Fetal Reprogramming and Senescence in Hypoplastic Left Heart Syndrome and in Human Pluripotent Stem Cells during Cardiac Differentiation

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Hypoplastic left heart syndrome (HLHS) is a severe cardiac malformation characterized by left ventricle (LV) hypoplasia and abnormal LV perfusion and oxygenation. We studied hypoxia-associated injury in fetal HLHS and human pluripotent stem cells during cardiac differentiation to assess the effect of microenvironmental perturbations on fetal cardiac reprogramming. We studied LV myocardial samples from 32 HLHS and 17 structurally normal midgestation fetuses. Compared with controls, the LV in fetal HLHS samples had higher nuclear expression of hypoxia-inducible factor-1α but lower angiogenic growth factor expression, higher expression of oncogenes and transforming growth factor (TGF)-β1, more DNA damage and senescence with cell cycle arrest, fewer cardiac progenitors, myocytes and endothelial lineages, and increased myofibroblast population (P < 0.05 versus controls). Smooth muscle cells (SMCs) had less DNA damage compared with endothelial cells and myocytes. We recapitulated the fetal phenotype by subjecting human pluripotent stem cells to hypoxia during cardiac differentiation. DNA damage was prevented by treatment with a TGF-β1 inhibitor (P < 0.05 versus nonhypoxic cells). The hypoplastic LV in fetal HLHS samples demonstrates hypoxia-inducible factor-1α up-regulation, oncogene-associated cellular senescence, TGF-β1–associated fibrosis and impaired vasculogenesis. The phenotype is recapitulated by subjecting human pluripotent stem cells to hypoxia during cardiac differentiation and rescued by inhibition of TGF-β1. This finding suggests that hypoxia may reprogram the immature heart and affect differentiation and development. (Am J Pathol 2013, 183: 720—734; http://dx.doi.org/10.1016/j.ajpath.2013.05.022)

Physiologic adaptations that enable a fetus to tolerate a period of intrauterine deprivation may result in permanent reprogramming of the pathways of organ development, with pathologic consequences in later life.1 The nature of this permanent reprogramming is poorly understood, particularly the interaction between environmental factors and developmental pathways. Several cardiac malformations are characterized by poor growth of cardiac chambers and major blood vessels, which impairs organ perfusion and oxygenation. An example is hypoplastic left heart syndrome (HLHS) in which the left-sided structures are poorly developed likely due to an underlying genetic defect. As a result, better oxygenated blood from the inferior vena cava fails to shunt right to left at the foramen ovale to reach the left ventricle (LV) and the aorta. Instead, the aorta and coronaries receive more deoxygenated blood by retrograde flow from the descending aorta into the hypoplastic ascending aorta to supply the head vessels and the coronaries. This results not only in lower oxygen saturation but also in lower coronary blood flow, further reducing oxygen delivery to the myocardium.2 The fetal blood oxygen dissociation curve is steep, and a difference of even 2 to 3 supported by an Ontario Ministry of Economic Development and Innovation GL2 award and SickKids Labatt Family Heart Centre Innovation Funds.
mm Hg in PO₂ can produce a change in oxygen content of as much as 1 to 1.5 mL/dL. We hypothesized that the reduced blood flow through the LV and the oxygenation and perfusion abnormalities in the coronary circulation that are critical for vascular growth may reprogram growth signals and reversibly or irreversibly impair future ventricular growth potential.³

We analyzed growth and differentiation pathways in HLHS by studying human fetal hearts during the second trimester, which is the most critical period for cardiac growth. We also studied human pluripotent—embryonic—stem cells (hPSCs), during cardiac differentiation to define the mechanisms of hypoxia-induced injury in fetal stage cardiac cells. Because the process of cardiac differentiation of hPSCs mimics the process of cardiogenesis, hPSCs provide a potential in vitro cellular model to study the effect of prenatal factors on cardiac differentiation.⁴,⁵ Overall, we found significant DNA damage accumulation and premature senescence, transforming growth factor (TGF)-β1—associated fibrosis, and impaired angiogenesis in fetal HLHS and in vitro in hPSC-derived cardiac lineages subjected to prolonged hypoxia. TGF-β1 inhibition promoted genomic stability and prevented a senescent phenotype.

Materials and Methods

Fetal Hearts

LV and right ventricle (RV) paired myocardial samples were obtained from 32 second-trimester fetuses with HLHS (median gestational age, 23 weeks; range, 20—27 weeks): 18 with moderate HLHS (ie, mitral and aortic valve stenosis and moderate LV and aortic hypoplasia) and 18 with severe HLHS (ie, mitral and aortic valve atresia and severe LV and aortic hypoplasia). We also studied 17 gestational age—matched control fetuses with no heart disease (median gestational age, 22 weeks; range, 19—27 weeks) (P = 0.38 versus HLHS). Samples from the LV and RV free wall were acquired from the pathology department at the time of fetal autopsies performed as soon as possible after pregnancy termination or fetal demise. Samples were processed for studies as described below. Results were compared between normal and HLHS hearts and LV and RV paired samples. All investigations were conducted according to the Declaration of Helsinki principles. Studies were approved by the Hospital for Sick Children and the Mount Sinai Hospital institutional review boards, and written informed consent was obtained from study participants (pregnant mothers) before inclusion in the study.

Immunohistochemistry

Myocardial tissues were sectioned (5 μm), deparaffinized, rehydrated, and heated in a steamed water bath with an epitope retrieval solution (Dako, Glostrup, Denmark). Cultured cells were fixed with 4% paraformaldehyde. Tissue sections (and fixed cells) were incubated overnight at 4°C using the following primary antibodies: i) growth factors: hypoxia-inducible factor (HIF)-1α, vascular endothelial growth factor (VEGF) (Abcam, Cambridge, MA), and anti—thymosin β4 (TB4) (Santa Cruz Biotechnology Inc, Santa Cruz, CA); ii) DNA damage—related γH2AX (Berthyl, Montgomery, TX), anti-p53 binding protein 1 (53BP1) (Abcam), and anti-p53 (phospho S6) (Abcam); iii) cell cycle progression markers: anti—cyclin-dependent kinase (CDK4) (Santa Cruz Biotechnology) and anti—Ki-67 (Chemicon, Billerica, MA); iv) differentiated cardiac markers: antisarcomeric myosin, MF20 (MABTrapGII; Pharmacia, Uppsala, Sweden), α-smooth muscle actin (SMA) (Abcam), cardiac troponin T (cTnT) (Abcam), and CD31 (Dako); v) capillary density: anti—von Willebrand factor (vWF) (Abcam); and vi) fibrosis-related double staining for anti—α-SMA (Abcam) and fibroblast-specific protein (FSP1) (Abcam), TGFB1 (Abcam), E-cadherin and transcription factor 21 (TCF21) (LSBio, Seattle, WA). Sections were visualized with anti-mouse, anti-rabbit, or anti-goat IgG antibodies conjugated with tetramethylrhodamine isothiocyanate and fluorescein isothiocyanate (Sigma-Aldrich, St. Louis, MO). Slides were mounted with Vectashield mounting medium with DAPI (Vector, Burlingame, CA) and examined on a Zeiss LSM510 META confocal microscope or Nikon ECLIPSE E1000 microscope and analyzed with Volocity software, version 5.0 (PerkinElmer Inc, Waltham, MA), or Open Lab software version 3 (PerkinElmer Inc). Also, for immunoperoxidase staining, Envision Doublestain System (Dako) was used, and the reaction was visualized by the enzyme substrate diaminobenzidine. Permanent red chromogen visualized the second reaction. Sections were counterstained with Mayer’s hematoxylin (Dako) or nuclear fast red (TACS assay kits, Trevigen, Gaithersburg, MD) and examined using light microscopy (Leica, Wetzlar GmbH, Bannockburn, ON, Canada). Fibrosis-associated collagen blue expression was evaluated using Masson’s trichrome staining.

Myocyte number, capillary number, and cardiac progenitor lineages were counted using a GSA Image Analyzer (GSA Bansemer & Scheel GbR, Rostock, Germany) to identify marker-positive cells with application of common threshold value and inversion of digital images and expressed as a percentage of the total cells. A similar approach was used to quantify cells positive for DNA damage markers and cell cycle progression markers. Capillary density was expressed as a ratio of capillary to myocyte numbers per section. Ten sections (10 fields per section) at ×400 magnification in each staining were used to calculate the mean density per high-power field. Only fields in the cross section were selected for capillary density quantification. Capillary density was compared between fetuses ≤22 weeks of gestational age and those >22 weeks of gestational age. Double immunostaining of γH2AX with cTnT, CD31, and α-SMA was performed to identify which lineages had greater DNA damage. The number of SMA⁺/FSP1⁺ double-positive cells was also measured.
Quantification of signal intensity and density was performed on the Cellomics Arrayscan VTI-HCS Reader (Thermo Scientific, Ottawa, ON, Canada), which provides high-content cellular imaging, and analyzed by Targeted Activation BioApplication (Thermo Scientific). Comparisons were made between LV from control hearts and LVs from moderate and severe fetal HLHS samples and between LV and RV from severe fetal HLHS samples.

β-Galactosidase (β-gal) activity was detected using senescence-galactosidase staining kit (Cell Signaling, Danvers, MA). Frozen myocardial sections were embedded and cryosectioned (5 μm). Slides were incubated with β-gal staining solution for 24 hours at 37°C. Sections were stained with nuclear fast red (TACS) and captured under a Leica DM LB2 microscope for development of blue color, and signal intensity was analyzed with Open Lab software.

Apoptosis was detected with the CardioTACS In situ Apoptosis Detection Kit (R&D System, Minneapolis, MN). 

Telomere lengths were determined by a terminal restriction fragment (TRF) kit (Roche Diagnostics, Quebec City, QC, Canada). Briefly, 1 to 2 μg of extracted genomic DNA samples were digested with Rsal and HinfI at 37°C for 2 hours and run on 0.8% agarose gels at 12 mAmp for 12 hours. DIG molecular weight marker was loaded on both sides of the samples. After denaturation and neutralization of the gel, digested DNA samples were transferred to Hybond-N+ membrane (GE Healthcare Biosciences, Pittsburgh, PA) overnight with 10× standard saline citrate buffer. The blot was then hybridized to telomere-specific digoxigenin-labeled probe for 3 hours at 42°C and washed with washing buffer. Chemiluminescent detection of hybridized telomeres was performed, and the mean TRF length was calculated according to manufacturer protocol and formula and reported as telomere lengths.

Table 1  Primers Used for Real-Time PCR

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<th>Gene target</th>
<th>Gene sequences</th>
<th>Amplicon size (bp)</th>
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<td>Reverse: 5′-GAAGGAGGAGTAGGTG-3′</td>
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<td>VEGF</td>
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<td></td>
<td>Reverse: 5′-CATCCGCTATGGTG-3′</td>
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<td>Forward: 5′-CAGGCCAGACCTCAGCTGTA-3′</td>
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<td></td>
<td>Reverse: 5′-GGTCTTCCCTCTGACATGT-3′</td>
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<tr>
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Real-Time PCR for Myocardial mRNA Expression

Total RNA was isolated using the RNeasy Fibrous Tissue Midi Kit (Qiagen, Valencia, CA) from frozen myocardial samples. Quality and integrity of total RNA were assessed on 1% formaldehyde-agarose gels. The target genes, primer sequences, and PCR amplicon size are outlined in Table 1. Relative mRNA expression of the following genes were measured using real-time PCR: growth factors and related genes: HIF1A, VEGF, TB4, and TGFB1; and tumor suppressors or oncogenes: p53, p16, and p18. GAPDH was used as the housekeeping gene to correct for differences in transcriptional regulation across samples. Brilliant II SYBR Green Q-PCR Master Mix (Stratagene, Cedar Creek, TX) was used for transcription and amplification using the ABI PRISM 7000 Thermocycler (AB Applied Biosystems, Valencia, CA). Real-time quantitation was performed using the iCycler iQ system (BioRad, Hercules, CA).

hPSCs

hPSC studies were approved by the Stem Cell Oversight Committee of Canada. HES2 cell lines from the National Stem Cell Bank (WiCells, Madison, WI) were differentiated into cardiac lineages using a published cardiac differentiation protocol (courtesy of Gordon Keller, McEwen Centre for Regenerative Medicine, Toronto, ON, Canada). Cells at day 17 of cardiac differentiation stage were passaged from suspension culture of embryoid bodies (EBs) to monolayer culture or studied as EBs. Cells were cultured in StemPro medium (Invitrogen, Carlsbad, CA) supplemented with 5 ng/mL of human bFGF and with 10 ng/mL of human VEGF during differentiation and without VEGF during hypoxia exposure. All factors were purchased from R&D Systems. Cultures were maintained in a 5% CO2/air environment. EBs or monolayer cultured cells that reached 50% to 60% confluence were exposed to hypoxia (1% O2, 5% CO2, and 75% N2) in a hypoxia chamber (Billups-Rothenberg Inc, Del Mar, CA) without supplementation of VEGF and FGF. StemPro media for 72 hours. Cells were studied using immunostaining, real-time PCR, electron microscopy, and flow cytometry, and results were compared between normoxic and control cells (5% CO2/air environment), cells subjected to 1% O2 for 72 hours, and cells treated with 10 μmol/L of SB431542, a TGF-β1 inhibitor (Tocris, Monrovia, CA).
during hypoxia exposure. For assessment, cells were fixed to measure immunofluorescence staining for markers (described earlier) and population doubling time. Real-time PCR for custom array was performed as described earlier. Briefly, total RNA was isolated using the RNeasy Fibrous Tissue Mini Kit (Qiagen) from frozen cells. A RT Profiler PCR custom array was designed (catalog no. CAPH-11134C; Qiagen) that included genes involved in pathways of cellular senescence, including the p53 pathway (TP53BP2, PTEN, MAPK14, HRA5), oxidative stress (SOD2, PRKCD), profibrotic (SNAIL), antifibrotic (BMP7, TAGLN), smooth muscle (CNN1), extracellular matrix (ECM) (COLIA2, COL3A1, COLIA1, ELN), ECM turnover enzymes (MMP2, MMP9, TIMP1), TGF superfamily (BMP7, TGFB1, SMAD2), epithelial-mesenchymal transition (AKT1, ILK, SMAD2, SNAIL, TGFB1), cell adhesion (CD44), and transcription factors (SRF, mTOR). RT² Real-Time SyBR Green PCR Mix was purchased from Qiagen. PCR was performed on 7700 ABI Prism 7700 sequence detector (AB Applied Biosystems) was measured by quantitative real-time PCR (qPCR) (described earlier). Beating rate was counted under an inverted microscope as beats per minute in control, hypoxia, and hypoxia plus TGF-beta incubation. Cells were then treated with primary antibodies, including rabbit polyclonal anti-CD31 (Abcam), mouse monoclonal anti-cTnT, CD31, and α-SMA was performed to identify which lineages had DNA damage. Telomere lengths were determined by a TRF kit (Roche Diagnostics). hTERT (AB Applied Biosciences). Data analysis was performed using the ∆∆CT method.

Double immunostaining of ph-P53 (Ser6) with cTnT, CD31, and α-SMA was performed to identify which lineages had DNA damage. Telomere lengths were determined by a TRF kit (Roche Diagnostics). hTERT (AB Applied Biosystems) was measured by quantitative real-time PCR (qPCR) (described earlier). Beating rate was counted under inverted microscope as beats per minute in control, hypoxia, and hypoxia plus TGF-β1 inhibitor, SB431542-treated EBs. Videos of beating EBs were taken using Handycam Camcorder (Sony, Toronto, ON, Canada).

Flow Cytometry

Differentiation stage day 20 EBs were dissociated to single cells after 72-hour normoxia, hypoxia, and hypoxia plus SB431542 incubation. Cells were then treated with primary antibodies, including rabbit polyclonal anti-CD31 (Abcam), mouse monoclonal anti-cTnT, CD31, α-SMA, S100A/FSP1, and γH2AX (phospho S139) (Abcam) for 40 minutes at 4°C, followed by 30-minute 37°C incubation with aliphophycocyanin-labeled anti-mouse and anti-rabbit secondary antibodies (BD Pharmingen, San Jose, CA) and phycoerythrin-conjugated cTnT, signal-regulatory protein α (SIRPA/CD172A), CD31, and platelet-derived growth factor receptor (PDGFR)-β (BD Pharmingen). Cells were also stained with fluorescein isothiocyanate, phycoerythrin, and aliphophycocyanin-labeled isotype-matched immunoglobulins (BD Pharmingen) and used as negative controls. Bromodeoxyuridine (BrdU) was added to growing EBs and incubated overnight. EBs were harvested and dissociated to single cells, fixed with 70% ethanol, and stained with a BrdU-labeled aliphophycocyanin BrdU Flow Kit (BD Pharmingen) for quantification of proliferating cells. For DNA content quantification of cell cycle check points, the cells were harvested, fixed with ethanol, and stained with propidium iodide in the presence of RNase. The cells were analyzed with the BDLSR II Flow Cytometer System and FlowJo software version 10.0.6 (BD Biosciences, San Jose, CA).

Endothelial and Smooth Muscle Lineages

Human umbilical vein endothelial cells (HUVECs) (Life-line, Cell Technology, Frederick, MD) were cultured in VascularLife EnGS Endothelial Cell Culture Medium (Life-line, Cell Technology) together with all of the components provided in the Low Serum Growth Supplement kit and the VascuLife EnGS LifeFactors kit (Lifeline, Cell Technology). Seventy-two-hour normoxia, hypoxia, and hypoxia plus SB431542 incubated HUVECs were used for flow cytometry to quantify DNA content and cell cycle analysis. SMC-enriched populations were derived from hPSC differentiation using a previously published SMC differentiation protocol that yields approximately 70% pure SMCs. For matrigel assay, 200 μL of undiluted matrigel (BD Gmbh, Heidelberg, Germany) was placed on 24-well plates and incubated for 1 hour at 37°C. Then 5 × 10⁴ HUVECs were reconstituted in 500 μL of culture medium and plated on the BD matrigel layer. Capillary tube formation was observed by converted microscope after incubation for an additional 24 hours at 37°C. Scores were given from 0 for individual cells separated to 5 for development of complex mesh-like structures.

Statistical Analysis

All results were expressed as means ± SD. Analysis was performed to measure differences between LV from control, moderate HLHS, and severe HLHS fetal hearts; paired LV and RV samples from severe HLHS; and cardiac lineages treated with normoxic, hypoxic, and hypoxia plus SB431542. The following variables were analyzed: number of cells with evidence of DNA damage, number of senescent cells, telomere length, cell proliferation index, myocyte density, capillary density, population doubling time, mRNA expression of cell cycling and growth factor genes, semiquantitative comparison by intensity of immunostaining, and flow cytometric quantitation of cardiac progenitors and differentiated lineages. Continuous variables were compared between HLHS and control samples using one-way analysis of variance and the nonparametric U-test. Differences were considered statistically significant at P < 0.05. Data were statistically evaluated using StatView software, version 5.0 (SAS Institute Inc, Cary, NC).

Results

LV Phenotype in Fetal HLHS Samples

We found strong nuclear localization of HIF-1α, the master regulator of the hypoxia response, in the LV in HLHS samples compared with control hearts using immunofluorescence (Figure 1A). Downstream angiogenic factors, VEGF, and TB4
were down-regulated on immunostaining (Figure 1A) and mRNA expression (Figure 1B). These differences were more marked in severe compared with moderate HLHS samples. Both VEGF and TB4 are important for cardiac progenitor recruitment, differentiation, and vasculogenesis.8

DNA Damage and Senescence

We assessed cell survival in the context of impaired growth factors by studying the DNA damage response. HLHS hearts had multiple green nuclear foci of γH2AX, a phosphorylated histone that binds to sites of double-stranded DNA breaks (Figure 2A). There were more γH2AX-positive cells in moderate (16% ± 4%) and severe HLHS samples (36% ± 6%) compared with controls (1 ± 1%) (P < 0.01 versus controls). Damaged cells had up-regulation of nuclear phospho-p53 (serine6), a tumor suppressor that induces cell cycle arrest to allow DNA repair to occur (Figure 2A). Failure of adequate DNA repair results either in apoptosis or senescence where the cell permanently exits the cell cycle and stops replicating. We found increased expression of the senescence marker β-gal (Figure 2A) but no apoptosis on TUNEL staining in the injured cells (Supplemental Figure S1A). Senescence was further confirmed through electron microscopy, which revealed nuclear heterochromatin foci in HLHS samples compared with compact nuclear chromatin in control LVs (Supplemental Figure S1B). These foci, called senescence-associated heterochromatin foci, represent a morphologic marker of cellular senescence.9

Cell Cycle Arrest

Senescence permits cell survival at the cost of cell replication, which can be seen through induction of cell cycle arrest in damaged cells. Cell cycle arrest in HLHS was evident by the decrease in the nuclear proliferation antigen Ki-67 and cyclin-dependent kinase CDK4 (G1-phase cell cycle progression activator) expression (P < 0.01 versus controls) (Figure 2, A and B). The up-regulation of the oncogene p53 and of the G1 cell cycle inhibitors p16 and p18 in HLHS (P < 0.05 versus controls) suggests that the cell cycle arrest and senescence was potentially mediated by oncogenes (Figure 2C). There was no telomere shortening in the LV of HLHS compared with the RV from the same hearts (Supplemental Figure S1, C and D), indicating that the senescence was stress induced as opposed to replicative senescence, which is associated with telomere shortening. We conclude that chronic hypoxia in fetal HLHS may be a factor in the observed severe DNA damage and cell senescence, resulting in reduced growth factor release in the microenvironment.

Endothelial Susceptibility to Genotoxic Injury

To determine which cell types were most susceptible to DNA damage, we performed double staining with γH2AX- and cardiac lineage—specific markers (ie, cTnT for cardiomyocytes, CD31 for endothelial cells (ECs), and SMA for SMCs). There were 90% ± 4% γH2AX+ ECs, 56% ±
2% γH2AX⁺ cardiomyocytes, and 18% ± 3% γH2AX⁺ SMCs in HLHS hearts versus 2% ± 1% γH2AX⁺ cells in controls (P < 0.01 versus controls) (Figure 3, A and B), indicating greatest susceptibility of ECs to genotoxic injury compared with other cardiac cell types.

Reduction Cardiac Progenitors and Differentiated Lineages

Not surprisingly, DNA damage was associated with reduced population of progenitor and differentiated cardiac lineages. Specifically, we found fewer Nkx2.5⁺ cardiac progenitors, and CD34⁺ endothelial progenitors (Figure 4A) and reduced mRNA expression of progenitor markers (P < 0.05 versus controls) (Figure 4B). The population of differentiated lineages (ie, MF20⁺ cardiomyocytes and vWF⁺ ECs) was also lower in HLHS samples versus controls. Interestingly, we found a higher proportion of SMA⁺ cells in severe HLHS hearts compared with control hearts (representative images are given in Figure 4A). Quantification by immunofluorescence confirmed lower myocyte and capillary numbers and lower capillary density in HLHS samples compared with controls (Figure 4C). In addition, capillary density was lower in advanced gestation HLHS fetuses (>22 weeks) compared to those ≤22 weeks of gestational age. This was seen in both moderate and severe HLHS hearts (Figure 4D). In contrast, SMA⁺ cells increased from 10% in control hearts to approximately 70% in HLHS hearts (P < 0.01 versus controls) (Figure 4E). We conclude that fetal HLHS was associated with depletion of cardiac progenitors and differentiated endothelial and myocyte lineages but with an increase in SMA-expressing cells and that the phenotype progressed with advancing gestation.
Increased Fibroblast Transformation

To determine the nature of SMA+ cells, we performed co-immunostaining with SMA and FSP1. FSP1 is expressed in mature fibroblasts and immature cells transitioning to mesenchymal lineages. Trichrome staining revealed extensive perivascular and interstitial fibrosis in HLHS hearts with an abundance of FSP1+ cells compared with controls (Figure 5, A and B). Co-immunostaining revealed a high proportion of SMA+/FSP1+ cells suggestive of proliferating myofibroblasts in areas of interstitial fibrosis in HLHS hearts (50% ± 2% in HLHS samples versus 2% ± 2% in controls, P < 0.01) (Figure 5, C and D). Importantly, the fibrosis was not driven by epithelial or endothelial-mesenchymal transition because E-cadherin, an epicardial marker, and TCF21/POD1, an epithelial-mesenchymal transition marker, expression was seen primarily in the epicardium and not the myocardium of both control and HLHS hearts (Supplemental Figure S2). We did however find co-localization of the profibrotic cytokine TGFB1 with FSP1 in HLHS hearts (Figure 5C) and TGF-β1 mRNA up-regulation by qPCR (P < 0.01 versus controls) (Figure 5E). This may suggest a role for TGF-β1 in myofibroblast proliferation and fibrosis.

LV versus RV Phenotype in HLHS

To exclude a generalized effect of intrauterine stress in HLHS, we studied paired LV and RV samples from the same hearts (10 fetal HLHS hearts and 10 control hearts). Up-regulation of DNA damage and senescence markers (γH2AX, phospho-p53, senescence-associated β-gal, and nuclear heterochromatin foci), and down-regulation of Ki-67 and CDK4 were restricted to the LV in HLHS hearts (Supplemental Figure S3, A and B). mRNA expression of the oncogenes p53, p16, and p18 was higher and capillary density was lower in the LV versus RV (Supplemental Figure S3, C and D). We conclude that the injury in HLHS is restricted to the LV, which may suggest a combination of genetic susceptibility and regional microenvironmental perturbations. Although the findings in human hearts are observational, together they raise the possibility that TGF-β1 up-regulation, DNA damage, and premature senescence contribute to the phenotype of fibrosis, reduced angiogenesis, and LV growth failure in HLHS.

Effect of Hypoxia in hPSC-Derived Cardiac Lineages

To examine the role of hypoxia and TGF-β1 up-regulation in mediating injury, we subjected hPSCs during cardiac differentiation to chronic hypoxia and assessed the ability of TGF-β1 inhibition to prevent injury. In brief, human embryonic HES2 cell lines were differentiated into cardiac lineages using an established cardiac differentiation protocol. This protocol yields 40% to 60% myocytes and 20% to 30% each of ECs and SMCs. To determine the degree of hypoxia that would reproduce the damaging effects seen in fetal hearts, we exposed hPSC-derived cardiac lineages to varying duration and severity of hypoxia. There was a dose-dependent response to hypoxia with an increase in γH2AX+ cells from 0.02% in controls, 8% with 5% O2 per 24 hours, 11% with 1% O2 per 24 hours, 16% with 1% O2 per 48 hours, and 26% with 1% O2 per 72 hours. All further experiments with hypoxia were performed in 1% O2 for 72 hours, and comparisons were made between normoxic or control cells, hypoxic cells, and hypoxic cells treated with TGF-β1 inhibitor.

DNA Damage and Senescence

Similar to fetal HLHS, hypoxia-treated cells had up-regulation of HIF-1α but not of the angiogenic growth
factor VEGF (Figure 6A). qPCR confirmed corresponding differences at the transcriptional level after hypoxia (Figure 6B). There was a marked increase in the DNA damage marker γH2AX and the senescence marker β-gal (Figure 7A) but no evidence of apoptosis (ie, TUNEL+ cells) (data not shown). The mRNA expression of the tumor suppressor oncogene p53 and the G1 cell cycle inhibitors p16 and p18 were also increased in hypoxic cells (Figure 7B). Therefore, hypoxia exposure induced DNA damage and senescence in hPSCs, thereby recapitulating the fetal HLHS phenotype.

Endothelial Susceptibility to Injury

To assess whether ECs were more susceptible to hypoxic injury, we performed double-staining with cardiac lineage markers and ph-p53 (Ser6). We found more extensive DNA damage in ECs with 77% p53+ ECs compared with 55% p53+ cardiomyocytes and 20% p53+ SMCs (Figure 7, C and D). We conclude that exposure of immature hPSC-derived cardiac lineages to hypoxia recapitulates the type of injury seen in fetal HLHS hearts, with ECs being more susceptible to injury followed by myocytes and then SMCs.

TGF-β1 Inhibition Reduces Cell Damage

To assess the role of TGF-β1 in rescuing hypoxia-induced injury, we treated the cells with 10 μmol/L SB431542, a TGF-β1 inhibitor, during hypoxia. SB431542 treatment prevented the up-regulation of the DNA damage marker 53BP1 and of the tumor suppressor ph-p53 (ser6) (Figure 8, A and B). Hypoxia also decreased proliferating BrdU+ cells from 21% to 14%; TGF-β1 inhibition prevented this decrease in proliferation (Figure 8, C and D). The hypoxia-induced increase in population doubling time (25 ± 3 hours in controls versus 49 ± 5 hours in hypoxia, P < 0.001) was similarly prevented by TGF-β1 inhibition (25 ± 5 hours, P < 0.001 versus hypoxia). To determine whether this loss of replication was related to stress-induced versus replicative senescence, we measured telomere length and telomerase expression levels [ie, telomerase reverse transcriptase (hTERT)], which were not different among controls, hypoxia-treated cells, and TGF-β1 inhibitor–treated cells (Figure 8, E and F). As expected, undifferentiated hESCs had the highest hTERT expression. We conclude that the decrease in replication potential is related to stress-associated premature senescence, which is telomerase independent and
potentially oncogene mediated rather than due to replicative senescence.\textsuperscript{11–14}

TGF-\(\beta\)1 Inhibition Preserves Myocyte and EC Populations

We performed flow cytometry to more precisely quantify the three primary cardiac lineages after exposure to hypoxia and assess TGF-\(\beta\)1 effects. Flow cytometry revealed a decrease in cTnT\(^+\) and SIRPA\(^+\) cardiomyocytes and in CD31\(^+\) ECs with hypoxia compared with normoxic controls (\(P < 0.05\) versus controls). SIRPA is a cell surface marker that was recently identified as a robust marker for cardiomyocytes derived from hPSCs.\textsuperscript{15} Conversely, we found an increase in SMA\(^+\) and PDGFR-\(\beta\)\(^+\) cells with hypoxia. PDGFR-\(\beta\) is a marker of mature SMCs (Figure 9, A and B). Treatment with SB431542 prevented the decrease in cardiomyocyte and EC populations and prevented the increase in SMA\(^+\) and PDGFR-\(\beta\)\(^+\) populations (Figure 9, A and B). We conclude that TGF-\(\beta\)1 inhibition can rescue hypoxia-induced decrease in cardiovascular lineages.

TGF-\(\beta\)1 Inhibition Reduces Fibroblast Transformation

Similar to fetal HLHS, hypoxia exposure was associated with a threefold increase in cells co-expressing SMA and FSP1 (yellow staining) (\(P < 0.01\) versus controls). This increase was attenuated with TGF-\(\beta\)1 inhibitor treatment (Figure 9, C and D). qPCR confirmed the threefold increase in TGF-\(\beta\)1 mRNA in hypoxic cells. (\(P < 0.01\) versus controls) (Figure 9E). We compared gene expression using a custom PCR array, with red color representing higher relative gene expression and green representing lower relative gene expression. The heat map (Figure 9F)
demonstrates hypoxia-induced up-regulation of senescence genes (TP53BP2, PTEN, MAPK14, HRAS), oxidative stress genes (SOD2, PRKCD), profibrotic genes (SNAIL), smooth muscle gene (CNN1), ECM genes (COL3A1, COL1A1, ELN) (except for COL1A2, which was reduced), remodeling enzyme genes (MMP2, MMP9, TIMP1), TGF superfamily genes (TGFB1, SMAD2), epithelial-mesenchymal transition genes (AKT1, ILK, SMAD2, SNAIL, TGFB1), cell adhesion gene (CD44), and transcription factors (SRF, mTOR). There was a down-regulation of anti-fibrotic genes (BMP7, TAGLN). Importantly, changes in gene expression seen with hypoxia were reversed with TGF-β1 inhibition (Figure 9F). We conclude that a hypoxia-induced increase in cellular and oxidative injury and fibroblast transformation during cardiac differentiation can be prevented by TGF-β1 inhibition.

TGF-β1 Inhibition Improves EB Contractility

To assess the functional effect of these changes, we assessed EB contractility by live imaging of beating EBs (Supplemental Videos S1, S2, and S3). EBs subjected to hypoxia had a marked decrease in beating rate from 64 ± 14 beats/min to 38 ± 7 beats/min with hypoxia (P < 0.01 versus controls). This was prevented by treatment with TGF-β1 inhibitor (51 ± 1 beats/min, P < 0.05 versus hypoxia). Overall, our findings confirm that chronic hypoxia can induce an in vitro cellular phenotype in hPSC-derived cardiac lineages that mimics the phenotype of fetal HLHS and that can be rescued by TGF-β1 inhibition.

Effect of Hypoxia on ECs and SMCs

To further assess lineage-specific susceptibility to hypoxia, we studied pure EC populations using HUVECs and SMC-enriched populations derived from hPSC differentiation. Hypoxia increased the G1/S phase of cell cycle arrest from 75% in controls to 86% with hypoxia (Figure 10, A and C). This increase was not seen in cells treated with a TGF-β1 inhibitor (P < 0.05 versus controls) (Figure 10, B and C). Hypoxia caused a smaller nonsignificant increase in G1/S cell cycle arrest in the SMC-enriched population derived from hPSCs compared with HUVECs (Figure 10, B and C). To assess the functional consequences of endothelial injury, we measured capillary tube formation by HUVECs in 3-dimensional matrigel assays and assigned scores from 0 for individual separated cells to 5 for development of complex mesh-like structures.7

Figure 7  Effect of hypoxia on DNA damage and oncogene up-regulation in hPSC-derived cardiac lineages. A: DNA damage–related marker γH2AX (green nuclear foci) and senescence marker β-gal (blue) is increased in hypoxic cells. Blue represents nuclear staining with DAPI. B: qPCR results revealed higher mRNA expression of the tumor suppressor oncogene p53 and the G1 cell cycle inhibitors p16 and p18 in hypoxic (gray bars) compared with control cells (black bars). C: Double immunostaining revealed co-localization of ph-p53 (green) with cTnT + myocytes (red), CD31 + endothelial cells (red), and SMA + SMCs (red), indicating DNA damage in all three lineages. Blue represents nuclear staining with TO-PRO-3. D: Cellomics quantification confirmed the higher number of ph-p53 + cardiac lineages in hypoxic cells (gray bars) compared with controls (black bars), with most severe injury in ECs followed by myocytes and then SMCs. *P < 0.01 versus controls; †P < 0.05 versus SMA + cells; ‡P < 0.05 versus cTnT + cells (n = 3 experiments in each group). Original magnification: ×1000 (γH2AX); ×600 (β-gal) (A); ×1000 (C).
Tube forming capacity in vitro was markedly reduced with hypoxia compared with controls (P < 0.01 versus controls) but was preserved with TGF-β1 inhibitor treatment (Figure 10, D and E). We conclude that ECs have a higher susceptibility to chronic hypoxia that causes endothelial senescence and cell cycle arrest and reduces tube forming capacity, whereas SMCs demonstrate a higher tolerance to chronic hypoxia.

**Discussion**

HLHS is a complex congenital heart malformation that is lethal without surgery. Survival requires multiple operations in the first 2 to 3 years of life to restore a physiologic circulation. This is associated not only with high mortality and morbidity but also with ventricular dysfunction and premature heart failure. Fetal interventions to restore LV flow are often unsuccessful or result in severe LV diastolic dysfunction that limits survival. Endocardial fibroelastosis is an important risk factor for poor outcomes after surgical repair of HLHS. The mechanism of this fibrosis and poor ventricular growth and function has been poorly understood. We hypothesized that although the underlying cause of HLHS may be genetic, the progressive abnormalities in LV growth and LV fibrosis during fetal life may be exacerbated by oxygenation and flow abnormalities.

Overall, our study suggests that the fetal LV in HLHS undergoes reprogramming with decrease in cardiac lineages and increase in fibroblast lineages possibly in an attempt to survive in a hostile environment at the cost of decrease in endothelial and myocyte lineages. The process involves TGF-β1 up-regulation, DNA damage, premature cell senescence, and loss of replication. The reduction in cardiac progenitors and differentiated myocytes and ECs and the increase in myofibroblasts likely promote tissue fibrosis, further affecting chamber growth and function. The recapitulation of these findings in hPSC-derived cardiac lineages exposed to hypoxia and prevention by TGF-β1 inhibition suggests an important role for hypoxia-induced TGF-β1 activation in mediating this injury. In particular, endothelial susceptibility to injury and senescence implicates impaired vasculogenesis as a key contributor to the observed phenotype.

The first important finding of our study was that of stress- or oncogene-associated senescence in malformed human fetal hearts, which has not been previously reported. Senescence occurs if genotoxic injury is too severe to permit adequate DNA repair and restore cellular integrity. Senescent cells, although functional, lack replicative capacity and fail to produce growth factors necessary to support organ growth. There was evidence of extensive cell senescence in fetal HLHS. The failure of senescent cells to produce VEGF and TB4 despite up-regulation of HIF-1α may have contributed to an adverse microenvironment that lacks the ability to support the survival, replication, and differentiation of cardiac progenitors, in particular ECs that are more susceptible to injury. Although an assessment of
the long-term effect of this reprogramming was beyond the scope of our study, severe and irreversible loss of replication potential during this critical fetal growth period is likely to significantly impair LV growth in the short term and LV function and regenerative capacity in the long term and may explain the failure of fetal intervention strategies to reverse injury and promote chamber growth.

The second important finding was the role of TGF-β1 in this phenotype. Damaged cells produce profibrotic cytokines such as TGF-β1, which in turn promote myofibroblast transformation of either endogenous fibroblasts or non-fibroblast populations (endothelial or epithelial cells). This process can further disrupt the architecture and function of the surrounding tissue. There was no evidence of epithelial or endothelial mesenchymal transition in the form of co-expression of epithelial markers such as E-cadherin or of endothelial markers with fibroblast markers, suggesting that the fibroblasts were most likely not of primary epicardial origin. We did however find an increase in cells co-expressing SMA and FSP1, a marker for proliferating myofibroblasts, along with co-expression of TGF-β1, suggesting that these myofibroblasts originated from tissue-specific fibroblasts that were induced by TGF-β1. An important aspect of this form of fibrosis unlike replacement fibrosis is the potential for reversibility once the stress is removed. A recent seminal article reported that introduction of cardiac differentiation factors into mouse hearts promoted transdifferentiation of fibroblasts into cardiomyocytes, highlighting the ability to alter fibroblast cell fate by providing appropriate transcription factors. This may explain why the myofibroblast population decreased after TGF-β1 inhibition in our in vitro cell cultures. Although the relatively extensive fibrosis observed in severe HLHS may not be reversible, our findings raise the interesting possibility that TGF-β1 manipulation may have the potential to reverse or reduce the progression of fibrosis at least in moderate HLHS. Besides its effect on fibrosis, studies in epithelial lineages have found that TGF-β1 induces cell cycle arrest by increasing the expression of CDK inhibitors and decreasing the expression of growth promoters, resulting in G1 arrest and premature senescence. To our knowledge, ours is the first study to demonstrate a similar association of TGF-β1-associated senescence and hypoxia in human cardiac tissues and cells.

The finding that TGF-β1 can prevent DNA damage is an exciting discovery. Cancer studies report that TGF-β1 can either promote genomic stability or instability, depending on the cellular context. In a normal cellular context with intact p53 function, TGF-β1 contributes to competent DNA repair and genomic stability. However, in cancers, TGF-β1 contributes to genomic instability. Our findings in injured cardiac cells parallel the findings in cancers because TGF-β1 up-regulation was associated with genomic instability, whereas TGF-β1 inhibition promoted genomic stability as
seen by reduced p53 activation and cell cycle arrest, thereby rescuing cells from a senescent fate. Although this effect may be detrimental in tumor cells, it appears to be a favorable response in cardiac cells by increasing the population of cardiac lineages. Nonetheless, a direct association between TGF-β1 and DNA damage response remains to be explored.

The third important finding was the potentially detrimental role of persistent HIF-1α up-regulation in fetal HLHS as a mediator of hypoxic injury. It is important to distinguish acute hypoxia (<24 hours) that transiently stabilizes HIF-1α to permit activation of the downstream hypoxia response machinery from persistent hypoxia that causes nuclear HIF-1α stabilization and a paradoxical maladaptive response. A role for HIF-1α in cardiac malformations has been reported before. Although some studies report HIF-1α down-regulation with congenital heart defects, others report that HIF-1α up-regulation is associated with cardiac and neural tube defects. Our findings support the latter possibility of the detrimental effect of chronic HIF-1α up-regulation on cardiac growth and future investigations should include a search for genomic defects in the HIF-1α signaling pathway as a potential factor in increasing the susceptibility of the fetal heart to genotoxic injury. Interestingly, TGF-β1-mediated DNA damage has been found in previous studies in mouse embryo fibroblasts to be influenced by oxygen levels because TGF-β1-induced DNA damage and senescence were partially rescued by normoxia. Our study suggests that hypoxia-induced TGF-β1 activation may be secondary to HIF-1α up-regulation.

The fourth novel finding was the susceptibility of vascular ECs to hypoxia-mediated DNA damage evident in both fetal HLHS and hPSC samples. In pure EC cultures of HUVECs, we observed increased G1/S cell cycle arrest, an effect that was less evident in a relatively pure SMC population. Although hypoxia-induced G1/S cell cycle arrest has been previously reported in other cell types, our study reports the unique susceptibility of immature ECs to hypoxia-induced cell cycle arrest, which is likely mediated by up-regulation of G1 cell cycle inhibitors such as p16 and p18. This finding differs from other studies that report increased angiogenesis in vitro after acute hypoxic exposure of ECs and may again reflect the differential effect of acute versus chronic hypoxia, with the latter overwhelming natural defense mechanisms, especially in the immature heart.

A fifth notable finding was the ability to recapitulate the fetal HLHS phenotype in hPSCs subjected to prolonged hypoxia during cardiac differentiation and to rescue this phenotype with TGF-β1 inhibition. In our study, conditions that mimicked fetal hypoxia resulted in a phenotype indistinguishable from fetal HLHS. Because the process of cardiac differentiation of hPSCs mimics the process of cardiogenesis,
hPSCs provide an excellent in vitro cellular model to study the effect of prenatal factors during cardiac differentiation, particularly because current cardiac differentiation protocols yield cardiac lineages that are in fetal stages of maturity.4,5,14 Lack of knowledge of injury pathways has hindered the development of medical therapies to promote ventricular growth and prevent early and late ventricular dysfunction.33,34 Our study suggests that the fetal growth and differentiation pathways are reprogrammed possibly to permit survival in an adverse environment but at the cost of organ growth and that interventions aimed at ventricular growth may need to be performed earlier before injury is irreversible and may need to target not just flow restoration but also the dysregulated biological pathways in HLHS.

Conclusion

We identified the unique susceptibility of the fetal heart and vasculature to DNA damage and premature senescence in association with impaired cardiac differentiation and vasculogenesis. These findings underscore the ability of intrauterine stressors to cause fetal reprogramming with deleterious consequences that can affect not only short-term cardiac growth but also long-term cardiovascular function and regenerative capacity. In addition, our ability to validate the hPSC-derived cardiac stem cell system as a potentially useful human in vitro cellular model to study signaling defects in fetal stage cells may have implications for its use in the study of environmental and teratogenic factors on cardiac development.

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Supplemental Data

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