

Iron deficiency impairs contractility of human cardiomyocytes through decreased mitochondrial function

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Aims

Iron deficiency is common in patients with heart failure and associated with a poor cardiac function and higher mortality. How iron deficiency impairs cardiac function on a cellular level in the human setting is unknown. This study aims to determine the direct effects of iron deficiency and iron repletion on human cardiomyocytes.

Methods and results

Human embryonic stem cell-derived cardiomyocytes were depleted of iron by incubation with the iron chelator deferoxamine (DFO). Mitochondrial respiration was determined by Seahorse Mito Stress test, and contractility was directly quantified using video analyses according to the BASiC method. The activity of the mitochondrial respiratory chain complexes was examined using spectrophotometric enzyme assays. Four days of iron depletion resulted in an 84% decrease in ferritin ($P < 0.0001$) and significantly increased gene expression of transferrin receptor 1 and divalent metal transporter 1 (both $P < 0.001$). Mitochondrial function was reduced in iron-deficient cardiomyocytes, in particular ATP-linked respiration and respiratory reserve were impaired (both $P < 0.0001$). Iron depletion affected mitochondrial function through reduced activity of the iron–sulfur cluster containing complexes I, II and III, but not complexes IV and V. Iron deficiency reduced cellular ATP levels by 74% ($P < 0.0001$) and reduced contractile force by 43% ($P < 0.05$). The maximum velocities during both systole and diastole were reduced by 64% and 85%, respectively (both $P < 0.001$). Supplementation of transferrin-bound iron recovered functional and morphological abnormalities within 3 days.

Conclusion

Iron deficiency directly affects human cardiomyocyte function, impairing mitochondrial respiration, and reducing contractility and relaxation. Restoration of intracellular iron levels can reverse these effects.

Keywords

Iron deficiency • Heart failure • Human cardiomyocytes • Contractility

Introduction

Iron deficiency is a highly clinical relevant co-morbidity, present in 40% of patients with chronic heart failure, even in non-anaemic

patients,^{1–4} and is related to impaired exercise capacity, reduced quality of life and a worse prognosis.^{5–8}

In addition to its key role in oxygen uptake and transport as a part of haemoglobin, iron has an important role in cellular oxygen

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storage and metabolism, redox cycling and as an enzymatic cofactor. Therefore, maintaining a normal iron homeostasis is crucial for cells that have a high energy demand such as cardiomyocytes.

Iron deficiency impairs functional status in heart failure patients independently of haemoglobin levels.⁹ In line with this, treatment with intravenous iron improves exercise capacity and symptoms in heart failure patients with iron deficiency, also when they are non-anaemic.^{10,11} Data from two small clinical studies in patients with heart failure and renal failure showed that intravenous iron improved left ventricular ejection fraction.^{12,13} Also, data from several animal studies demonstrated that cardiac iron deficiency induced by cardiomyocyte specific deletion of the transferrin receptor (*TfRc*) hepcidin (*HAMP*), or iron-regulatory proteins leads to impaired cardiac function and increased mortality.^{14–17} These effects are independent of systemic haemoglobin levels.

No studies have assessed the consequences of iron deficiency in human cardiomyocytes. We determined the effects of iron deficiency on human embryonic stem (hES) cell-derived cardiomyocytes. Since mitochondria are the key sites of cellular iron utilization and ATP production, we focused on mitochondrial function and contractility. Subsequently, we assessed whether iron supplementation was able to reverse the phenotype inflicted by iron deficiency.

Methods

Cell culture

HUES9 hES cells (Harvard Stem Cell Institute) were maintained in Essential 8 medium (A1517001; Thermo Fisher Scientific, Waltham, MA, USA) on a Geltrex-coated surface (A1413301; Thermo Fisher Scientific); medium was refreshed daily. Cells were incubated under controlled conditions with 37°C, 5% CO₂ and 100% atmospheric humidity. Differentiation to cardiomyocytes was achieved as described previously.¹⁸ Briefly, hES cells were dissociated with 1x TrypLE (12604-021; Thermo Fisher Scientific) for 4 min and plated as single cells in Essential 8 medium containing 5 μM Y26732 (S1049, Selleck Chemicals, Houston, TX, USA); Essential 8 medium (without Y26732) was refreshed daily. Once cultures reached 80% confluency, cells were washed with phosphate buffered saline (PBS) and differentiation was initiated (day 0) by culturing cells in RPMI1640 medium (21875-034, Thermo Fisher Scientific) supplemented with 1x B27 minus insulin (Thermo Fisher Scientific) and 6 μM CHIR99021 (13122, Cayman Chemical, Ann Arbor, MI, USA). At day 2, cells were washed with PBS and medium was refreshed with RPMI1640 supplemented with 1x B27 minus insulin and 2 μM Wnt-C59 (5148, Tocris Bioscience, Bristol, UK). From day 4, medium was changed to CDM3 medium as described by Burrige *et al.*¹⁹ and was refreshed every other day as cardiomyocyte maintenance medium. This resulted in cultures with >90% spontaneously contracting cardiomyocytes at day 8–10. To further enrich these cultures, starting from day 12, differentiated cardiomyocytes were cultured in glucose-free RPMI1640-based (11879, Thermo Fisher Scientific) CDM3 medium supplemented with 5 mM sodium DL-lactate (CDM3L; L4263, Sigma-Aldrich, St Louis, MO, USA) for 6–10 days.¹⁹ This resulted in >99% pure spontaneously beating cardiomyocytes. Experiments were typically started at day 24.

Iron chelation and restitution

In order to deplete the intracellular iron pool, cells were treated with 30 μM deferoxamine (DFO; D9533, Sigma-Aldrich) in CDM3 medium, which was added to cells at 0.1 mL/cm².²⁰ To restore intracellular iron levels, cells were incubated with CDM3 medium supplemented with 5 μg/mL partially saturated transferrin (T8158, Sigma-Aldrich). During experiments, medium was refreshed daily for all conditions.

Statistical analysis

Experimental groups consisted of at least three biological replicates and technical duplicates were used. Data shown are representative for three independent experiments and are expressed as means ± standard error of the mean. Differences between two groups were assessed by Student's *t*-test, while comparisons between three or more groups were assessed by one-way ANOVA followed by Bonferroni post-hoc test. Kruskal–Wallis test was used to compare the difference between groups with non-parametric variances followed by Dunn's post-hoc test. A *P*-value of <0.05 was considered statistically significant.

For remaining methods, see supplementary material online, *Methods S1* and *Table S1*.

Results

Induction of iron deficiency in stem cell-derived cardiomyocytes

To characterize the generated human cardiomyocytes, cells were stained for cardiac markers and cardiac-specific gene expression was determined. Differentiated cardiomyocytes stained positive for α-actinin and cardiac troponin T, showing a clear cross-striation pattern that is a hallmark of cardiomyocytes (online supplementary *Figure S1A*). Cardiac genes were found to be activated exclusively in differentiated cardiomyocytes whereas expression of pluripotency genes was exclusively found in hES cells (online supplementary *Figure S1B*). The observation that these cardiomyocytes show spontaneous contraction verifies stem cell differentiation towards cardiomyocytes.

To determine iron levels, intracellular ferritin levels were used as a proxy for cellular iron status. Incubating cardiomyocytes with the iron chelator DFO for 4 days resulted in 84% reduction in ferritin levels (*P* < 0.0001; *Figure 1A*). Iron depletion for more than 4 days resulted in cell death.

Gene expression analysis showed that expression levels of genes involved in iron uptake [*TfRc*, solute carrier family 11 member 2 (*SLC11A2*) and solute carrier family 39 member 14 (*SLC39A14*)] significantly increased in concert with a decrease of ferritin levels (*Figure 1B*). Additionally, iron depletion was associated with increased gene expression levels of ferritin heavy chain 1 (*FTH1*), ferritin light chain (*FTL*), 5'-aminolevulinic synthase 1 (*ALAS1*) and heme oxygenase 2 (*HMOX2*) (online supplementary *Figure S2*). Furthermore, iron deficiency resulted in increased protein levels of hypoxia-inducible factor 1 alpha (*HIF1α*), indicating a hypoxic cellular response (*Figure 1C*).

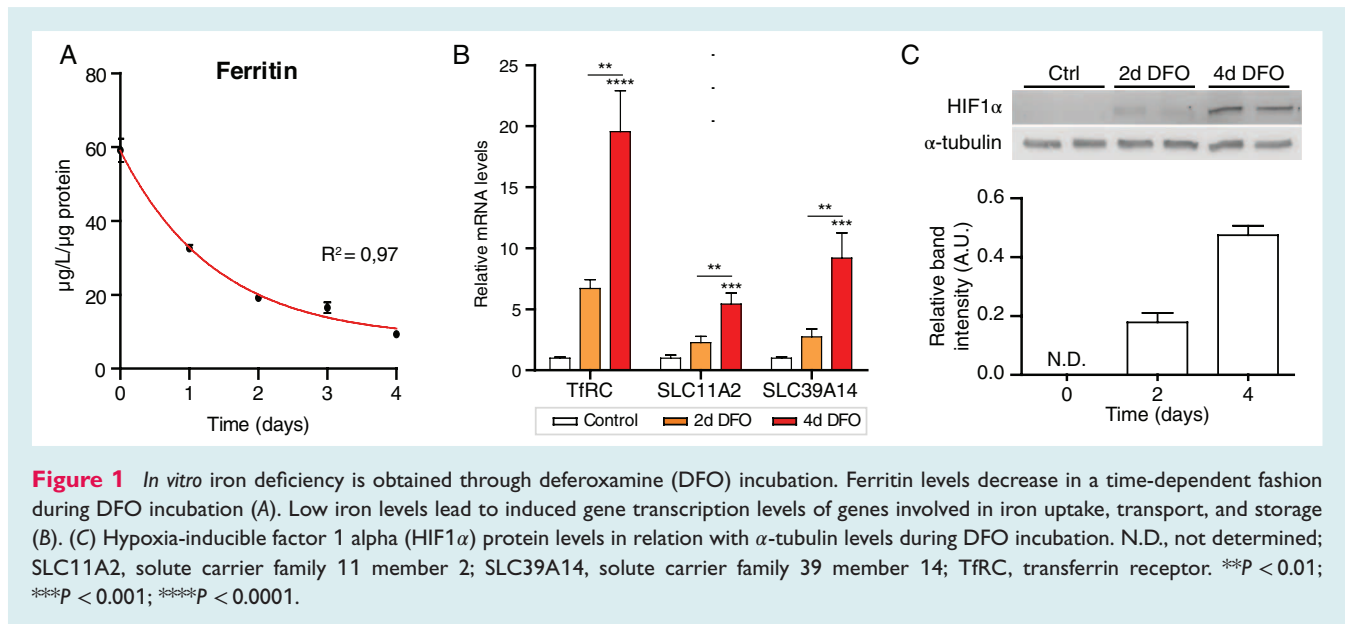


Figure 1 *In vitro* iron deficiency is obtained through deferoxamine (DFO) incubation. Ferritin levels decrease in a time-dependent fashion during DFO incubation (A). Low iron levels lead to induced gene transcription levels of genes involved in iron uptake, transport, and storage (B). (C) Hypoxia-inducible factor 1 alpha (HIF1 α) protein levels in relation with α -tubulin levels during DFO incubation. N.D., not determined; SLC11A2, solute carrier family 11 member 2; SLC39A14, solute carrier family 39 member 14; TfRC, transferrin receptor. ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

Iron deficiency leads to mitochondrial dysfunction

To determine global mitochondrial function, first total cellular ATP levels were measured. ATP levels decreased gradually with the duration of DFO incubation (Figure 2A). After 2 days of iron depletion ATP levels were reduced by 46% and after 4 days by 74% (both $P < 0.001$). To assess which specific elements of the electron transport chain were affected by iron deficiency, iron depleted cardiomyocytes were analysed with a Seahorse Mito Stress test (Figure 2B). Cardiomyocytes treated with DFO for 2 and 4 days showed reduced basal respiration [41% ($P < 0.01$) and 79% ($P < 0.0001$) reduction compared to untreated cardiomyocytes, respectively]. Injection of oligomycin inhibited ATP synthase-linked respiration, which was 73% in control cardiomyocytes and 63% ($P = 0.098$) in cardiomyocytes treated for 2 days with DFO, while cardiomyocytes treated for 4 days exhibited an ATP-linked respiration of 30% ($P < 0.0001$ compared to control, Figure 2C). Subsequent addition of the uncoupler carbonyl cyanide *p*-tri-fluoromethoxy-phenyl-hydrazone (FCCP) induced mitochondria to function at maximum capacity. Figure 2C demonstrates that only control cardiomyocytes were able to increase the oxygen consumption rate (OCR) above baseline values, indicating a respiratory reserve. All cardiomyocytes treated with DFO lacked this reserve regardless of the severity of iron depletion. To determine whether mitochondrial dysfunction could lead to further metabolic imbalance, the expression of key genes involved in (anaerobic) glycolysis or fatty acid metabolism was determined (online supplementary Figure S3). Iron-deficient cardiomyocytes showed decreased expression of acetyl-CoA carboxylase 1 and 2 (ACACA and ACACB, respectively) and ATP citrate lyase (ACLY), while glycolysis genes pyruvate kinase (PKM), hexokinase II (HK2) and lactate dehydrogenase A (LDHA), but not glucose transporter 4 (GLUT4) were upregulated during iron deficiency. Additionally, PPAR γ expression was increased during iron deficiency. This

further indicated the metabolic switch from fatty acids to glycolysis as a response to increased HIF1 α activity. Increased levels of LDHA is indicative of anaerobic glycolysis. Lipids were stained with Nile Red in iron-deficient cardiomyocytes (online supplementary Figure S4A). Indeed, iron deficiency resulted in lipid droplet formation, which was also confirmed by electron microscopy (online supplementary Figure S4B). To study mitochondrial function in more detail, the activity of complexes I–V was determined individually. During iron deficiency, complexes I and II showed the first signs of aberrant function after 2 days of DFO treatment; 4 days of DFO treatment also significantly reduced complex III activity levels. No changes were observed in complexes IV and V.

Transferrin-bound iron rescues iron-deficient cardiomyocytes

To rescue iron-deficient cardiomyocytes, physiological transferrin-bound iron was added after 4 days of DFO treatment. We found that transferrin-bound iron was able to restore ferritin to baseline levels after 2 days of supplementation (Figure 3A). After iron restitution, expression levels of genes involved in iron uptake (TfRC, SLC11A2 and SLC39A14) were significantly lower compared to iron-deficient cardiomyocytes, albeit higher than in control cardiomyocytes (Figure 3B). Expression levels of *FTH1*, *FTL*, *ALAS1* and *HMOX2* remained significantly increased compared to untreated controls (online supplementary Figure S5).

Analysis of cellular respiration demonstrated that transferrin-bound iron treatment resulted in improved mitochondrial function compared to DFO treatment (Figure 3C). Transferrin-treated cardiomyocytes showed improved basal respiration. In addition, ATP-linked respiration was restored after addition of transferrin-bound iron to DFO-treated cardiomyocytes (Figure 3D, left panel). Furthermore, transferrin-treated cardiomyocytes had regained a respiratory reserve, reaching 262.1% of baseline OCR, whereas OCR in iron-deficient cardiomyocytes was not

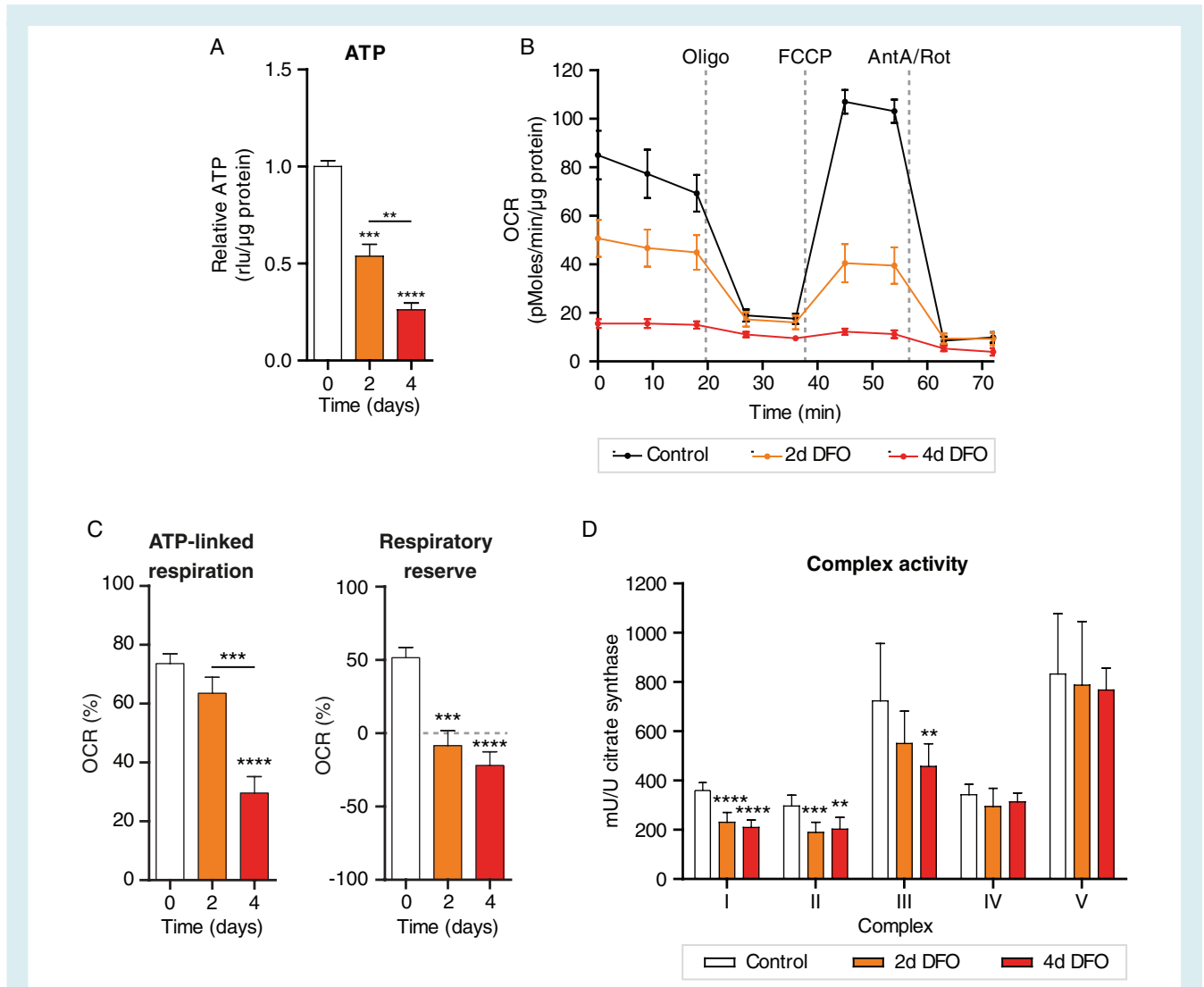


Figure 2 The effect of iron deficiency on mitochondrial function. Decreasing levels of intracellular iron correlate with ATP levels (A). Representative traces for control cardiomyocytes and cardiomyocytes treated with deferoxamine (DFO) for 2 and 4 days in a Mito Stress test (B). Effects of iron deficiency on ATP-linked respiration and respiratory reserve are shown in (C). The enzymatic activity of each individual mitochondrial complex was analysed (D). OCR, oxygen consumption rate. ***P* < 0.01; ****P* < 0.001; *****P* < 0.0001.

increased further by the injection of FCCP (Figure 3D, right panel). Furthermore, addition of transferrin-bound iron to iron-deficient cardiomyocytes eliminated HIF1α protein levels in iron-deficient cardiomyocytes (Figure 3E), and fully restored ATP levels (Figure 3F).

Additionally, to ascertain whether the observed mitochondrial dysfunction was the result of altered localization or a reduced number of mitochondria, cardiomyocytes were stained for the mitochondrial membrane marker translocase of outer mitochondrial membrane 20 (TOM20) and TOM20 protein levels were determined by western blot. Mitochondrial localization was found to be aberrant in iron-deficient cardiomyocytes, as opposed to the perinuclear localization in control cardiomyocytes. TOM20 protein levels did not differ significantly between control cardiomyocytes and iron-deficient cardiomyocytes (online supplementary

Figure S6). Furthermore, mitochondria of iron-deficient cardiomyocytes were typically found to be swollen and contained electron dense inclusions (Figure 4A). To determine which chemical elements were most abundant in these inclusion bodies, energy dispersive X-ray (EDX) analysis was performed (Figure 4B). Interestingly, the observed electron dense inclusion bodies contained low amounts of phosphorus as opposed to high levels of nitrogen and sulfur, suggesting that protein with a high sulfur content aggregated in iron-deficient mitochondria.

Contractile function is impaired in iron-deficient cardiomyocytes

Iron deficiency resulted in a 2.1% fractional area change (FAC) compared to 3.5% FAC of control cardiomyocytes (*P* < 0.05), while

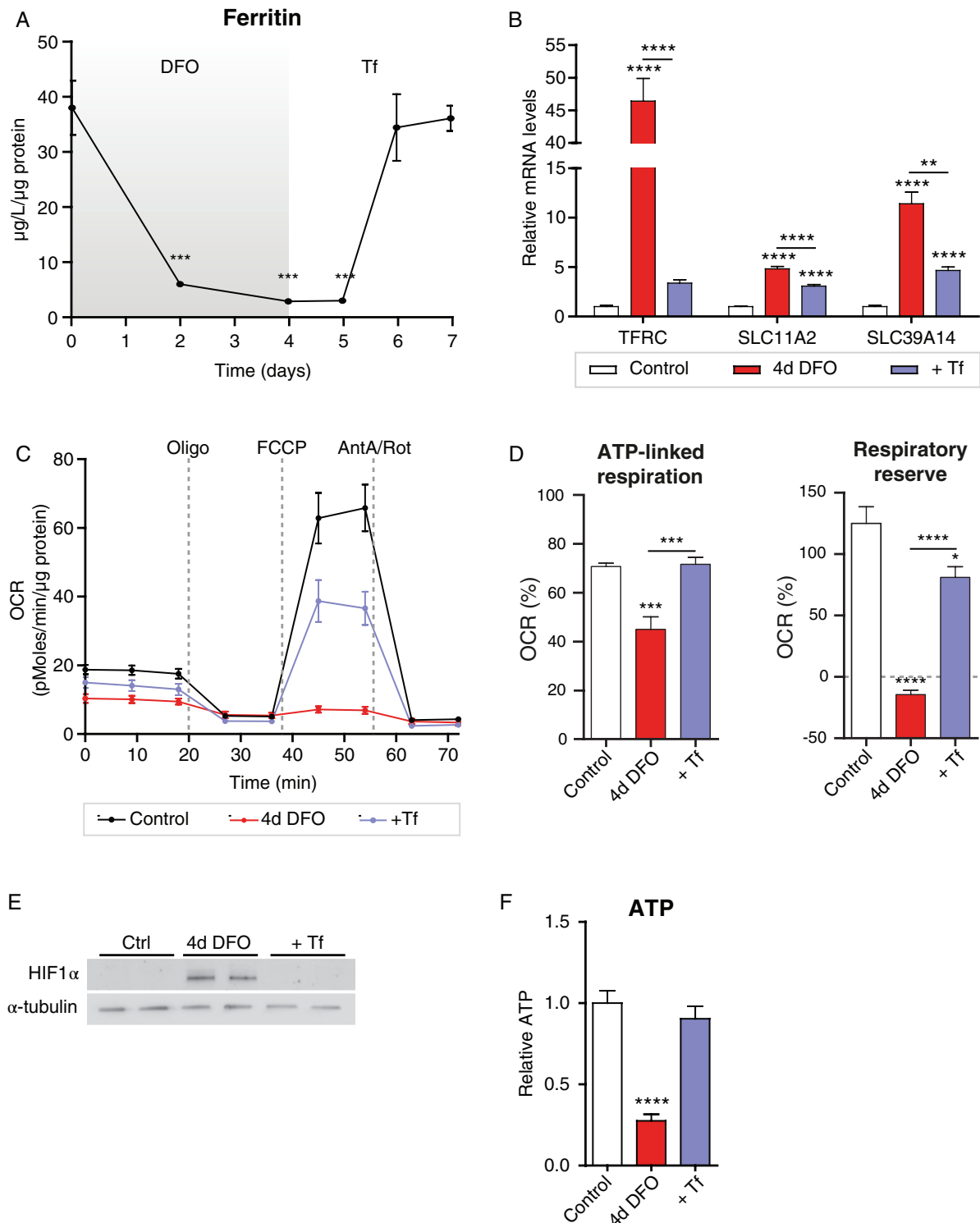


Figure 3 Effects of iron depletion are reversible by transferrin (Tf) administration. Following Tf-bound iron supplementation, levels of ferritin (A), gene expression (B), mitochondrial respiration (C), of which ATP-linked respiration and respiratory reserve shown in detail (D), hypoxia-inducible factor 1 alpha (HIF1 α) protein (E), and ATP (F) were mostly found to be restored. AntA, antimycin A; FCCP, carbonyl cyanide *p*-tri-fluoromethoxy-phenyl-hydrazine; OCR, oxygen consumption rate; Oligo, oligomycin; Rot, rotenone. * $P < 0.05$; *** $P < 0.001$; **** $P < 0.0001$.

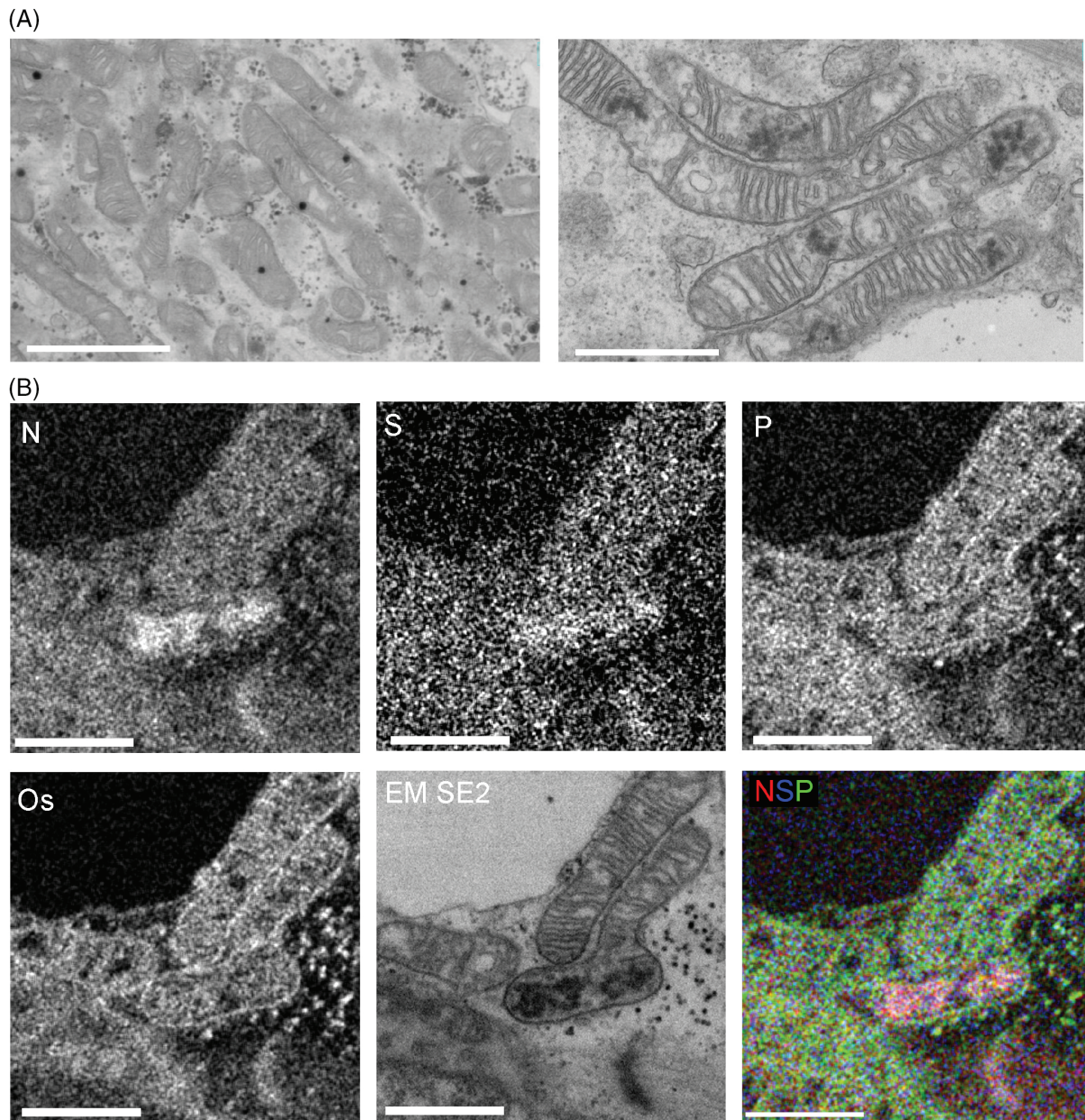


Figure 4 Mitochondrial morphology is also affected by iron deficiency. Mitochondria in iron-deficient cardiomyocytes appear swollen and contain electron dense inclusion bodies (A), which were found to contain nitrogen and sulfur based on energy dispersive X-ray (EDX) analysis (B). Scale bar = 1 μ m. DFO, deferoxamine.

the subsequent addition of transferrin-bound iron could reverse the FAC to 4.46% ($P = 0.19$ vs. control; *Figure 5*, and in more detail in online supplementary *Figure S7* and *video S1* and *S2*). Systolic maximum velocity (V_{max}) was significantly reduced to 0.33% FAC per 20 ms under iron-deficient conditions compared to 0.91% FAC per 20 ms ($P < 0.001$), which was reversible by addition of transferrin-bound iron to 0.97% FAC per 20 ms. Cardiomyocyte relaxation (diastolic V_{max}) was significantly reduced to 0.11% FAC per 20 ms in iron-deficient cardiomyocytes, compared to 0.77% FAC per 20 ms ($P < 0.001$) and improved after addition

of transferrin-bound iron to 0.40% FAC per 20 ms ($P < 0.01$), but remained impaired compared to control ($P < 0.05$).

The endoplasmic reticulum forms vacuole-like structures during iron deficiency

During iron chelation, cardiomyocyte morphology changed dramatically (online supplementary *Figure S8*). Vacuoles became apparent after 3 days of DFO incubation, while most prominent

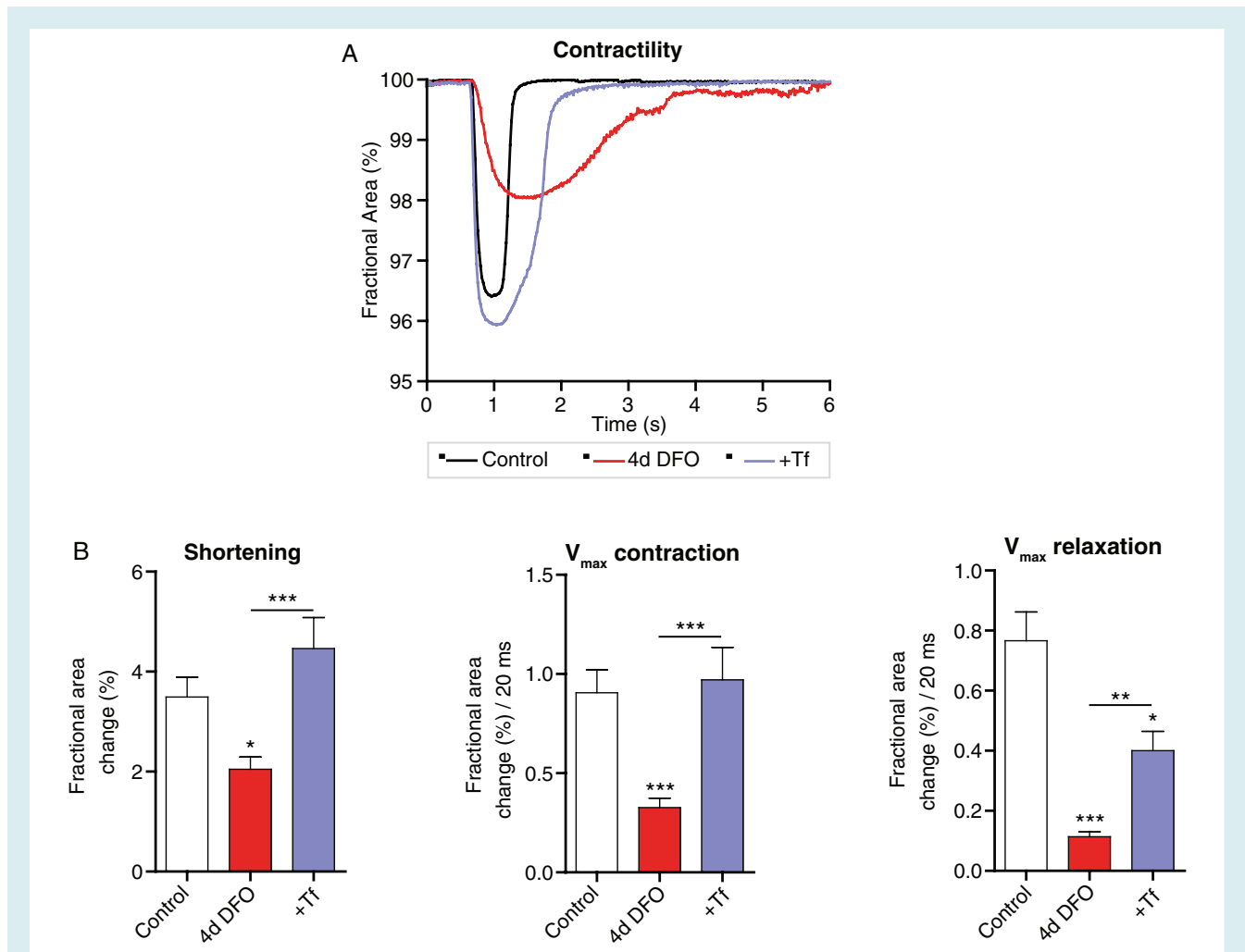


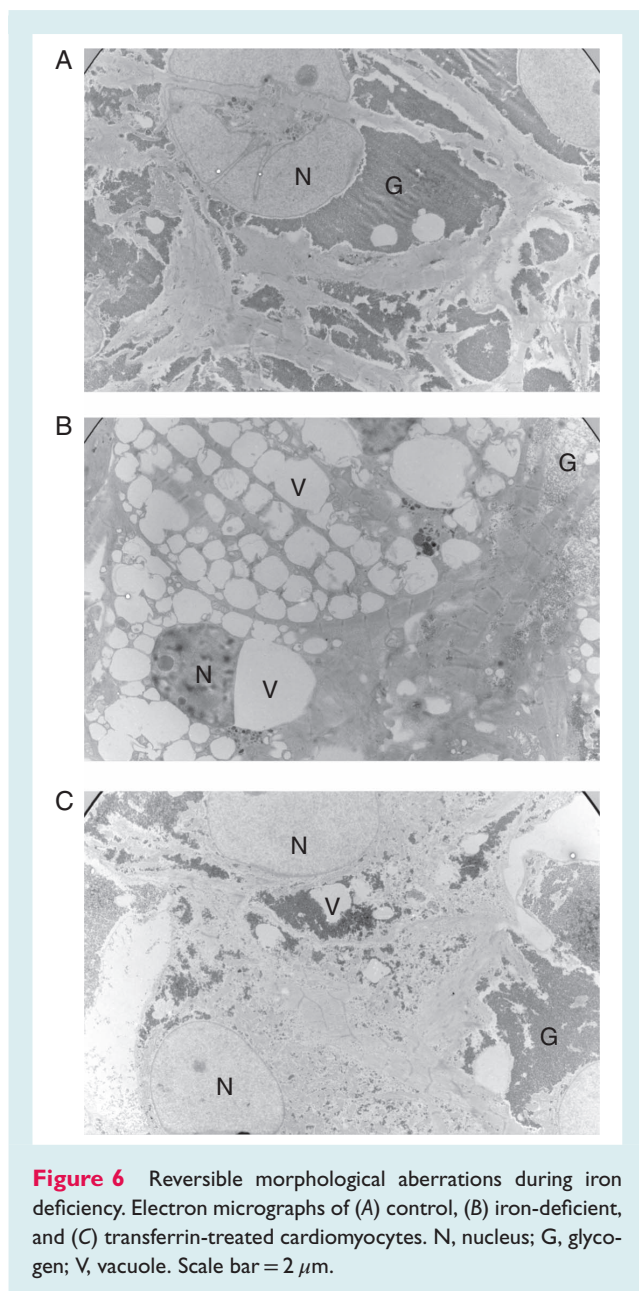
Figure 5 Low iron levels resulted in reduced contractile force and impaired systolic and diastolic velocity. The fractional area change (FAC) of a single contraction for each condition (A) show that iron deficiency impairs contractile force (B). FAC, and maximum systolic and diastolic velocities (V_{max}) are affected by low iron levels, but are restored upon addition of transferrin (Tf)-bound iron. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. DFO, deferoxamine.

after 4 days of DFO incubation. To identify the subcellular structures from which these vacuoles originated, control cardiomyocytes and cardiomyocytes after 4 days of DFO incubation were examined at electron microscopy level (Figure 6 and online supplementary Figure S9). Both conditions showed physiological mitochondrial structures as well as defined sarcomeric structures. Additionally, both conditions showed vast amounts of glycogen in the cytosol. Iron-deficient cardiomyocytes contained vacuoles with clear contents. Based on electron microscopy analysis, increased autophagy or lysosomal activity could be excluded as causes for vacuoles at this scale. One striking observation was the recurring formation of large perinuclear vacuoles, which suggested that the endoplasmic reticulum (ER) was severely affected. To determine to what extent ER stress plays a role in vacuole formation, an ER-linked FLIPPER probe was expressed in cardiomyocytes. Vacuoles were GFP-positive, demonstrating ER morphology (online supplementary Figure S9C). Gene expression analysis of various

ER stress-related genes further indicated that iron-deficient cardiomyocytes had increased levels of ER stress (online supplementary Figure S10). After the addition of transferrin-bound iron, the vacuoles disappeared, restoring morphology as observed with electron and light microscopy (Figure 6 and online supplementary Figure S11; full data via: <http://www.nanotomography.org>).

Discussion

Independent of its effects on haemoglobin, iron deficiency negatively impacts exercise capacity, symptoms and prognosis of patients with heart failure.^{1,5–8} We therefore hypothesized that low levels of intracellular iron result in impaired function of cardiomyocytes, possibly due to compromised mitochondrial respiration. In the present study, we demonstrate that iron deficiency in human cardiomyocytes provokes a hypoxic response and results



in mitochondrial dysfunction, low levels of ATP and impaired contractility and relaxation. After restoring iron levels, these effects are reversible. Using an *in vitro* model with cultured human stem cell-derived cardiomyocytes, we provide insights into the cellular effects of iron deficiency.

This study utilizes the iron chelator DFO to induce cellular iron deficiency. DFO is one of the most used iron-chelating agents approved for clinical use.²¹ *In vivo*, it chelates excess iron by binding free iron in the bloodstream, whereas it is taken up by cardiomyocytes via endocytosis *in vitro*.²² Once internalized, DFO efficiently chelates iron and subsequently depletes the cellular iron pool (i.e. ferritin-bound iron). In our experiments, medium was refreshed daily to prevent DFO from reaching an equilibrium with saturated DFO and to maximize chelation kinetics. In addition

to DFO, we tested multiple other iron-chelating agents, including deferasirox, deferiprone, dexrazoxan, PIH, bipyridyl. However, in our experiments, DFO was found to be most effective. In response to iron depletion, cardiomyocytes induce a gene expression pattern that greatly promotes iron uptake and transport. The obtained model of iron deficiency may be more severe than what can be expected in iron-deficient patients and may therefore not be directly translatable to the *in vivo* pathophysiology. However, direct comparison is difficult as circulating ferritin levels are assessed in patients while we measured cellular ferritin levels. The cellular and circulation systems might be subjected to separate and different regulatory mechanisms.

Low iron levels resulted in significantly reduced levels of ATP, which suggests mitochondrial dysfunction. The remaining levels of ATP are mainly produced by other mechanisms, such as anaerobic glycolysis and phosphocreatine conversion. We have shown that iron-deficient cardiomyocytes undergo a metabolic switch from fatty acid oxidation to anaerobic glycolysis. However, we have not determined a possible imbalance between the respiratory chain and the citric acid cycle. In conditions with a sufficient environmental partial oxygen tension, iron-deficient cardiomyocytes are unable to transport and utilize sufficient amounts of oxygen. In itself, reduced oxygen transport may account for mitochondrial dysfunction by inhibition of complex IV, whereas oxidative phosphorylation in general is hampered by aberrant redox cycling as a result of iron deficiency. Interestingly, only the activity of mitochondrial complexes I–III, which all contain iron–sulfur (Fe–S) clusters, were affected by DFO treatment, while the activity of the exclusively heme-based complexes IV and V remained unaltered. This observation confirms data of a previous study reporting low levels of cytosolic non-heme iron and increased levels of cytosolic and mitochondrial heme in cardiac tissue of patients with advanced heart failure.²³ Additionally, these data are in line with those from Rensvold *et al.*²⁴ that showed comparable mitochondrial function under iron-deficient conditions. Moreover, Rensvold *et al.* observed decreased levels of complexes I, II and IV following 24 h of 100 μM DFO incubation, whereas we demonstrate that complexes I–III show a reduced enzymatic activity after DFO incubation. Reduced protein levels of these complexes may support our observation of reduced complex activity. Finally, Melenovsky *et al.*²⁵ also showed a decreased activity of mitochondrial complex I and III in heart failure patients. Gene expression levels of the genes encoding *ALAS1* and *HMOX2* are both increased during iron deficiency. These genes encode for proteins with antagonistic functions; the underlying regulatory mechanism with regard to heme conservation remains unclear.

After restoring the ferritin levels with supplemented transferrin-bound iron, the effects of iron deficiency on the cardiomyocytes regarding iron metabolism, HIF1 α protein levels, ATP production, and mitochondrial respiration could be mostly restored. These observations indicate that the cellular effects of iron deficiency are highly reversible. In clinical trials, it has already been shown that iron deficiency can be reversed. CONFIRM-HF and FAIR-HF both show improvements in exercise capacity and symptoms.^{26,27} In case of the FAIR-HF, these improvements were already observed 4 weeks after the initial dose of intravenous iron.²⁷ Interestingly, after iron restitution, genes transcribing

ferritin light and heavy chains *ALAS1* and *HMOX2* remain induced (online supplementary Figure 5). These genes were found to be active in other forms of stress as well, indicating that these effects are not specific for iron deficiency, but rather are induced by various stress responses (e.g. hypoxic responses, reduced ATP levels, and ER stress).^{28–30}

Iron-depleted cardiomyocytes generate less force compared to untreated controls. Impaired contractile function could be fully restored by the addition of transferrin-bound iron with regard to FAC and systolic V_{max} , while cardiomyocyte relaxation only partially restored. These findings suggest that diastolic function is affected more permanent than systolic force and velocity. Ultimately, low levels of intracellular iron result in a diminished diastolic function *in vitro*, confirming observations from clinical cases.³¹

Morphological examination of the iron-depleted cardiomyocytes revealed swollen mitochondria containing electron dense material, as well as vacuole formation. Interestingly, mitochondrial dysfunction is observed in concert with morphological abnormalities. Previous studies found inclusion bodies in iron-deficient mitochondria, an observation that is strikingly similar to our observations.³² These inclusion bodies were found to be rich in sulfur and nitrogen, but not phosphorus, excluding the presence of DNA. These findings may indicate that Fe-S cluster remnants form aggregates with associated proteins. Additionally, the primary source of these vacuoles is the ER, as indicated by electron and light microscopy. The ER plays a major role in stress responses in general, which seems to be excessive during severe iron deficiency. ER stress-related genes were found to be induced during iron deficiency, which links iron deficiency to ER stress.³³ Furthermore, we show that lipid handling and homeostasis are severely disrupted by iron deficiency, which has been shown previously.³⁴

Our data further emphasize the potential negative effects of an impaired cardiac iron metabolism on the heart directly and independently of systemic iron, or heme, levels. Similar results were reported by *in vitro* and animal studies for intracellular iron status in skeletal muscle, showing deranged mitochondrial morphology with an impaired oxidative metabolism, impaired activity of mitochondrial complexes I and II, decreased Fe-S cluster synthesis and a shift to anaerobic glycolysis.²⁴ These cellular effects might be relevant when considering that strategies targeting the hepcidin/ferroportin axis are being developed. These agents lower hepcidin levels and increase ferroportin [solute carrier family 40 member 1 (*SLC40A1*)] expression, thereby increasing systemic iron levels and availability. However, this axis is also present and functional in the heart.¹⁵ An increased cardiac *SLC40A1* expression leads to iron export and lower intracellular iron levels in the cardiomyocyte, which might be counterproductive.⁹ Importantly, cardiac *HAMP* and *SLC40A1* expression might be subject to regulation independent of their systemic counterparts.

In conclusion, cellular iron deficiency results in a reduced activity of Fe-S cluster-based complexes in the mitochondria of human cardiomyocytes and is associated with impaired mitochondrial respiration and morphology, ATP production and contractility. These effects can be reversed by supplementation of iron. Our

study provides mechanistic insights into how treatment of iron deficiency may lead to improved cardiac function.

Supplementary Information

Additional Supporting Information may be found in the online version of this article:

Methods S1. Supplementary methods.

Table S1. Primer sequences for quantitative real-time polymerase chain reaction.

Figure S1. Cardiomyocyte differentiation from human embryonic stem cells.

Figure S2. Genes associated with iron storage and metabolism are upregulated in iron deficiency.

Figure S3. Metabolism switches from fatty acid oxidation to anaerobic glycolysis.

Figure S4. Lipid droplets in iron-deficient cardiomyocytes.

Figure S5. Genes associated with general stress response remained activated after iron restitution.

Figure S6. Iron deficiency does not reduce the number of mitochondria.

Figure S7. Effects on contractile function of iron deficiency.

Figure S8. Vacuole formation in response to severe iron deficiency.

Figure S9. The endoplasmic reticulum is enlarged during severe iron-deficient states.

Figure S10. Iron deficiency induces endoplasmic reticulum stress.

Figure S11. Vacuoles dissipate when iron levels are restored.

Video S1. Time lapse images of typical cardiomyocyte contractions *in vitro*.

Video S2. Time lapse images of iron-deficient cardiomyocytes showing impaired contractile function and aberrant morphology.

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