CW or IR-Dye 680RD, secondary antibodies (LI-COR Biosciences).

BrdU Incorporation Assay

An APC BrdU flow Kit (BD Pharmigen) was used to analyze cell proliferation. At day 14, EBs received a 10 µM pulse of BrdU for 1 h. After incubation, EBs were enzymatically dissociated to obtain a single cell suspension, washed and resuspended in 100 µl of BD Cytofix/Cytoperm (BD Pharmigen). After 20 min of incubation on ice, cells were washed by centrifugation in BD Perm/Wash Buffer (BD Pharmigen, US). The cells were then resuspended in BD Cytoperm Permeabilization buffer for 10 min, washed and re-fixed. Cells were treated with DNAase (300 µg/ml) for 1 h at 37 °C. Finally, cells were washed and incubated with the specific primary antibodies against BrdU (APC-anti BrdU) and α-MHC (AB-2147781, DSHB). After incubation, primary antibodies were washed and a secondary antibody conjugated with FITC against mouse IgG was used to detect the anti-α-MHC primary antibody. Cells were analyzed by flow cytometry using a FACS Aria II (Becton Dickinson) and results were analyzed using BD FACSDiva software.

LIF EASIA Analysis

LIF concentrations were determined using the Biosource LIF/HILDA enzyme amplified sensitivity immunoassay (EASIA, Thermo fisher scientific KAC1351). Non-contractile and contractile areas were manually separated and seeded in 24-well plates. Then, plates were incubated for 4 days at 37 °C in a humidified 5% CO_2 incubator. After incubation, cell culture supernatants were collected, centrifuged, and stored at −20 °C until analysis. The amount of substrate turnover was determined by measuring light absorbance. LIF concentrations are expressed as the mass of cytokine per ml of culture supernatant per 100 µg of total protein.

Statistical Analyses

All results are expressed as mean ± SEM. For XTT, qPCR, flow cytometry, and oligomers detection experiments; one-way ANOVAs followed by Dunnet's multiple comparisons tests were used to detect significant differences among treatments ($p < 0.05$). Student's paired t test was used to determine significant differences between means for western blot, cell viability, BrdU incorporation assay ($p < 0.05$). All statistical analysis was performed using GraphPad Prism 6 software.

Results

LIF Is Expressed During Human Cardiac Development

To address whether LIF is present and to what extent during human cardiomyogenesis, we performed immunohistochemistry assays on paraffin embedded sections of human embryonic hearts at different developmental stages (Fig. 1). At week 8, we observed that LIF is expressed in both atrial (Fig. 1a) and ventricular (Fig. 1b) myocardium while no detectable expression is observed in endothelial cells or in the epicardium. At weeks 11 and 15, LIF is present in the ventricular and atrial myocardium but is absent in endocardial and epicardial cells (Fig. 1d, e). Surprisingly at week 17, LIF expression is restricted to the atrial myocardium (ventricular area showed none or little immunoreactivity; Fig. 1g, h). We note that gp130 (at week 17 of development; Fig. 1i) is widely expressed in the heart, both in the myocardium and in the endothelial cells associated with blood vessels, coincident with expression of STAT3 (Fig. 1j). Endothelial cells present in blood vessels show the strongest expression of STAT3. Alpha-myosin heavy chain (α-MHC) staining was used as lineage marker (Fig. 1c, f). Our results show a widespread expression in the developing

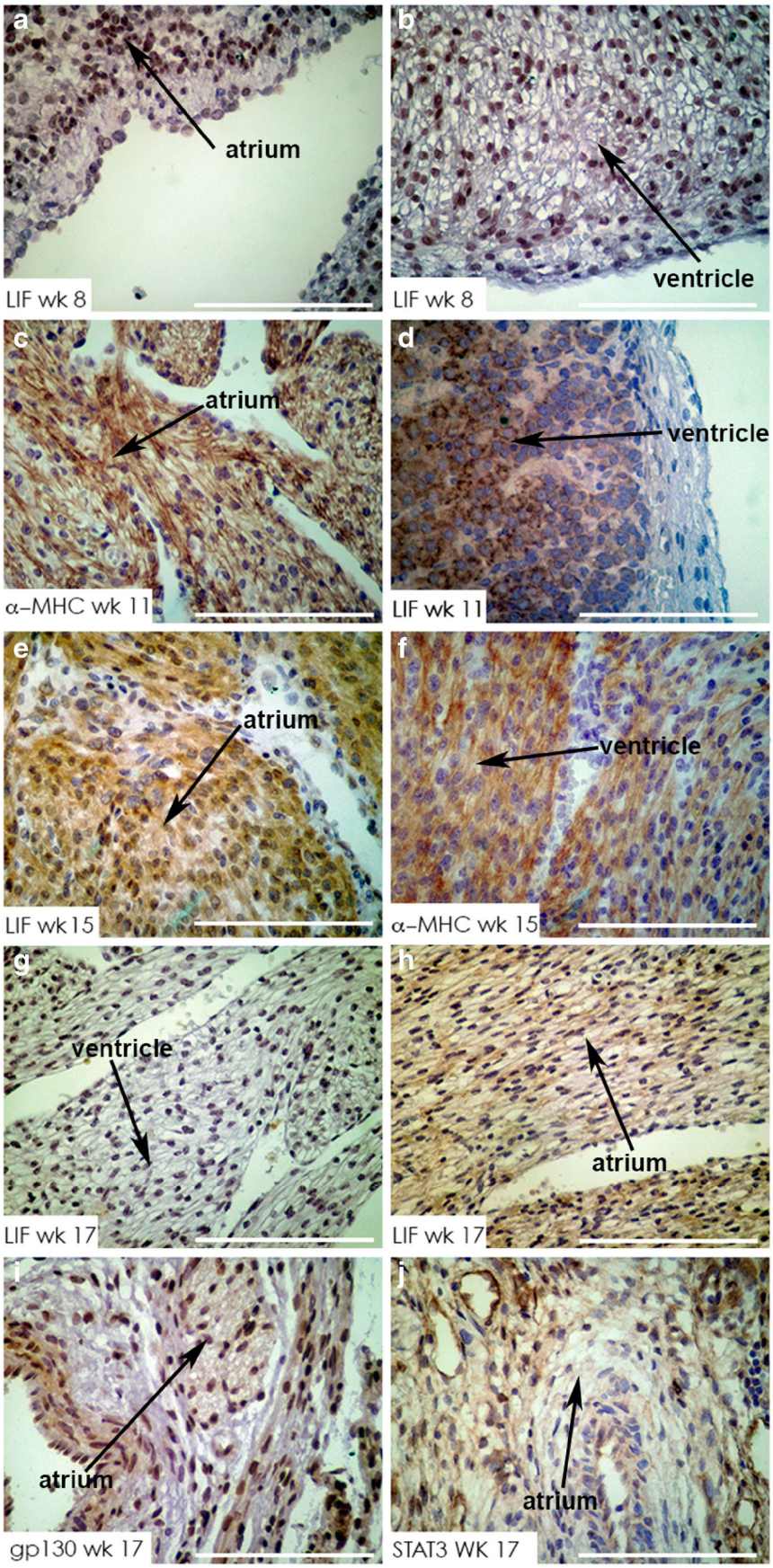

Fig. 1 LIF is present in the developing heart. Immunohistochemical staining of sections made from developing human hearts stained positive for LIF. The cytokine is widely present in the atrial and ventricular myocardium (**a**, **b**, **d**, **e**), but absent in endocardial and epicardial cells in week 8 through 15. α-MHC was used as lineage marker (**c**–**f**). LIF expression is restricted to the atrial myocardium at week 17 (**g**–**h**). Both gp130 and STAT3 show a wide expression in developing human hearts (**i**, **j**). Scale bar = 100 µm, photographed at ×400

heart and therefore suggest a role for LIF and its receptor in cardiac development.

In Vitro Cardiac Differentiation of hESCs

hESCs represent a unique *in vitro* model to partly reproduce human cardiogenesis. Therefore, we modified a protocol to obtain cardiac cells from hESCs and study the role of LIF during cardiac differentiation. The differentiation protocol used in the present study is a combination of two previously reported protocols [20, 21]. Mesodermal commitment was induced in EBs by the addition of Activin A (3 ng/ml), basic fibroblast growth factor (bFGF; 5 ng/ml), and bone morphogenetic protein 4 (BMP4; 10 ng/ml). These three factors are key developmental signaling molecules that play a crucial role during early cell fate decisions and, in a concerted manner, drive the cells toward the mesoendoderm lineage [22]. Mesoderm induction became evident between days 1 and 4. To induce cardiac mesoderm, we treated EBs with IWR-1 (5 μM), a potent inhibitor of the Wnt/β-catenin pathway, the same day in which EBs were attached to gelatin-coated wells (day 4). Inhibition of the canonical Wnt pathway has been earlier described to promote cardiac specification of the mesoderm progenitor cell population [20, 23]. From day 5 and onward, EBs culture media was supplemented with bFGF (10 ng/ml), VEGF (5 ng/ml), and IWR-1 (5 μM). Spontaneously beating areas within EBs appeared approximately from day 8 onwards (Fig. 2a).

To evaluate cardiac differentiation in our model, reverse transcription qPCR analyses were performed. We determined the expression level of characteristic genes from PSCs, the three germ layers (ectoderm, endoderm, and mesoderm) and from cardiac cells. As shown in Fig. 2b, the levels of Oct-4 and Nanog markedly decreased as differentiation proceeded. Expression of early mesoderm markers, such as T/Brachyury and Mesp-1, peaked on day 4 and decreased thereafter, consistent with mesoderm and early cardiac induction. Expression of Islet-1 mRNA was detectable from day 4 onwards and continued to increase until day 14. Cardiac markers transcripts cardiac troponin T (cTnT), α-MHC and Nkx-2.5 showed maximum expression levels on day 14, consistent with the presence of cardiac cells. We also evaluated expression of specific ectoderm and endoderm genes in order to assess the specificity of our differentiation protocol. Pax6 and Nestin, both early neuronal precursor markers, did not show significant changes in their expression levels during the differentiation process, which correlates with the absence of neural structures. In contrast, induction of endoderm markers Sox17 and AFP was observed. This could be due to the induction of mesoendodermal cells and the formation of an outer layer of primitive endoderm cells in the EB structure [24, 25].

Next, we quantified the number of cells expressing cardiac proteins by flow cytometry. As shown in Fig. 2c, nearly 20% of the cells showed expression of cTNT and α-MHC. Expression of cardiac proteins was also evaluated by immunofluorescence microscopy (Fig. 2d). Beating EBs resulted immunoreactive to Nkx2.5 and cTnT. Prominently, their expression was restricted to the contractile areas. Expression of cTnT and α-MHC was also evaluated by immunohistochemistry assays on paraffin embedded sections of beating EBs at day 14 of cardiac differentiation (supplemental Fig. 1a, b, f).

LIF Expression During Cardiac Differentiation of hESCs

It has been previously reported that gp130 and LIF receptors are expressed in adult and neonatal murine CMs. Hence, we evaluated the expression of LIF and its receptors during in vitro cardiac differentiation of hESCs. Although the cardiac differentiation protocol used in the present work was able to increase the yield of cardiac cells, it did not generate a homogeneous cardiac cell population. Therefore, we evaluated the expression of LIF, gp130, and LIFR transcripts in both cardiac and non-cardiac cell population. To do so, contractile and non-contractile areas were mechanically separated at day 14 of differentiation. RT-qPCR analyses revealed that LIF, LIFR, and gp130 mRNA levels are higher in non-contractile areas than in contractile ones (Fig. 3a). Accordingly, Western blot analysis revealed that LIF levels were higher in non-contractile areas than in contractile counterparts (Fig. 3c). Expression of LIF was also detected by immunohistochemistry assays on paraffin embedded sections of beating EBs at day 14 of cardiac differentiation (EBs were also inmunoreactive for gp130 and STAT3, supplemental Fig. 1c–e). To assess whether LIF was secreted into the culture media, we performed a solid-phase enzyme-amplified sensitivity immunoassay (EASIA) to quantify the amount of this cytokine in contractile and non-contractile cell culture supernatants. After 4 days in culture, we observed a threefold higher concentration of LIF in non-contractile cell culture supernatants than in contractile cells ones (Fig. 3b). In summary, these findings support the presence of an active form of LIF being synthesized and secreted during differentiation of PSCs into cardiac cells.

Effect of LIF During in Vitro Cardiac Differentiation of hESCs

We analyzed the effect of adding two different concentrations of LIF (10 or 100 ng/ml) at different time points during the cardiac differentiation protocol. LIF was added on day 4 (once mesoderm commitment has been established), day 8 (consistent with the appearance of the first beating areas), or

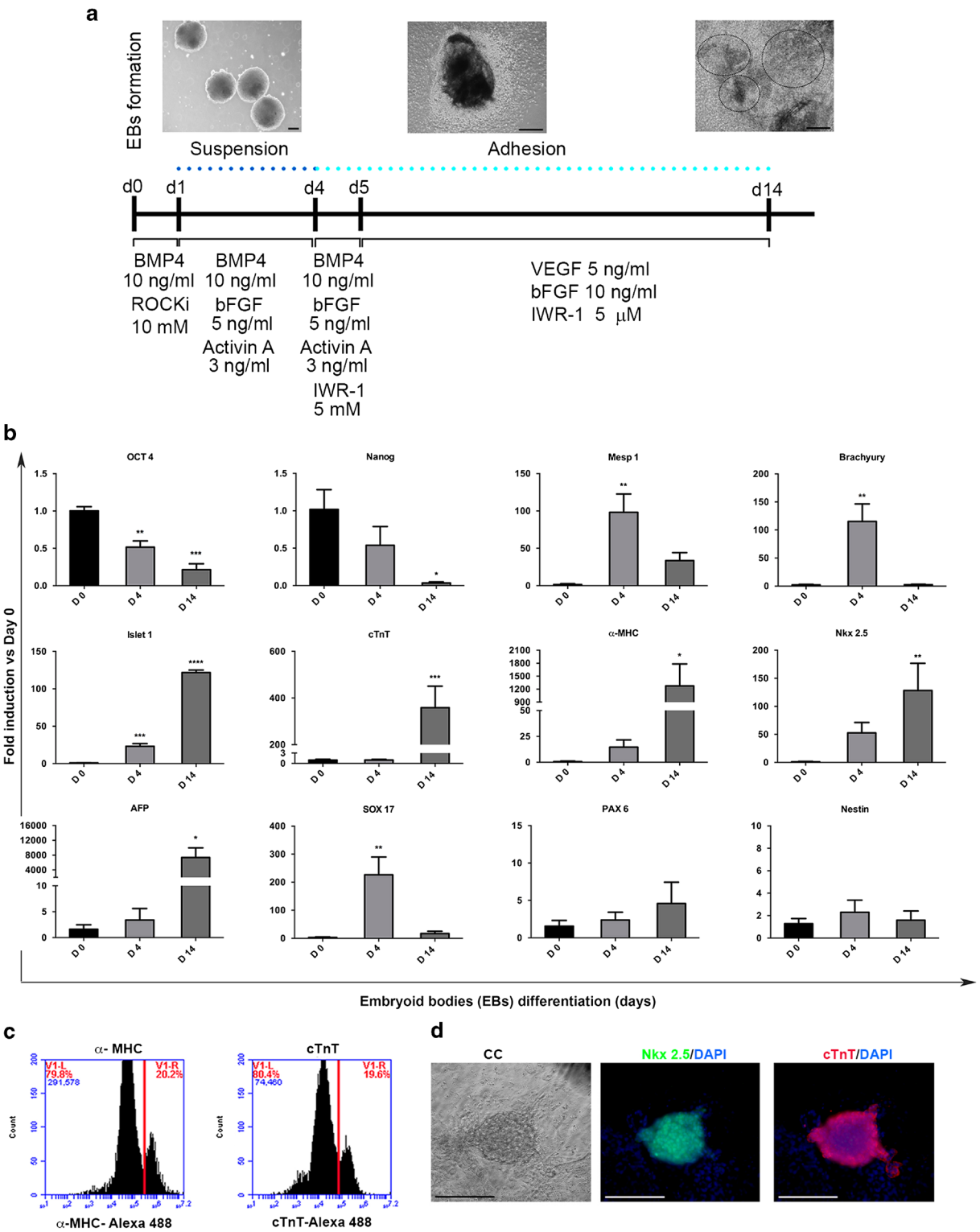

Fig. 2 In vitro cardiac differentiation. **a** Schematic representation of the in vitro differentiation protocol using morphogens that promote cardiac differentiation. **b** mRNA levels of stemness (Oct-4 and Nanog), mesoderm (T/Brachyury, Mesp-1, and Islet-1), cardiac cells (cTnT, α-MHC, and Nkx2.5), endoderm (α-fetoprotein, Sox-17), and ectoderm (pax-6, nestin) markers were analyzed by quantitative RT-PCR at days 0, 4, and 14 of the differentiation protocol. Rpl7 expression was used as normalizer. Graphs show mRNA fold induction relative to day 0. The mean ± SEM from three independent experiments are shown ($*p < 0.05$). **c** Efficiency of the cardiac differentiation protocol evaluated by flow cytometry. Cells were labeled using anti-cTnT or anti-α-MHC and detected using AlexaFluor-488 secondary antibodies. **d** Immunostaining of contractile EBs showing expression of cardiac transcription factor Nkx2.5 and structural protein cTnT. Nuclei were counterstained with DAPI (blue). Scale bar 100 μm

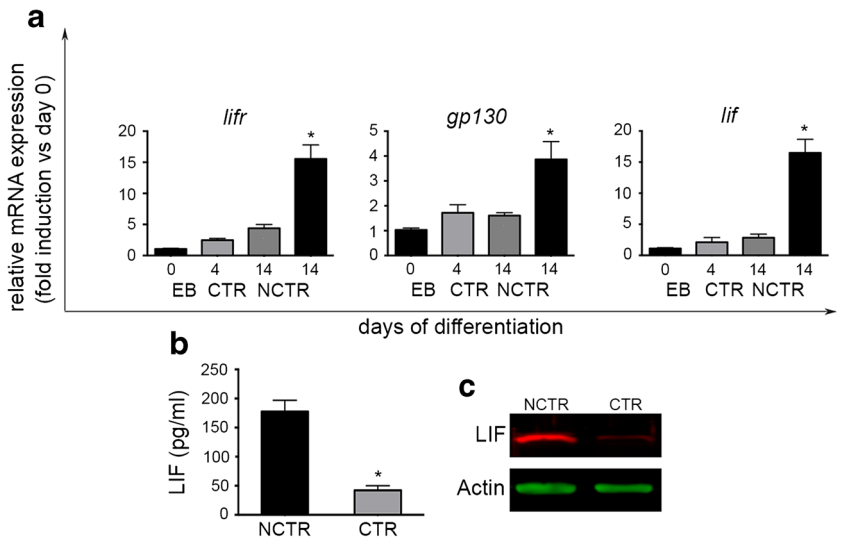

Fig. 3 LIF expression profile during cardiac differentiation of hESCs. **a** Quantitative RT-PCR analyses of LIFR, gp130, and LIF during cardiac differentiation. At day 14, contractile (CTR) and non-contractile (NCTR) areas were analyzed separately. Rpl7 expression was used as normalizer. Graph shows mRNA fold induction relative to day 0. The mean ± SEM. from three independent experiments are shown (*p<0.05). **b** LIF concentrations in NCTR and CTR cell culture supernatants quantified after 4 days by a LIF-EASIA kit (*$p < 0.05$). **c** A representative western blot image of NCTR and CTR samples showing LIF expression. Actin was used as loading control

on day 10 (when contractile areas were already established). On day 14, we found that there were no significant differences in the percentage of beating EBs generated under any of the experimental conditions (data not shown). Next, we analyzed the number of α-MHC expressing cells by flow cytometry. In all cases, we observed a general increase in the number of α-MHC+cells. Particularly, when LIF was added at day 8 at 100 ng/ml, we observed a significant increase (twofold) in the relative amount of α-MHC+ cells (Fig. 4a). Previous reports [7] showed that during gastrulation of murine embryos LIF expression is practically zero and that overexpression of LIF during this stage, inhibits in vivo mesoderm specification. In agreement with this result, adding LIF from day 0 of the cardiac differentiation protocol led to a decrease in the relative number of cardiac cells (ctrl: 0.99±0.21 vs LIF: 0.72±0.13).

The increase in the number of CMs when LIF is added at day 8 could be attributed either to differences in the proliferation rate or in the death rate of cardiac cells. We determined the proliferation rate of α-MHC+ cells by a bromodeoxyuridine (BrdU) incorporation assay. At day 14, cells were pulsed with BrdU (10 µM) and then analyzed by flow cytometry to quantify the percentage of BrdU+/α-MHC+ cells in both experimental conditions (absence or presence of LIF 100 ng/ml from day 8 onward). No significant differences were found between the S-phase (DNA synthesis) populations of control and treated cells (Fig. 4b). Therefore, we can conclude that LIF is not affecting the proliferation rate of CMs.

LIF Exerts an Anti-apoptotic Effect on Cardiac Cells

Throughout the cardiac differentiation of hESCs into CMs various cell types are generated, including mesoendodermal and mesenchymal cells. The coexistence of a non-homogeneous cell population emerging during cardiac differentiation prompted us to elucidate whether LIF is signaling directly on CMs or indirectly through the adjacent cells that were co-generated concomitantly with the CMs, which in turn may induce a protective effect on them. To this end, we used the stable cell line H9-hTnTZ-PGZ-D2 (H9-cTnT, WiCell, US). This cell line harbors the Zeocin resistance gene downstream the cTnT promoter and allows the generation of a population of cardiac cells to near homogeneity (supplemental Fig. 2).

We assessed the levels of cell death in H9-cTnT-derived CMs (Zeocin resistant) cultured in the presence or absence of LIF from day 8 onwards and quantified the percentage of surviving cells using a XTT/PMS vital dye assay. We found that LIF-treated cells showed approximately a 25% increase in viability (Fig. 4c). To further investigate the cause of this increase in cell viability, we measured the presence of oligonucleosomal DNA fragmentation, a typical hallmark of apoptosis. We quantified the abundance of DNA fragments with an enzyme-linked immunosorbent assay (ELISA). As shown in Fig. 4d, cytoplasmatic extracts from Zeocin-resistant EBs treated with LIF showed a reduction in the percentage of DNA oligomers (approximately 25%), suggesting that LIF is attenuating CMs death by apoptosis.

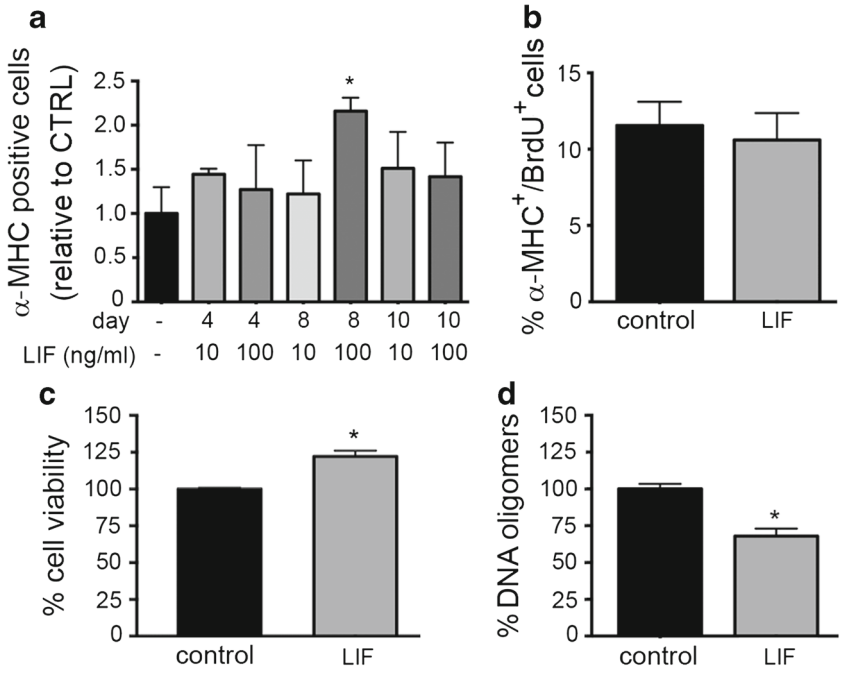

Fig. 4 Effect of LIF during in vitro cardiac differentiation of hESCs. **a** Quantitative data of α-MHC+ cells analyzed by FACS. Results are expressed as percentage of LIF-treated α-MHC+ cells with respect to untreated α-MHC+ cells (control), which was set to 1. Bars represent the mean ± S.E. of three different experiments ($p = 0.0062$ by ANOVA; $^*p < 0.05$ by Dunnet's test for subgroup analysis). **b** Quantification of α-MHC+/BrdU+ cells analyzed by FACS showing the proportion of LIF-treated (+LIF) or untreated (control) CMs residing in S phase. Data from triplicates is shown as mean ± SEM. **c** Cell viability was measured by the XTT/PMS vital dye assay at day 14. Results are presented as the percentage of the viability of untreated CMs; ($^*p < 0.05$). **d** CMs (day14) were collected, and DNA oligomer levels were measured by immunoassay. Results are presented as the percentage of DNA oligomers of untreated CMs. Each bar represents the mean ± SEM of three independent experiments performed in triplicates ($^*p < 0.05$).

Similarly, using a terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay, we quantified by flow cytometry that H9-derived CMs exposed to LIF exhibit lower levels of DNA breaks (30%) than untreated counterparts (supplemental Fig. 3). These experiments support the hypothesis that LIF is protecting developing CMs by reducing apoptosis.

LIF Reduces the Levels of Pro-apoptotic Protein BAX

The Bcl-2 protein family regulates the mitochondrial apoptotic pathway. Bcl-2 itself is known to belong to a family of apoptosis regulatory genes, which may either be death antagonists (e.g., Bcl-2, Bcl-xL, Bcl-w, Bfl-1, Mcl-1, and A1) or death agonists (e.g., Bax, Bak, Bcl-xS, Bad, Bid, Bik, and Hrk) [26]. The ratio of pro-apoptotic versus anti-apoptotic Bcl-2 family members is a critical determinant of apoptosis susceptibility. Thus, to determine if LIF induces changes in the ratio of pro- versus anti-apoptotic factors in our model, we assessed the levels of Bax (pro-apoptotic) and Bcl-xL (anti-apoptotic) mRNAs expressed by CMs exposed to LIF. Quantitative RT-PCR analyses revealed that in both experimental conditions CMs display similar expression levels of Bcl-xL. However, LIF-treated CMs exhibit significant lower levels of Bax mRNA than untreated counterparts (Fig. 5a). This expression pattern was further confirmed by Western Blot analysis (Fig. 5b).

Previously, it was reported that the molecular mechanisms downstream of gp130 activation, predominantly the JAK/STAT pathway, exert a cytoprotective effect on adult CMs [8, 27]. Additionally, cardiac-specific STAT3-deficient mice have a higher degree of apoptosis of CMs, resulting in an increased infarct size and consequently decreased cardiac function [26]. Therefore, to gain insights into the transduction pathways required for the observed protective effect exerted by LIF, we decided to evaluate the contribution of STAT3 activation on LIF. To do so, we utilized a new generation chemical inhibitor, STATTIC V, that selectively disrupts active STAT3 dimers [28]. We confirmed that STATTIC V pretreatment (20 µM during 60 min) prevents STAT3 activation by LIF (supplemental Fig. 4). The incubation with STATTIC V did not alter the percentage of cTnT-GFP+ cells generated at day 14 of cardiac differentiation (Fig. 5c). Importantly, when the STAT3 inhibitor was added to the LIF-containing culture media, the percentage of cTnT-GFP+ cells was significantly reduced to approximately the same as control treatment (Fig. 5c).

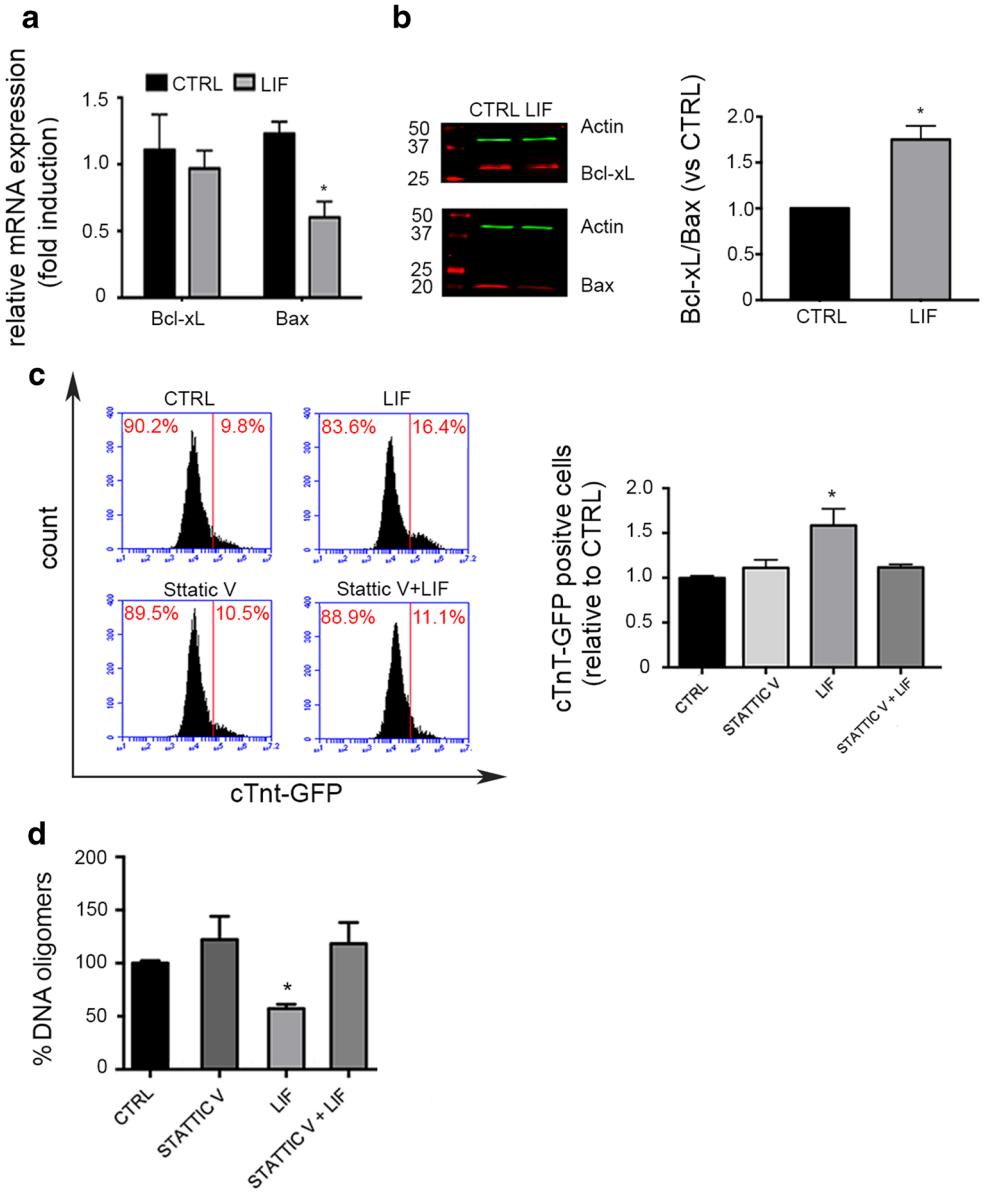

Fig. 5 LIF exerts an anti-apoptotic effect on cardiac cells. **a** mRNA levels of Bcl-xL and Bax were analyzed by quantitative RT-PCR at 14 of the differentiation protocol in CM exposed to LIF (+LIF) or untreated (control). Rpl7 expression was used as normalizer. Graphs show mRNA fold induction relative to day control. The mean ± S.E. from three independent experiments are shown (*$p < 0.05$). **b** A representative Western Blot image of +LIF and CTRL samples showing Bcl-xL and Bax expression. Actin was used as loading control. **c** (left) Representative histograms of flow cytometry analysis of H9-cTnT+ cells after cardiac differentiation protocol. Untreated (control, top left panel), LIF treated (top right panel), only treated with STATTIC V (bottom left panel) or with STATTIC V + LIF (bottom right panel). (right) cTnT-GFP positive cells quantification. The mean ± S.E. from three independent experiments are shown (*$p < 0.05$). **d** Untreated, LIF treated, only treated with STATTIC or with STATTIC+LIF cells were harvested, and cytoplasmatic DNA oligomers were quantified by immunoassay. Results are presented as DNA oligomers fold induction versus untreated control cells. Each bar represents the mean ± SE of three independent experiments performed in triplicate (*$p < 0.05$)

To further investigate whether the inhibition of LIFR/STAT3 axis would affect the anti-apoptotic action of LIF, we quantified the amounts of DNA oligomers present in CMs exposed to STATTIC V and LIF. We found that STAT3 inhibition impaired LIF protection (Fig. 5d). As a whole, these results indicate that LIF lowers the apoptotic rate of hESCs derived CMs through STAT3 trans-activation.

Discussion

Cytokines of the IL-6 family exhibit a wide range of biological functions on adult CMs. It is known that CMs can produce LIF, CT-1, and IL-6 in response to stress stimuli [29]. There is evidence of the beneficial role of LIF in preventing heart damage and facilitating myocardial repair in myocardial models of I/R via an autocrine/paracrine effect; this can mediate short- and long-term myocardial responses preventing CMs death by apoptosis and stimulating a hypertrophic grow response. Also, it has been previously reported that LIF is expressed in the murine embryonic heart at very high levels prior to the end of the waves of cardiomyocytes mitotic divisions [30]. However, it is not clear whether LIF (or other cytokine from IL-6 type family) has a relevant contribution in the differentiation, growth or survival of CMs during human embryonic development.

In the present work, we report that during the first months of gestation LIF exhibits a differential pattern of expression

throughout the developing human heart. Importantly, we observed that LIF is present in the myocardium in both atrial and ventricular regions at weeks 8, 11, and 15 of gestation (Fig. 1a, b, e, d). LIF was absent both in the embryonic epicardium and endocardium. While other studies demonstrated the presence of LIF in endothelial cells in different tissues [31, 32], we were not able to detect LIF in cardiac endothelial cells. Consequently, a localized and temporal expression of LIF in the developing heart suggests a specific role in this early embryonic stage and a specific role in the cardiac cell population. Taking this into consideration, we analyzed the effects of LIF in an in vitro model of cardiac differentiation from hESCs. Previously, Daheron et al. [33] have shown that despite activation of STAT3 by LIF signaling through LIFR and GP130, human LIF cannot sustain self-renewal capacity, and pluripotency in human cells. In agreement with this, we detected LIFR and gp130 transcripts in WA09 hESC line undergoing serum-induced (data not shown) or cardiac directed differentiation [34, 35]. However, it is interesting to note that LIFR and gp130 transcripts increased after the onset of differentiation, suggesting an active signaling through this pathway in this model.

We investigated the effects of LIF at different time points during the cardiac differentiation of hESCs. We found that the addition of recombinant LIF from day 8 onwards reduces apoptosis without affecting proliferation of hESCs undergoing cardiac differentiation. Interestingly, after 14 days of differentiation, we detected the presence of endogenous LIF in cells forming the contractile and non-contractile areas (45–175 pg/ml, respectively) that was being secreted into the culture media. The relatively large concentration of exogenous LIF (100 ng/ml) that was required in our experiments to achieve a protective effect on hESC-derived CMs may suggest that throughout the tested time points, LIFR is under-saturated by the endogenous LIF produced either by hESC- CM and/or co-generated adjacent cells. In fact, we found a higher expression of LIF on non-cardiac cells (Fig. 3). Hence, in this model of cardiac differentiation from pluripotent stem cells, it is possible that LIF signaling is provided by mesenchymal cells that co-differentiate during this process.

Frequently, the ability to reach an apoptotic threshold is regulated by the endogenous expression levels of critical pro- and anti-apoptotic factors. In this regard, we found that CMs exposed to LIF from day 8 onwards display similar levels of anti-apoptotic protein Bcl-xL and lower levels or pro-apoptotic factor Bax than untreated counterparts. The protective effect elicited by LIF was reverted by the specific STAT3 inhibitor STATTIC V treatment, suggesting that STAT3 activation modulates the apoptotic threshold of hESCs undergoing cardiac differentiation. Conversely, previous data describe that LIF upregulates bcl-x gene expression via STAT1-binding cis element in rat CMs [8]. As no abnormality was reported in the heart in either bcl-x-deficient mice [36] or STAT1- disrupted mice [37, 38], it is possible that this signaling pathway may be activated during stress but not during heart development.

Previous reports demonstrated that LIF confers cytoprotection to cardiac myocytes and that cardiac fibroblasts express LIF [39], together with our results on LIF expression and secretion from contractile versus not contractile areas, we can suggests that LIF expressed by not contractile cells in the heart during development can exert a paracrine effect on CMs preventing cell death during embryonic development, thus ensuring the correct formation of cardiac tissue [39–41]. Consistent with these results, Bader and his colleagues [30], suggested that LIF contributes, along with numerous factors, to the proper formation and maintenance of murine heart; and that the presence of LIF in the myocardium during development modulates cardiomyogenesis by controlling the maintenance of the differentiated state of the CMs.

Our findings highlight the importance of identifying molecules that are normally present during human cardiac development and how their posterior application during in vitro differentiation can help improve the yield of cardiac differentiation protocols. Preventing apoptosis in developing CMs warrants further research as a way to increase the final absolute number of CMs obtained from a given differentiation procedure. We therefore consider that adding LIF in cardiac differentiation protocols could be an effective way to increase CMs yield by diminishing the levels of programmed cell death.

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Compliance with Ethical Standards

Conflict of Interests The authors declare that they have no conflict of interest.

Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors.

References

1. Taupin, J.L., Pitard, V., Dechanet, J., Miossec, V., Gualde, N., & Moreau, J.F. (1998). Leukemia inhibitory factor: part of a large ingathering family. *International Reviews of Immunology*, *16*(3–4), 397–426.
2. Rathjen, P.D., Toth, S., Willis, A., Heath, J.K., & Smith, A.G. (1990). Differentiation inhibiting activity is produced in matrix-associated and diffusible forms that are generated by alternate promoter usage. *Cell*, *62*(6), 1105–1114.

3. Godard, A., Heymann, D., Raher, S., Anegon, I., Peyrat, M.A., Le Mauff, B., et al. (1992). High and low affinity receptors for human interleukin for DA cells/leukemia inhibitory factor on human cells. Molecular characterization and cellular distribution. *The Journal of Biological Chemistry*, *267*(5), 3214–3222.
4. Fischer, P., & Hilfiker-Kleiner, D. (2008). Role of gp130-mediated signalling pathways in the heart and its impact on potential therapeutic aspects. *British Journal of Pharmacology*, *153 Suppl 1*, S414–S427.
5. Yoshida, K., Taga, T., Saito, M., Suematsu, S., Kumanogoh, A., Tanaka, T., et al. (1996). Targeted disruption of gp130, a common signal transducer for the interleukin 6 family of cytokines, leads to myocardial and hematological disorders. *Proceedings of the National Academy of Sciences of the United States of America*, *93*(1), 407–411.
6. Sheng, Z., Pennica, D., Wood, W.I., & Chien, K.R. (1996). Cardiotrophin-1 displays early expression in the murine heart tube and promotes cardiac myocyte survival. *Development*, *122*(2), 419–428.
7. Conquet, F., Peyriéras, N., Tiret, L., & Brûlet, P. (1992). Inhibited gastrulation in mouse embryos overexpressing the leukemia inhibitory factor. *Proceedings of the National Academy of Sciences of the United States of America*, *89*(17), 8195–8199.
8. Fujio, Y., Kunisada, K., Hirota, H., Yamauchi-Takihara, K., & Kishimoto, T. (1997). Signals through gp130 upregulate bcl-x gene expression via STAT1-binding cis-element in cardiac myocytes. *The Journal of Clinical Investigation*, *99*(12), 2898–2905.
9. Kunisada, K., Tone, E., Fujio, Y., Matsui, H., Yamauchi-Takihara, K., & Kishimoto, T. (1998). Activation of gp130 transduces hypertrophic signals via STAT3 in cardiac myocytes. *Circulation*, *98*(4), 346–352.
10. Nelson, S.K., Wong, G.H., & McCord, J.M. (1995). Leukemia inhibitory factor and tumor necrosis factor induce manganese superoxide dismutase and protect rabbit hearts from reperfusion injury. *Journal of Molecular and Cellular Cardiology*, *27*(1), 223–229.
11. Yang, X., Cohen, M.V., & Downey, J.M. (2010). Mechanism of cardioprotection by early ischemic preconditioning. *Cardiovascular Drugs and Therapy*, *24*(3), 225–234.
12. Van Vliet, P., Wu, S.M., Zaffran, S., & Pucéat, M. (2012). Early cardiac development: a view from stem cells to embryos. *Cardiovascular Research*, *96*(3), 352–362.
13. Poon, E., Kong, C.W., & Li, R.A. (2011). Human pluripotent stem cell-based approaches for myocardial repair: from the electrophysiological perspective. *Molecular Pharmaceutics*, *8*(5), 1495–1504.
14. Zou, Y., Takano, H., Mizukami, M., Akazawa, H., Qin, Y., Toko, H., et al. (2003). Leukemia inhibitory factor enhances survival of cardiomyocytes and induces regeneration of myocardium after myocardial infarction. *Circulation*, *108*(6), 748–753.
15. Thomson, J.A., Itskovitz-Eldor, J., Shapiro, S.S., Waknitz, M.A., Swiergiel, J.J., Marshall, V.S., et al. (1998). Embryonic stem cell lines derived from human blastocysts. *Science*, *282*(5391), 1145–1147.
16. Romorini, L., Scassa, M.E., Videla Richardson, G., Blüguermann, C., Jaquenod de Giusti, C., Questa, M., et al. (2012). Activation of apoptotic signalling events in human embryonic stem cells upon Coxsackievirus B3 infection. *Apoptosis*, *17*(2), 132–142.
17. Scassa, M.E., Jaquenod de Giusti, C., Questa, M., Pretre, G., Richardson, G.A.V., Bluguermann, C., et al. (2011). Human embryonic stem cells and derived contractile embryoid bodies are susceptible to Coxsakievirus B infection and respond to interferon Ibeta treatment. *Stem Cell Research*, *6*(1), 13–22.
18. García, C.P., Videla Richardson, G.A., Romorini, L., Miriuka, S.G., Sevlever, G.E., & Scassa, M.E. (2014). Topoisomerase I inhibitor, camptothecin, induces apoptogenic signaling in human embryonic stem cells. *Stem Cell Research*, *12*(2), 400–414.
19. Wu, L., Bluguermann, C., Kyupelyan, L., Latour, B., Gonzalez, S., Shah, S., et al. (2013). Human developmental chondrogenesis as a basis for engineering chondrocytes from pluripotent stem cells. *Stem Cell Reports*, *1*(6), 575–589.
20. Willems, E., Spiering, S., Davidovics, H., Lanier, M., Xia, Z., Dawson, M., et al. (2011). Small-molecule inhibitors of the Wnt pathway potently promote cardiomyocytes from human embryonic stem cell-derived mesoderm. *Circulation Research*, *109*(4), 360–364.
21. Yang, L., Soonpaa, M.H., Adler, E.D., Roepke, T.K., Kattman, S.J., Kennedy, M., et al. (2008). Human cardiovascular progenitor cells develop from a KDR+ embryonic-stem-cell-derived population. *Nature*, *453*(7194), 524–528.
22. Vallier, L., Touboul, T., Chng, Z., Brimpari, M., Hannan, N., Millan, E., et al. (2009). Early cell fate decisions of human embryonic stem cells and mouse epiblast stem cells are controlled by the same signalling pathways. *PLoS One*, *4*(6), e6082.
23. Lanier, M., Schade, D., Willems, E., Tsuda, M., Spiering, S., Kalisiak, J., et al. (2012). Wnt inhibition correlates with human embryonic stem cell cardiomyogenesis: a structure-activity relationship study based on inhibitors for the Wnt response. *Journal of Medicinal Chemistry*, *55*(2), 697–708.
24. Bader, A., Gruss, A., Höllrigl, A., Al-Dubai, H., Capetanaki, Y., & Weitzer, G. (2001). Paracrine promotion of cardiomyogenesis in embryoid bodies by LIF modulated endoderm. *Differentiation*, *68*(1), 31–43.
25. Lough, J., & Sugi, Y. (2000). Endoderm and heart development. *Developmental Dynamics*, *217*(4), 327–342.
26. Wong, W.W.L., & Puthalakath, H. (2008). Bcl-2 family proteins: the sentinels of the mitochondrial apoptosis pathway. *IUBMB Life*, *60*(6), 390–397.
27. Hirota, H., Chen, J., Betz, U.A., Rajewsky, K., Gu, Y., Ross, J. Jr., et al. (1999). Loss of a gp130 cardiac muscle cell survival pathway is a critical event in the onset of heart failure during biomechanical stress. *Cell*, *97*(2), 189–198.
28. Schust, J., Sperl, B., Hollis, A., Mayer, T.U., & Berg, T. (2006). Stattic: a small-molecule inhibitor of STAT3 activation and dimerization. *Chemistry & Biology*, *13*(11), 1235–1242.
29. Passier, R., Denning, C., & Mummery, C. (2006). Cardiomyocytes from human embryonic stem cells. *Handbook of Experimental Pharmacology*, *174*, 101–122.
30. Bader, A., Al-Dubai, H., & Weitzer, G. (2000). Leukemia inhibitory factor modulates cardiogenesis in embryoid bodies in opposite fashions. *Circulation Research*, *86*(7), 787–794.
31. Grosset, C., Jazwiec, B., Taupin, J.L., Liu, H., Richard, S., Mahon, F.X., et al. (1995). In vitro biosynthesis of leukemia inhibitory factor/human interleukin for DA cells by human endothelial cells: differential regulation by interleukin-1 alpha and glucocorticoids. *Blood*, *86*(10), 3763–3770.
32. Liu, X., Tseng, S.C.G., Zhang, M.C., Chen, S.Y., Tighe, S., Lu, W.J., et al. (2015). LIF-JAK1-STAT3 Signaling delays contact inhibition of human corneal endothelial cells. *Cell Cycle*, *14*(8), 1197–1206.
33. Dahéron, L., Opitz, S.L., Zaehres, H., Lensch, M.W., Lensch, W.M., Andrews, P.W., et al. (2004). LIF/STAT3 Signaling fails to maintain self-renewal of human embryonic stem cells. *Stem Cells*, *22*(5), 770–778.
34. Aghajanova, L., Skottman, H., Strömberg, A. M., Inzunza, J., Lahesmaa, R., & Hovatta, O. (2006). Expression of leukemia inhibitory factor and its receptors is increased during differentiation of human embryonic stem cells. *Fertility and Sterility*, *86*(4 Suppl), 1193–209.

35. Skottman, H., Strömberg, A.M., Matilainen, E., Inzunza, J., Hovatta, O., & Lahesmaa, R. (2006). Unique gene expression signature by human embryonic stem cells cultured under serum-free conditions correlates with their enhanced and prolonged growth in an undifferentiated stage. *Stem Cells*, *24*(1), 151–167.
36. Motoyama, N., Wang, F., Roth, K.A., Sawa, H., Nakayama, K., Nakayama, K., et al. (1995). Massive cell death of immature hematopoietic cells and neurons in Bcl-x-deficient mice. *Science*, *267*(5203), 1506–1510.
37. Durbin, J.E., Hackenmiller, R., Simon, M.C., & Levy, D.E. (1996). Targeted disruption of the mouse Stat1 gene results in compromised innate immunity to viral disease. *Cell*, *84*(3), 443–450.
38. Meraz, M.A., White, J.M., Sheehan, K.C., Bach, E.A., Rodig, S.J., Dighe, A.S., et al. (1996). Targeted disruption of the Stat1 gene in mice reveals unexpected physiologic specificity in the JAK-STAT signaling pathway. *Cell*, *84*(3), 431–442.
39. Wang, F., Trial, J., Diwan, A., Gao, F., Birdsall, H., Entman, M., et al. (2002). Regulation of cardiac fibroblast cellular function by leukemia inhibitory factor. *Journal of Molecular and Cellular Cardiology*, *34*(10), 1309–1316.
40. Nakajima, S., Tanaka, T., Umesaki, N., & Ishiko, O. (2003). Leukemia inhibitory factor regulates cell survival of normal human endometrial stromal cells. *International Journal of Molecular Medicine*, *11*(3), 353–356.
41. Cullinan, E.B., Abbondanzo, S.J., Anderson, P.S., Pollard, J.W., Lessey, B.A., & Stewart, C.L. (1996). Leukemia inhibitory factor (LIF) and LIF receptor expression in human endometrium suggests a potential autocrine/paracrine function in regulating embryo implantation. *Proceedings of the National Academy of Sciences of the United States of America*, *93*(7), 3115–3120.