

Myeloid Cell Replacement Therapy Improves Function in Friedreich Ataxia Mice by Intercellular Mitochondrial Transfer

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Abstract

Friedreich's ataxia (FA) is a mitochondrial disease caused by frataxin deficiency, leading to neurodegeneration and cardiomyopathy. Currently, there are no effective therapies for FA. Our study investigated the potential of myeloid cell replacement using bone marrow-derived cells in the YG8-800 mouse model. Combining Busulfan myeloablation, Colony-stimulating factor 1 receptor inhibition, and bone marrow transplantation, we achieved almost complete microglia and tissue macrophage replacement. This replacement facilitated mitochondrial transfer to various CNS cells, enhancing ATP synthesis and oxidative phosphorylation and improving the mice's growth and neurobehavioral performance. Similarly, replacing macrophages restored function and increased mitochondrial activity in the heart. In vitro studies showed that transferring intact mitochondria partially restored respiratory capacity in FA cells, which had a higher uptake, suggesting a specific compensatory mechanism for mitochondrial acquisition. These findings suggest that myeloid cell replacement can promote metabolic recovery in FA through mitochondrial transfer and provide a new approach to treating FA and other mitochondrial disorders.

Introduction

Friedreich's ataxia (FA) is a multi-systemic disorder characterized by slowly progressive ataxia with onset usually before age 25 years. About two-thirds of people with FA develop cardiomyopathy, up to 30% diabetes, and around 25% show atypical clinical features^{1,2}. The disease is caused by a deficiency in frataxin (FXN) due to biallelic pathogenic variants in the *FXN* gene. FA's most common pathogenic variant is an expanded GAA repeat in intron 1 of *FXN*³. The GAA repeats lead to the silencing of the promoter, thereby reducing FXN expression. GAA repeat length contributes to disease severity, with lengths greater than 66 being disease-causing, and most expanded alleles in affected individuals range from 600 to 1,200 repeats³⁻⁵.

FA is fundamentally a mitochondrial disease. FXN is an essential protein enriched in mitochondria with its highest expression in dorsal root ganglia (DRG), spinal cord, cerebellar dentate nuclei, cerebral cortex, pancreas, heart, liver, and skeletal muscle, reflecting the affected organs in FA^{6,7}. FXN is involved in the assembly of mitochondrial iron-sulfur clusters, which are cofactors for Krebs cycle proteins as well as in multiple respiratory complexes. FXN deficiency results in abnormal accumulation of intra-mitochondrial iron, defective mitochondrial respiration, and overproduction of oxygen free radicals, resulting in cellular damage⁸. Accordingly, therapeutic strategies targeting mitochondria function and reducing oxidative stress have been a big focus in this disease.

The manifestations of FA in the nervous system are primarily attributed to neurodegeneration, but neuroinflammation also plays a role⁹. In the CNS, activated microglia are the primary producers of reactive oxygen species and appear activated in brain regions implicated in FA neuropathology¹⁰⁻¹³. Multiple studies have identified defects in FXN-deficient microglia, including increased phagocytic activity, heightened inflammatory responses, and loss of homeostatic functions, some preceding

neurodegeneration¹⁴⁻¹⁷. Similarly, macrophage activation and resultant systemic inflammation¹⁸ potentially contribute to the development of cardiomyopathy^{19,20} and insulin resistance²¹. These findings suggest that replacing affected microglia and macrophages could mitigate the progression of the disease.

Currently, there are no disease-modifying therapies for FA. Treatment focuses on symptomatic management and addressing mitochondrial redox and iron homeostasis²². Gene therapy using adeno-associated viruses (AAVs) and genome editing are being explored in preclinical studies, showing promising results in boosting frataxin levels and addressing either heart or nerve function²³⁻³⁶. However, challenges remain, such as the feasibility of efficiently targeting multiple cell types, maintaining appropriate frataxin levels, improving delivery methods, and their long-term safety^{37,38}. Bone marrow (BMT) or hematopoietic stem cell transplantation (HSCT) has been used for decades to treat multi-systemic diseases with neurodegeneration as a hallmark symptom³⁹. BMT has shown encouraging therapeutic effects in YG8sR FA mice^{40,41}. The rationale is that bone marrow-derived cells replace tissue myeloid cells, providing healthy microglia-like cells (MGLCs) in the CNS and dorsal root ganglia, as well as macrophages in the heart and muscle, where they cross-correct affected cells⁴²⁻⁴⁵. However, previous studies used high doses of whole-body irradiation for pre-transplant conditioning, achieving low MGLC replacement, which, combined with concerns about its unfavorable risk-to-benefit ratio, has tempered the enthusiasm about its application for FA. Nevertheless, a hematopoietic cell-based therapy is worth investigating as it offers several advantages. HSCT could be implemented immediately using allogeneic transplants and autologous transplantation strategies could be developed to enhance safety^{35,46,47}. Compared to AAVs, a cell-based therapy provides an alternative for those ineligible for AAV due to the high seroprevalence of AAV-neutralizing antibodies and avoids the potential toxicity risks associated with high doses, particularly in the DRG^{48,49}.

The mechanisms responsible for recruiting hematopoietic-derived cells to the CNS are not yet known, but myeloablative conditioning of the recipient is necessary^{42,50}. Current conditioning methods in HSCT result in slow and low engraftment of MGLCs in the CNS^{51,52}, significantly reducing HSCT's therapeutic efficacy for conditions like FA⁵³⁻⁵⁶. To improve the success of a hematopoietic stem cell-based approach for neurological disorders, we recently developed a conditioning regimen using FDA-approved drugs that rapidly and robustly replace microglia in the CNS and macrophages in the heart⁵⁷. This regimen combines busulfan myeloablation and the Colony-stimulating factor 1 receptor (CSF1R) inhibitor PLX3397. In progranulin-deficient mice, this approach successfully restored biochemical and neurological abnormalities⁵⁷. Other microglia replacement protocols have also been effective in several murine models of neurological diseases^{52,58-60}. We posited an optimized microglia/macrophage replacement protocol would enhance the efficacy of transplantation for FA. Ultimately, a clear demonstration of the benefit of this approach would support the clinical development of a hematopoietic stem cell-based therapy for FA.

To explore the potential of myeloid cell replacement therapy for FA, we utilized the YG8-800 mouse model. This model, derived from the YG8sR strain, possesses a human FXN gene with approximately 800 GAA trinucleotide repeats, making it the best existing model as it replicates critical features of the human disease^{17,61-63}. We achieved nearly complete replacement of microglia and macrophages in the YG8-800 mice through a combination of busulfan, PLX3397, and BMT. Following microglia replacement, we observed mitochondrial transfer, enhanced ATP synthesis, and oxidative phosphorylation across various CNS cell types. These changes significantly improved overall health and neurobehavioral performance in the treated mice. Similarly, macrophage replacement restored cardiac function and improved mitochondrial activity in the heart, underscoring the therapy's effectiveness. Additionally, we provide compelling evidence of intercellular mitochondrial transfer from donor myeloid cells in vivo and in vitro, effectively enhancing metabolic activity in FA cells.

Results

Highly Efficient Microglia Replacement and Mitochondria Transfer to Brain Cells in YG8-800 Mice

We recently described a strategy that achieves robust, rapid, and persistent myeloid cell replacement by bone marrow-derived cells throughout most tissues, including CNS and heart. This regimen combines two approved drugs: Busulfan, the myeloablative agent used in the clinic, with the CSF1R inhibitor PLX3397 (Pexidartinib)⁵⁷. To assess whether this regimen could achieve high engraftment of bone marrow-derived microglia-like cells (MGLCs) and improve neurological manifestations in FA, we tested it in FA mice. Several mouse models of FA have been developed, each showing differences in frataxin reduction, symptom severity, affected tissues, and age of onset⁶⁴. One of the most recently developed models is YG8-800, a knockout for the mouse *Fxn* rescued from lethality by a human FXN YAC transgene harboring ~800 GAA repeats. YG8-800 mice display more severe symptoms than other human YAC transgenic models like YG8sR, and it is currently considered the most accurate model of human FA^{17,61-63}. Mice were conditioned with Busulfan over four days (days -4 to -1, 100 mg/kg/day), transplanted (day 0), and administered PLX3397 (100 mg/kg/day) over six days by oral gavage 15 days post bone marrow transplant (BMT, Figure 1A). Donor bone marrow with wild-type levels of mouse FXN was derived from a double transgenic reporter mouse line that expresses a cytoplasmic enhanced green fluorescent protein (GFP) and a mitochondrial-targeted far-red fluorescent protein (mKate2), allowing us to track donor cells and their mitochondria in vivo and in vitro.

To assess the engraftment of MGLCs in the CNS and their therapeutic effect in YG8-800 mice, we compared three experimental conditions: YG8-800 mice receiving YG8-800 bone marrow (FA + FA), YG8-800 mice receiving GFP/mKate2 or double-positive (DP) bone marrow (DP+FA), and wild-type mice receiving GFP/mKate2 or double-positive (DP) bone marrow (DP+WT) (Figure 1B). Engraftment rates of DP cells in peripheral blood (PB) and brain were analyzed five months post-BMT by measuring the percent of GFP+ cells using flow cytometry. FA and WT mice showed high chimerism in the PB, 82.7%

and 94%, respectively (Figures 1C and Extended Data 1). The combination of Busulfan and PLX3397 achieved a very high proportion of GFP+ CD45+CD11b+ MGLCs in the brain of FA (82.2% $\pm$ 4.03) and WT mice (94.6% $\pm$ 2.67) (Figures 1D and E, and Extended Data 2). In the periphery and brain, the chimerism was slightly lower in YG8-800 mice compared to WT mice ($p < 0.05$). Histological analysis of transplanted FA and WT brains revealed a widespread and homogeneous distribution of bone marrow-derived GFP+/mKate2+ cells throughout the brain, accounting for 22-28% of all nucleated cells (Figures 1F and G). These findings demonstrate the effectiveness of our regimen and confirm high microglia replacement in YG8-800 mouse brains.

To investigate the possibility of mitochondrial transfer, we prepared single-cell suspensions from mice under all experimental conditions and analyzed them using flow cytometry (Figure 1H). We used the mKate2 signal to monitor mitochondrial movement while the GFP marked the transplant-derived donor cells. Putative mitochondrial recipient cells were identified for the lack of GFP expression and the presence of the mKate2 (GFP-/mKate2+) (Figure 1I). A significantly higher percentage of GFP-/mKate2+ cells was observed in YG8-800 mice compared to wild-type mice (12.7% vs. 4.19%, $p < 0.0001$, Figure 1J). We further analyzed the percentage of mKate2+ cells across different CNS cell types using cell type-specific surface markers, including endogenous microglia (CD45+/CD11b+/GFP-), astrocytes (ACSA-2+), oligodendrocytes (O4+), and neurons (CD90+, also known as Thy1). The fraction of mKate2+ cells varied between 15% and 30% across all four cell types. In YG8-800 mice, the highest percentage of mKate2+ cells was observed in endogenous CD45+/CD11b+ microglia, followed by CD90+ neurons. A similar pattern was seen in WT mice, although the increase in mKate2+ cells in microglia was not statistically significant (Figure 1K). In line with the flow cytometry results, high-resolution imaging of brain sections revealed mKate2+ signals, often without co-localization with GFP, suggesting that the signal was transferred to other cells (Figure 1L).

High Hematopoietic Reconstitution and Enhanced Mitochondria Transfer in YG8-800 Mice

To investigate the differences in hematopoietic reconstitution in YG8-800 mice, we performed a detailed analysis of cell lineages in the bone marrow and spleen five months post-transplantation, using flow cytometry. The specific lineage markers included CD45+ for all hematopoietic cells, CD11b+/Ly6C+ for myeloid cells, CD4+/CD8a+ for T cells, and CD19+ for B cells (Extended Data 1). In the bone marrow, the proportions of CD11b+/Ly6C+ cells were 59.2%, 60.3%, and 61.6% in the FA+FA, DP+FA, and DP+WT groups, respectively. The proportions of CD19+ cells were 19.5%, 23.0%, and 22.0%, while CD4+/CD8a+ T cells represented 4.9%, 4.1%, and 4.2% of the population (Figure 2A). None of these values were statistically significant.

YG8-800 mice exhibited similar engraftment levels compared to wild-type mice, as measured by the frequency of GFP+ donor-derived cells across all these populations. The chimerism rates were 81.6% in the DP+FA group and 89.1% in the DP+WT group, revealing a slight decrease in donor cell chimerism in

YG8-800 mice, which was observed in the peripheral blood ($p = 0.02$, Figures 2B and 1B). This high level of donor cell engraftment was consistently high in myeloid, T, and B cells: CD11b⁺/Ly6C⁺ (84% vs. 88%), CD4⁺/CD8a⁺ (77% vs. 83%), and CD19⁺ (79% vs. 86%). However, only in CD19⁺ B cells was donor cell chimerism lower, suggesting that the small difference in total chimerism is likely due to this cell type (Figure 2B). Similar findings for cell frequencies, donor cell engraftment, and differentiation were observed in the spleen, where, as expected, there was a higher proportion of lymphocytes (B and T cells) compared to the bone marrow (Figure 2C-D). As in the bone marrow, there was a small but statistically significant decrease in the proportion of donor-derived cells (CD45⁺), likely attributable to CD19⁺ B cells.

To evaluate mitochondrial transfer, we quantified the fraction of mKate2⁺/GFP⁻ cells in all groups. Notably, the proportion of mKate2⁺ cells within the GFP⁻ recipient population in the bone marrow was nearly three times higher in YG8-800 mice compared to wild-type mice (29.2% vs. 10.6%, Figures 2E and F). Further analysis to characterize the mKate2⁺/GFP⁻ cells in the different hematopoietic cell types revealed that CD19⁺ B cells had the greatest proportion of these cells (50.8% and 56.1% for DP+FA and DP+WT, respectively), followed by CD11b⁺/Ly6C⁺ myeloid cells (18.9% and 16.8%), and the lowest levels in CD4⁺/CD8a⁺ T cells (2.8% and 1.9%) (Figure 2G). Similar findings were observed in the spleen, where there was a three-fold increase in mitochondrial transfer in YG8-800 mice and an increased transfer in CD19⁺ cells (Figure 2H-J). Compared to the bone marrow, mitochondrial transfer in the spleen was even more pronounced in CD19⁺ cells, likely due to the higher frequency of these cells in the spleen.

Microglia Replacement Improves Health and Neurobehavior in YG8-800 Mice

YG8-800 mice exhibit poor growth, alopecia, and neurological abnormalities^{17,61,62}. YG8-800 mice that received autologous cells (FA+FA) displayed more pronounced alopecia than wild-type mice transplanted with wild-type DP cells (DP+WT). In contrast, YG8-800 mice transplanted with DP cells expressing wild-type levels of frataxin (DP+FA) showed significant improvements in alopecia (Figure 3A). We tracked body weight for approximately five months after transplantation (8 to 30 weeks of age) across these three groups to evaluate growth. We also included two additional control groups of unmanipulated FA and WT mice to account for the effects of conditioning and transplantation on weight and behavior. The WT group showed the expected weight gain pattern, with the highest trajectory, closely followed by the DP+WT group (27.3g $\pm$ 2 vs 26.0g $\pm$ 1.5 at week 29 for WT and DP+WT, respectively). The slightly lower weight trajectory initially observed in the DP+WT group was not significantly different from that of WT mice by week 30, possibly indicating an initial effect of BU+PLX conditioning that did not affect the long-term weight gain. The growth patterns of unmanipulated FA and FA+FA mice were similar and both significantly depressed, demonstrating the effects of the expanded human transgene on growth in this model (18.2g $\pm$ 0.5 for FA and 17.8g $\pm$ 0.7 for FA+FA). In contrast, DP+FA mice exhibited a 50% recovery in weight loss, reaching approximately 22.5 $\pm$ 0.7 g (Figure 3B). Microglia replacement with DP cells improved survival rates in these mice, increasing it from 50% in unmanipulated FA and FA+FA mice to 86% in DP+FA mice (Figure 3C).

Per previous reports, motor abnormalities in YG8-800 mice become apparent at 26 weeks. We assessed each group's neuro-motor deficits and muscle strength at 30 weeks of age. At this age, YG8-800 mice showed impairments in spontaneous locomotion, coordination, and muscle strength that improved with microglia replacement but did not completely normalize (Figure 3D-K). Compared to WT and WT+DP mice, FA and FA+FA mice showed impaired coordination in the crossbeam test, showing increased time to cross the beam and slips (Figure 3D-E). These differences were evident, with affected mice struggling to cross, relying heavily on the thin support of the crossbeam. FA and FA+FA mice also exhibited reduced spontaneous locomotion in the activity chamber compared to WT and DP+WT mice, as indicated by decreased ambulatory distance, vertical rearings, reduced time spent in the center zone, and increased time spent in the periphery zone. All these parameters were partially improved after microglia replacement in DP+FA mice (Figure 3F-J). In the wire-hanging test, the neuromuscular function was also reduced in FA and FA+FA, who often almost fell immediately after hanging. The treated mice also improved the wire-hang test (Figure 3K). Notably, unmanipulated WT and DP+WT mice behaved similarly in all neurobehavioral assays, supporting previous observations that microglia replacement does not affect neurobehavior⁵⁷ (Figure 3D-K).

Improved ATP Synthesis and Oxidative Phosphorylation Following Mitochondrial Uptake in CNS cells

To explore the identity of cells acquiring the mKate2+ signal and the biochemical impact of mitochondrial uptake in CNS cells, we dissociated brain cells from DP+FA mice. These cells were sorted into GFP- recipient populations based on mKate2 positivity (GFP-/mKate2+ vs. GFP-/mKate2-), followed by single-cell RNA sequencing (scRNA-seq, Figure 4A). The scRNA-seq analysis allowed us to identify cell types without bias and measure gene expression to assess the cells' metabolic status. We sequenced 9,151 mKate2+ cells and 3,196 mKate2- cells, combining data from three mice. Principal component analysis (PCA) showed that mKate2+ and mKate2- cells were distributed across most identified cell clusters, indicating that uptake of mKate2+ was not limited to a specific cell type (Figure 4B). Using known transcriptional signatures of CNS cells (Supplementary Table 1), we categorized the cells in each sample into 12 subpopulations (clusters), including choroid plexus epithelial cells (CPC), endothelial cells, oligodendrocytes, microglia, neurons, ependymal cells, astrocytes, macrophages, olfactory ensheathing glia (OEG), pericytes, arachnoid barrier cells (ABC), and oligodendrocyte precursor cells (OPC) (Figure 4C, Extended Data 3 and 4). The cellular composition was comparable between mKate2+ and mKate2- populations (Figure 4D).

To evaluate transcriptional changes in response to mitochondrial uptake and identify shared gene expression changes across all cell types, we conducted an unbiased differential gene expression (DGE) analysis between mKate2+ and mKate2- cells in the 12-cell populations. Using a significance threshold of $p < 0.05$, we found that 8,068 out of 32,285 genes showed significant expression changes in at least one cell type after mitochondrial transfer. Of these, 7,102 genes also met a 20%-fold-change threshold (Figure 4E, Supplementary Table 2). To pinpoint genes with consistent transcriptional changes across

cell types, we calculated a Common Expression Score (CES), which was determined by subtracting the number of cell types where a gene is downregulated from the number where it is upregulated. A positive CES indicates a gene is commonly upregulated, while a negative CES indicates downregulation across multiple cell types (Supplementary Table 2).

We first examined the top commonly upregulated genes with a CES of 6 or higher (indicating upregulation in at least six cell types). In mKate2+ cells, genes associated with ATP synthesis and oxidative phosphorylation (e.g., *Cox4i1*, *Ndufa13*, *Ndufa4*, *Atp5g1*, *Ndufb11*, *Cox7b*), inflammation (e.g., *Ttr*, *Apoe*, *Igfbp2*, *Enpp2*), and cellular homeostasis (e.g., *Manf*, *Glr3*, *Hspa5*, *Pebp*) were notably upregulated (Figure 4F, Supplementary Table 2). To further understand the biological pathways represented by the commonly upregulated genes, we performed pathway and process enrichment analysis on genes with CES ≥ 6 (50 genes). The resulting network map highlighted clusters related to ATP synthesis and oxidative phosphorylation, encompassing 24 of the 50 genes, with specific enrichments for oxidative phosphorylation ($\log_{10}(P) = -17$), proton transmembrane transport ($\log_{10}(P) = -6$), and Complex IV ($\log_{10}(P) = -4$), underscoring processes involved in cellular energy production (Figure 4G, Supplementary Table 3). Other enriched processes included protein localization to the membrane, transmembrane transport regulation, and insulin-like growth factor transport. Conversely, DGE and pathway analysis for commonly downregulated genes (CES < -6) in mKate2+ cells indicated that the most frequently downregulated genes were associated with mRNA processing, RNA stabilization, protein folding, and translation (26 out of 50 genes, Extended Data 5, Supplementary Table 4). Together, these findings suggest that the uptake of mKate2+ is indicative of mitochondrial transfer, which subsequently induces beneficial biochemical changes in FA cells, particularly in processes related to oxidative phosphorylation and ATP production, which are known to be impaired in FA.

Since most upregulated genes following mitochondrial uptake were involved in ATP synthesis and oxidative phosphorylation, we surveyed all known genes related to these processes. We observed upregulation in several subunits from Complex I (*Ndufb5*, *Ndufb7*, *Ndufc2*, *Ndufa11*, *Ndufb11*, *Ndufa4*, *Ndufa13*), Complex III (*Uqcrb*, *Uqcrh*, *Uqcrq*, *Uqcr11*, *Uqcr10*), Complex IV (*Cox5a*, *Cox8a*, *Cox7b*, *Cox4i1*), and Complex V (*Atp5j*, *Atp5l*, *Atp5h*, *Atp5o*, *Atp5d*, *Atp5g1*) across most brain cells after mitochondrial transfer (Figure 4H and Extended Data 6).

We also reviewed transcriptomic data from FA cellular models to determine if the transcriptional changes following mitochondrial uptake aligned with improvements in FA-related profiles⁶⁵⁻⁷¹. Although no universal signature was found across all cell types, two genes—*Prdx2* and *Prdx5*—were frequently downregulated in prior studies and showed upregulation in several cell types following mitochondrial uptake, suggesting potential improvement. Antioxidant defense genes (*Prdx2*, *Prdx5*, *Sod1*, and *Sod2*) and Krebs cycle genes (*Aco2* and *Mdh1*) were upregulated in mKate2+ cells, indicating a partial recovery in gene expression, as these genes are downregulated in FA skeletal muscle cells⁷¹. Furthermore, several genes associated with transcriptional and translational repression, which are usually upregulated in FA (*Ehmt2*, *Eif2ak4*, *Paip2b*, *Suds3*, and *Tcf25*), were downregulated in at least one cell type, further supporting a partial improvement (Extended Data 7).

Neurons, Astrocytes, Microglia, and Oligodendrocytes Increase Transcription of Energy Production Genes Following Mitochondrial Uptake

We investigated gene expression differences based on mitochondrial acquisition status (mKate2+/mKate2-) in specific cell types by conducting differential gene expression (DGE) analysis and gene set enrichment analysis (GSEA) for each cell type separately (Figures 5A-H and Extended Data 8 and 9). GSEA evaluated the enrichment of 7,713 gene sets (pathways) derived from the gene ontology biological process collections in the Mouse MSigDB. This analysis ultimately identified 855 pathways with significant changes ($p < 0.05$ and $q < 0.25$) in at least one cell type (Supplementary Table 5).

All major CNS cell types—neurons, astrocytes, microglia, and oligodendrocytes—showed substantial transcriptional changes following mitochondrial acquisition (Figures 5A-H). In neurons, several nuclear genes involved in ATP synthesis and oxidative phosphorylation, such as *Cox4i1*, *Ndufv1*, *Atp5o*, and *Ndufa9*, along with mitochondrial DNA genes (*mt-Cytb* and *mt-Co2*), were upregulated following (Figure 5A). GSEA of all DEGs in neurons identified 17 significantly upregulated and four downregulated pathways (Supplementary Table 5). Notably, the upregulated pathways are mainly related to ATP synthesis, electron transport chain, and oxidative phosphorylation, followed by pathways involved in DNA binding regulation and proteostasis (Figure 5B). Astrocytes also showed enhanced expression of genes involved in ATP synthesis and oxidative phosphorylation (e.g., *Atp5g1*, *Atp5k*, *Cox4i1*, *Ndufa11*, and *Ndufa13*). In these cells, upregulation of *Ttr*, *Ptgds*, *Igf1bp2*, and *Chchd10* may contribute to neuroprotection and cellular recovery (Figure 5C). Consistent with DGE findings, pathway analysis indicated increased pathways related to ATP synthesis, oxidative phosphorylation, and cytoplasmic translation (Figure 5D). Microglia displayed similar patterns to astrocytes, with upregulation of DEGs in ATP synthesis and oxidative phosphorylation. In addition, the upregulation of *Arl6ip1*, *Igf1bp2*, and *Cybb* suggests modulation of pathways related to cell survival and inflammatory responses (Figure 5E and F). In oligodendrocytes, DEGs overlapped with those in other cell types; however, pathway analysis showed a higher normalized enrichment score in pathways related to sodium regulation and pyramidal neuron differentiation and function, along with other energy derivation-related processes (Figures 5G and H). These findings suggest that mitochondrial transfer may partially restore energy production and support other biochemical pathways essential for normal cellular function across various cell types.

We examined 855 pathways that exhibited significant changes in at least one cell type to identify commonly altered pathways. To identify common pathways, we calculated the Common Pathway Score (CPS) by subtracting the number of cell types with downregulated pathways from those with upregulated ones. A higher CPS indicates that the pathways are more enriched across multiple cell types. We found 33 pathways with common enrichment following mitochondrial uptake, with CPS values ranging from 3 to 8 (Figure 5I). The analysis of these commonly enhanced pathways, consistent with our DGE findings and previous studies on mitochondrial transport, revealed enrichment in pathways related to ATP synthesis and oxidative phosphorylation, including proton transmembrane transport, ATP metabolic

process, and oxidative phosphorylation. Eight pathways, with CPS scores ranging from -3 to -5, showed downregulation after mitochondrial uptake (Figure 5J). Many of these pathways are related to RNA splicing and processing (e.g., RNA processing, RNA splicing via transesterification reaction, mRNA processing) and translation (e.g., translation initiation, cytoplasmic translation, peptide biosynthetic process). Previous GSEA analyses in human FA skeletal muscle and fibroblasts have highlighted enrichment in pathways associated with RNA splicing, RNA processing, RNA binding, and translation^{67,71}. The downregulation of these pathways in our data suggests a potential amelioration of the FA disease phenotype following mitochondrial uptake.

Macrophage Replacement Restores Cardiac Function in YG8-800 Mouse Hearts

Cardiomyopathy affects about two-thirds of individuals with FA, starting as hypertrophic and progressing to dilated cardiomyopathy and heart failure, a common cause of death⁷²⁻⁷⁴. YG8-800 mice have signs of hypertrophic cardiomyopathy that emerge by six months of age⁶¹⁻⁶³. We first examined the reconstitution of macrophages in the YG8-800 mouse hearts following transplantation. Mice conditioned with Busulfan/PLX3397 and subsequently receiving BMT showed high engraftment of donor-derived GFP+/mKate2+ macrophages within the heart. Notably, frequent mKate2 signals were observed that did not co-localize with GFP (Figure 6A).

We evaluated cardiac function using two-dimensional (2D) echocardiography to measure parameters such as left ventricular dimensions (end-diastolic and end-systolic diameters), ejection fraction, fractional shortening, wall thickness, and chamber volumes. Comparisons were made across FA (unmanipulated), FA sham (FA+FA), treated (FA+DP), healthy (WT+DP), and WT (unmanipulated) control groups (Figure 6B). FA mice transplanted with cells expressing wild-type levels of frataxin exhibited significant improvements in cardiac function, including increased left ventricular ejection fraction (LVEF, Figure 6C), fractional shortening (FS, Figure 6D), and left ventricular posterior wall thickness at end-diastole (LVPWd, Figure 6E), along with reduced left ventricular end-diastolic volume (LV-vol-s, Figure 6F).

To investigate the molecular mechanisms underlying the therapeutic effects of macrophage replacement in the FA heart, we performed bulk RNA sequencing (RNA-seq) of whole heart tissue. This study included three groups: FA+FA (n=3), DP+FA (n=3), and DP+WT (n=2). PCA of gene expression profiles revealed distinct clustering, with DP+FA and DP+WT samples grouping closely together, while FA+FA samples formed a separate cluster, indicating significant differences in gene expression patterns (Figure 6G). To further explore the genes and pathways involved, we compiled a set of 52 marker genes associated with cardiac phenotypes in FA murine and cellular models^{30,31,75-77} (Supplementary Table 6). Z-scores were computed from FPKM values across all samples for these genes. In line with our PCA findings, hierarchical clustering of the expression of these 52 genes for the three conditions clustered the DP+FA samples closer to the DP+WT samples, indicating more similar gene expression profiles than those of the affected FA+FA mice (Extended Data 10A).

A detailed analysis of gene expression profiles of these 52 genes across the experimental groups revealed significant modulation in pathways associated with hypertrophic cardiomyopathy, heart failure, fibrosis, iron metabolism, β -oxidation and glycolysis, electron transport, and the TCA cycle (Figure 6H-I, Supplementary Table 6 and Extended Data 10). Consistent with prior studies, the FA+FA group exhibited marked upregulation of genes linked to hypertrophic cardiomyopathy and heart failure, such as *Acta1*, *Actn1*, and *Igf1*, alongside heart failure markers *Nppb*, *Aldh1a3*, and *Gdf15*, suggesting cardiac stress and heart failure. Importantly, most of these genes showed significant reductions in the DP+FA group, most indistinguishable from the DP+WT group (Figure 6H and I and Extended Data 10B). Fibrosis markers, including *Postn* and *Bag3*, were significantly elevated in FA+FA, reflecting active extracellular matrix remodeling, but were reduced in DP+FA. Nrf2 activation in the heart regulates the expression of antioxidant genes like *Nqo1* and *Sod2*, which reduce oxidative stress, protect against ischemia-reperfusion injury, mitigate cardiac remodeling, and improve cardiac function⁷⁸. In the DP+WT group, the upregulation of these Nrf2 targets compared to FA+FA suggests enhanced oxidative stress defense and protection against cardiac damage (Figure 6H and I and Extended Data 10B). Disruptions in iron metabolism were also evident in FA+FA mice, characterized by decreased expression of *Slc40a1* and *Isca1* and increased expression of *Slc25a37* and *Trf* (Figure 6I and Extended Data 10B). These patterns were improved in the DP+FA group, suggesting improved iron homeostasis. Furthermore, genes involved in β -oxidation and glycolysis, commonly downregulated in FA such as *Hadha* and *Hadhb*, were upregulated in DP+FA, indicating metabolic reprogramming to meet energy demands (Figure 6I and Extended Data 10B). Collectively, these findings underscore the cardiac dysfunction and metabolic disturbances in FA YG8-800 mice and indicate substantial improvements across multiple pathways important for cardiac function in the DP+FA group.

To unbiasedly assess the treatment effects, we also performed differential gene expression analysis between FA+FA and DP+FA mice. This analysis identified 822 significantly differentially expressed genes ($p < 0.05$), with 349 exhibiting a fold change (FC) $> 20\%$ and 473 displaying an FC $< -20\%$ (Supplementary Table 7 and Extended Data 10C). Gene set enrichment analysis (GSEA) of all differentially expressed genes revealed consistent enrichment (FDR < 0.05) of pathways associated with aerobic respiration, ATP synthesis, mitochondrial respiration, and mitochondrial function in treated samples (Figure 6J). These findings suggest a notable recovery of mitochondrial function in heart tissue upon macrophage replacement.

Intercellular Mitochondrial Transfer Restores Metabolic Function in FA Cells

To investigate whether mitochondrial transfer is a mechanism through which tissue myeloid cells restore mitochondrial function in FA cells, we established a co-culture system consisting of donor macrophages and recipient FA cells. We generated double-positive (DP) macrophages by differentiating bone marrow cells from GFP/mKate2 mice using a cytokine cocktail including M-CSF and GM-CSF, and skin fibroblasts from wild-type WT and YG8-800 mice. In these co-cultures, fibroblasts and macrophages were

distinguishable due to differences in cell and nuclear size, and they were easily separated based on their different attachment properties. Fluorescence microscopy revealed the presence of mKate2+ signals in fibroblasts, suggestive of mitochondrial uptake (Figure 7A and B). After 96 hours of co-culture, WT fibroblasts exhibited a low level of mitochondrial uptake, approximately 2%, while FA fibroblasts showed a 10-fold higher uptake, around 20% (Figure C). This observation suggests that FA fibroblasts have a compensatory mechanism that facilitates mitochondrial uptake.

To confirm that intact mitochondria, rather than mRNA or protein from the mitochondrially targeted mKate2 transgene, were being transferred, donor macrophages were labeled with a mitochondrial tracker that fluoresces upon accumulating in the mitochondrial membrane. Flow cytometry was then used to assess mitochondrial transfer to fibroblasts. Fibroblasts and macrophages were distinguished based on their light scattering properties (forward scatter [FSC] and side scatter [SSC]) and the expression of the myeloid marker CD11b (Figure 7D-F). At 48 hours, a distinct mKate2+/GFP- fibroblast population (Q1) emerged, representing ~10% of the total cells. By 72 hours, this population increased to ~20% (Figure 7D). Flow cytometry confirmed that all mKate2+/GFP- cells were CD11b-negative and exhibited light-scattering properties consistent with fibroblasts, ruling out the possibility that this population arose from macrophages that had lost GFP expression (Figure 7E). The complete co-localization of the mitochondrial tracker and mKate2 signals indicates the transfer of intact mitochondria (Figure 7F and J). Similar results were seen in patient-derived fibroblasts with CD90 expression, confirmed by flow cytometry (Figure 7G-J) and imaging (Figure 7K and Extended Data 11). These findings demonstrate that mitochondrial transfer occurs and involves mitochondria rather than transgene mRNA or protein alone.

To assess the impact of intercellular mitochondrial transfer on cellular respiration, we analyzed the oxygen consumption rate (OCR) across four different cell populations purified by fluorescence-activated cell sorting: WT fibroblasts (WT monoculture), mouse FA fibroblasts (FA monoculture), mKate2- FA fibroblasts (FA mito co-culture), and mKate2+ FA fibroblasts sorted from co-cultures (FA mito+ co-culture). As expected, FA fibroblasts exhibited significantly lower OCR levels than WT fibroblasts, indicating impaired mitochondrial function (Figure 7L). OCR was significantly enhanced in FA mito+ fibroblasts, which received mitochondria via co-culture, particularly during maximal respiration, as demonstrated by the response to FCCP. In contrast, FA mito- fibroblasts (co-cultured cells that did not receive mitochondria) showed no such improvement (Figure 7L). Quantitative analysis revealed a significant increase in maximal respiration in FA mito+ fibroblasts compared to FA fibroblasts and FA mito- fibroblasts ($p < 0.0001$, Figure 7M). However, WT fibroblasts retained the highest maximal respiration capacity, indicating only partial recovery. Similarly, spare respiratory capacity was significantly improved in FA mito+ fibroblasts compared to FA fibroblasts and FA mito- fibroblasts ($p < 0.0001$), although it remained below the levels observed in WT fibroblasts (Figure 7N). These results demonstrate that intercellular mitochondrial transfer from macrophages partially restores mitochondrial function and respiratory capacity in FA cells.

Discussion

Our findings demonstrate that replacing microglia and tissue-resident macrophages with bone marrow-derived cells expressing wild-type FXN levels leads to significant metabolic restoration and improved function in FA mice. By employing an optimized conditioning regimen that uses Busulfan and the shortest reported post-transplant course of CSF1R inhibition, we reproducibly achieved high and stable microglia replacement by MGLCs throughout the CNS and macrophages in the heart. Our previous studies have demonstrated similar successes in the neuroretina and spinal cord⁵⁷. This protocol has strong clinical applicability. Busulfan is already utilized in HSCT for neurometabolic disorders. Additionally, PLX3397, administered at 1/100 to 1/200 of the human dose, is the only FDA-approved CSF1R inhibitor with established safety data⁷⁹. Comparisons with unmanipulated FA and WT mice suggest the lack of long-term toxicities, as shown by growth and neurobehavioral outcomes. However, short-term toxicities would be anticipated and warrant further investigation. These findings underscore the importance of the conditioning regimen in HSCT/BMT for FA.

Previous studies evaluating the efficacy of BMT utilized YG8R mice^{40,41}. Compared to the YG8-800 line used in this study, the YG8R model carries copies of the human FXN gene with shorter expansions (approximately 82 and 190 GAA repeats), exhibits higher levels of FXN expression, and presents a milder, less reproducible phenotype^{61,80,81}. Furthermore, irradiation was employed as a conditioning regimen, which is not clinically applicable for FA. Aside from its associated toxicities, irradiation leads to low, variable, and slow MGLC engraftment in the CNS, even at high doses. This limitation has prompted the use of CSF1R inhibitors (CSF1Ri) to improve cell recruitment from the bone marrow^{52,82-86}. Our studies build upon these previous findings and support using a hematopoietic cell-based therapy for FA. By using a more suitable model for evaluating disease-modifying therapies, we showcase the potential of CSF1Ri in enhancing microglial replacement and convincingly demonstrate the potential outcomes that high levels of microglia and tissue macrophage replacement can achieve in FA.

Several mechanisms may explain how replacing tissue myeloid cells improves symptoms in FA. Research has identified significant defects in FXN-deficient microglia, including increased phagocytic activity, heightened inflammatory responses, and a loss of homeostatic functions¹⁴⁻¹⁷. Collectively, these abnormalities likely drive neuroinflammation and neurodegeneration in FA, suggesting that simply replacing dysfunctional microglia could slow disease progression. A similar mechanism may occur in the heart and other tissues, where macrophage activation likely contributes to systemic inflammation and the development of cardiomyopathy^{9,20}. Indeed, blood samples from FA patients show pro-inflammatory signatures, indicating that addressing these inflammatory processes through HSCT could improve symptoms^{18,65}. While inflammation certainly plays a role in the pathophysiology of FA, it does not completely account for it, which could explain why myeloid cell replacement therapy offers only partial benefits.

Myeloid cells may also help FA through intercellular mitochondrial transfer (IMT). Numerous studies have shown that cells can export mitochondria to FA-relevant cells under normal and pathological conditions⁸⁷⁻⁹⁰. Mitochondrial transfer is critical in cardiomyocytes to maintain functional connectivity

and support mitochondrial fitness⁹¹⁻⁹³. IMT also promotes cardiac recovery in pathological conditions by reducing infarct size, preventing apoptosis, and enhancing cardiomyocyte function⁹⁴⁻⁹⁷. Similarly, IMT has been demonstrated between neurons and microglia and under oxygen or glucose deprivation in CNS cells, mitigating cellular damage⁹⁸⁻¹⁰². Beyond tissue repair, IMT has been shown to impact tumor resistance, immune regulation, and inflammation control¹⁰³⁻¹⁰⁶.

Following transplantation, we observed mKate2 signals in various cell types in the CNS and heart and transcriptomic changes consistent with mitochondrial recovery, all supporting mitochondrial uptake. Using donor myeloid cells with mitochondria labeled with a membrane-bound reporter, we confirmed the transfer of intact mitochondria, which partially restored respiratory capacity in FA cells. Interestingly, *in vivo* and *in vitro*, mitochondrial uptake was approximately 10-fold more prevalent in FA cells, suggesting a specific compensatory mechanism upregulated to facilitate mitochondrial acquisition. This aligns with prior studies showing enhanced uptake with mitochondrial stress¹⁰⁷. These findings support the hypothesis that intercellular cross-correction by myeloid cells can occur through IMT.

Several distinct mechanisms of IMT have been reported^{87,88}. In the context of FA, transient cellular connections, known as nanotubules, and cell fusion have been proposed^{40,41}. Other pathways include the transfer of free mitochondria or their encapsulation within extracellular vesicles. Despite not being fully understood, IMT has been clinically explored in patients with large-scale mitochondrial DNA deletion syndromes and for cardiac ischemia^{108,109}. Transfer of healthy mitochondria between cells has therapeutic potential, but further research is needed to understand the mechanisms involved, especially in the context of myeloid cell replacement.

Our transcriptomic analyses of single cells in the CNS and heart revealed increased gene expression and upregulation of pathways related to ATP synthesis, oxidative phosphorylation, and the electron transport chain. Although FXN-deficient cells do not exhibit a universal transcriptional signature⁶⁵⁻⁷¹, several commonly downregulated genes—such as *Prdx2*, *Prdx5*, *Sod1*, and *Sod2*—were upregulated in various CNS cells after mitochondrial uptake. Similarly, in the heart, we analyzed 52 marker genes associated with cardiac phenotypes in FA murine and cellular models^{30,31,75-77}. This analysis revealed significant modulation of pathways linked to hypertrophic cardiomyopathy, heart failure, fibrosis, iron metabolism, β -oxidation, glycolysis, electron transport, and the TCA cycle. These findings underscore the metabolic disturbances in YG8-800 mice and demonstrate substantial improvements across multiple pathways critical for CNS and cardiac function following myeloid cell replacement.

Our findings demonstrate that myeloid cell replacement with those expressing wild-type FXN can significantly restore metabolism and improve tissue function in FA. However, like other investigational therapies for FA, this approach presents several challenges. Although promising, the restoration achieved is only partial and carries potential risks. The safety of this approach can be improved through autologous transplantation strategies^{35,46,47} and by exploring less genotoxic conditioning regimens¹¹⁰. Its efficacy can be enhanced through engineered FXN over-expression or improving cross-correction

capacity. The future of FA treatment may require combining multiple therapies with unique effectiveness and risk-benefit profiles tailored to the disease stage at the intervention time. Importantly, mitochondrial transfer provides a mechanism for cellular cross-correction, representing a therapeutic opportunity not only for FA but also for other mitochondrial diseases.

Materials And Methods

Mouse experimentation

Mice were housed under a 12:12-h dark/light cycle, temperature (20-22°C), and humidity (30-70%)-controlled environment. Sterile food and water were provided ad libitum in the animal facilities at Stanford University. All experiments were conducted in compliance with the National Institutes of Health institutional guidelines and were approved by the Stanford University Administrative Panel on Laboratory Animal Care (IACUC 33941). Experiments were conducted using female mice to minimize variability due to sex differences, particularly in bioinformatics analyses such as bulk and single-cell RNA-seq. At the end of each study, mice were deeply anesthetized with a Ketamine/Xylazine mixture (80 mg/kg Ketamine/16 mg/kg Xylazine, intraperitoneally) and underwent transcatheter perfusion with 1X phosphate-buffered saline (PBS-1X, Fisher Scientific 10-010-023).

Mouse conditioning

Adult (8-10-week-old) C57BL/6J mice (Jax strain #000664) and YG8-800 mice: Fxn^{em2.1Lutzy} Tg(FXN)YG8Pook/800J (Jax strain #030395) were conditioned with Busulfan (Sigma-Aldrich 14843) and administered intraperitoneally with busulfan (25 mg/kg/day) for 4 days, totaling 100 mg/kg, prior to transplant, as described in each study. PLX3397 (Pexidartib, MedChemExpress HY-16749) was administered by gavage at a dose of 100 mg/kg/day. The PLX3397 powder was dissolved in 100% DMSO and stored in aliquots at -80°C. For administration, the PLX3397 stock was diluted in a 1:1 mixture of Polyethylene glycol Mn 400 (PEG400, Sigma-Aldrich 202398) and PBS-1X, pH 7.4, without calcium or magnesium (Fisher Scientific 10-010-023). The administration schedule and treatment period are detailed in the figure legend (Figure 1A). The optimized PLX3397 conditioning regimen involved administering the drug by oral gavage for 6 days (600 mg/kg, 100 mg/kg/day), starting 15 days post-bone marrow transplantation.

Transplantation of total bone marrow

Bone marrow was harvested from adult (8-12-week-old) double-positive mice generated in our lab by crossing C57BL/6-Tg(CAG-EGFP) (Strain # 006567) and C57BL/6-Tg(CAG-mito:mKate) (Strain # 032188) mice, and from YG8-800 mice: Fxn^{em2.1Lutzy} Tg(FXN)YG8Pook/800J (Strain # 030395). Total bone marrow cells were collected by flushing the tibiae and femurs with PBS-1X (Fisher Scientific 10-010-023) containing 4U/mL Heparin (Sigma-Aldrich H3149-500KU). After collection, the bone marrow cells were filtered through a 30 µm cell strainer, washed twice with PBS-1X and resuspended in 100 µL of PBS-1X

(1.5×10^8 cells/ μ L). Mice were transplanted with total bone marrow cells via intravenous injection into the retro-orbital sinus (1.5×10^7 cells/mouse) 24 hours after busulfan conditioning.

Flow cytometry analyses of cells isolated from mouse hematopoietic tissues

Mice were euthanized at designated time points for the analysis of donor chimerism in various tissues. Deep anesthesia was induced using a Ketamine/Xylazine mixture (80 mg/kg Ketamine/16 mg/kg Xylazine, intraperitoneally). Following transcardial perfusion with PBS-1X, tibiae, femurs, spleen, brain, and heart were harvested. Bone marrow cells (BM, from tibiae and femurs) and splenocytes (SP) were isolated in RPMI (Thermo Fisher Scientific 61870127) supplemented with 10% FBS, 4U/mL Heparin (Sigma Aldrich H3149-500KU) and 0.2 U/mL Deoxyribonuclease I (Worthington Biochemical Corporation LS002007) and filtered through a 30 μ m cell strainer. Erythrocytes were lysed using the RBC lysis buffer (Thermo Fisher Scientific 00-4333-57). Afterward, the cells were washed, resuspended in FACS-BL buffer, and maintained on ice until further processing. For flow cytometry staining, cells were blocked for 10 minutes with 10% vol/vol Mouse BD Fc Block™ (clone 2.4G2 BD Biosciences) and then stained in the dark for 30 min using the following antibodies: anti-mouse CD45.2 BV650 (clone 104 Biolegend), anti-mouse Ly6C PE-Cy7 (clone RB6-8C5 Biolegend), anti-mouse/human CD11b (clone M1/70 Biolegend), anti-mouse TER-119 AF700 (clone TER-119, Biolegend), anti-mouse CD4 APC (clone RM4-5 Biolegend), anti-mouse CD8a APC (clone 53-6.7 Biolegend), anti-mouse CD19 APC/Cy7 (clone 6D5 Biolegend), and dead cells were stained (Live/dead Fixable Blue Dead Cell Stain Kit; Invitrogen). The cells were then washed and resuspended in FACS-BL buffer. Stained cells were acquired using a BD FACSAria II cell sorter and conducted with BD FACSDiva software. Flow cytometry data were analyzed using FlowJo software (FlowJo, LLC).

Dissociation of Brain Tissue and Flow Cytometric Analysis of Brain Cells

Brain tissue dissociation was performed using a modified protocol from the Adult Brain Dissociation Kit (Miltenyi Biotec 130-107-677). Mice were deeply anesthetized with a Ketamine/Xylazine mixture and underwent rapid decapitation to minimize brain tissue damage. The extracted brains were washed three times with ice-cold Dulbecco's phosphate-buffered saline (D-PBS) containing calcium, magnesium, glucose, and pyruvate (Thermo Fisher Scientific 14287080). Tissue pieces were collected by centrifugation at 400 g for 5 minutes and dissociated with enzyme mixes 1 and 2 in gentleMACS C Tubes (Miltenyi Biotec 130-093-237) for 30 minutes using the gentleMACS Octo Dissociator with Heaters (Miltenyi Biotec 130-096-427), according to the manufacturer's instructions. Digested samples were quenched with ice-cold D-PBS and filtered through a 70 μ m cell strainer. After debris and red blood cell removal, following the manufacturer's instructions, the cell pellet was washed with FACS-BL and stored at 4°C for further processing. For flow cytometry staining, all brain cell pellets were resuspended in 10% vol/vol Mouse BD Fc Block™ (clone 2.4G2 BD Biosciences) for 10 minutes and stained in the dark for 30 min with the following antibodies:

anti-mouse CD45 PE-Cy7 (clone 30F11 Biolegend), anti-mouse/human CD11b-BV650 (clone M1/70 Biolegend), anti-mouse TER-119 AF700 (clone TER-119, Biolegend), anti-mouse CD90.2; Thy1.2 BV421 (clone 30-H12 Biolegend), anti-mouse ACSA-2 APC (Miltenyi Biotec 130-116-245), anti-mouse/human/rat O4 PE (Miltenyi Biotec 130-117-357), and dead cells were stained (Live/dead Fixable Blue Dead Cell Stain Kit; Invitrogen). Following staining, the cells were washed and resuspended in FACS-BL buffer. All the stained brain cells were acquired with a BD FACS Aria II cell sorter and BD FACSDiva software. Flow cytometry data were analyzed using FlowJo software (FlowJo, LLC).

Histological analyses

Brain and heart tissues were collected following transcardial perfusion with cold PBS-1X, then fixed overnight in 4% paraformaldehyde solution in PBS (Santa Cruz Biotechnology sc-281692). The fixed samples were washed once with PBS-1X and cryoprotected by transferred to a 30% sucrose solution in PBS overnight. The tissues were embedded in Tissue-Tek optimal cutting temperature compound (OCT, Fisher Scientific 4585) and sectioned at 20 μ m using a cryostat (Leica, Wetzlar, Germany, CM3050). Tissues sections were stored at -20°C until further use. For imaging transplant-derived GFP+ cells in the brain and heart, slides were washed once with PBS-1X supplemented with 1 mM CaCl_2 and 0.5mM MgCl_2 (PBS-1X++), counterstained with Hoechst 3342 (1:1000 dilution in PBS-1X, Thermo Fisher Scientific PI62249), and mounted in Aqua Poly/Mount (Polysciences 18606-20) for fluorescent microscopy.

Cell engraftment images in the brain and heart were visualized and captured using the BZ-X800 all-in-one fluorescence microscope and BZ-X800 software (Keyence, Itasca). To assess mitochondrial transfer in the brain, tissue slides were prepared for confocal imaging. Images were acquired using a 63X objective on a confocal laser scanning microscope (Zeiss LSM 800). Image editing and analysis were performed using ZEN software (Zeiss). The number of GFP+ cells was quantified using 20X composite images of an entire brain sagittal section, with slides de-identified both before and after image acquisition to ensure unbiased quantification. Based on the acquired images, the engraftment rate was quantified as the ratio of GFP+/DAPI-positive cells. Quantification was performed using ImageJ software, and the data were normalized to the selected area

Behavior study in YG8-800 mice

Mice were housed in groups under a reversed light cycle (8:30 am Light OFF-8:30 pm Light ON), and behavior tests were conducted during the dark cycle at the Stanford's Behavioral and Functional Neuroscience Laboratory (SBFN) by an experimenter who was blinded to the experimental conditions. Behavioral assessments were performed at 20 and 22 weeks after bone marrow transplantation, corresponding to the age of 7-8 months for each group.

Cross Beam Test

The Cross Beam Test was conducted following the protocol described by Luong et al¹¹¹. The test apparatus consisted of a 1-meter-long, 2 cm-wide Plexiglass beam with a central platform measuring 0.66 cm in both width and height. Mice were placed at one end of the beam and allowed to traverse to the opposite end. During the task, the time taken to cross the beam and the number of foot slips (when the mouse's foot lost grip off the beam) were recorded. Each mouse underwent three trials, and the average values for each parameter were calculated for analysis. To ensure consistency, the experimental setup and test conditions were standardized across all trials.

Activity Chamber

The locomotor assessment was conducted in an Open Field Activity Arena equipped with Activity Monitor Software-811 (Med Associates Inc., St. Albans, VT. Model ENV-515) and infrared detectors arranged in three planes, housed within a sound-attenuating chamber (Med Associates Inc., St. Albans, VT. MED-017M-027). The testing arena dimensions were 43cm (L) x 43cm (W) x 30cm (H), and the sound-attenuating chamber measured 74cm (L) x 60cm (W) x 60cm (H). Mice were placed in one corner of the testing arena and allowed to freely explore for 10 minutes, while an automated tracking system tracked their movements. Key parameter, including total distance traveled, velocity, rearing frequency, and times spent in periphery versus the center of the arena, were analyzed. The periphery was defined as the area within 5 cm of the arena wall. After each trial, the arena was cleaned with 1% Virkon solution to prevent cross-contamination.

Hanging wire test.

The mice were weighed before the test. The four-limb hanging test was based on Kondziela's inverted screen test where the mouse grasped a wire screen that was subsequently inverted¹¹². The time the mouse maintained limb tension to counteract its body weight was recorded. The chronometer was started immediately after the screen was inverted over the cage, and the duration the mouse remained suspended was noted, with a maximum limit of 300 seconds. Three hanging trials were conducted, with a least a 2-minute interval between each test. The longest duration the mouse hung from the screen was recorded for analysis.

Echocardiography

Echocardiographic assessments were conducted on all experimental mouse groups at 20 and 22 weeks after bone marrow transplantation, corresponding to the age of 7-8 months for each group. Mice were anesthetized using 0.5-1% isoflurane, and heart function was assessed using the FUJIFILM VisualSonics Vevo 2100 ultrasound system equipped with the MS 550D probe. B-mode videos and M-mode images were acquired to assess systolic and diastolic functions. Key parameters, including Ejection Fraction (EF), Fractional Shortening (FS), Left Ventricular Volume in Systole (LVVsys), and Left Ventricular Posterior Wall Systolic (LVPWS) were quantified for each animal.

Primary macrophage and fibroblast cultures

Primary macrophages were isolated from the bone marrow of 10-week-old DP (GFP/mKate2+) mice. Bone marrow cells were obtained by flushing the tibias and femurs with PBS-1X (Fisher Scientific, 10-010-023) containing 4 U/mL heparin (Sigma-Aldrich, H3149-500KU). The resulting cell suspension was filtered through a 30 µm cell strainer and washed twice with PBS. The isolated cells were then cultured in DMEM/F12 medium (Gibco, 10378-016) supplemented with 5% FBS, 1% Penicillin-Streptomycin-Glutamine (Gibco 10378-016), and 25 ng/mL M-CSF at 37°C with 5% CO₂ for 7 days. After the incubation period, the medium was replaced with fresh culture medium containing 2% FBS, 1% PS, 25 ng/mL M-CSF, and 50 ng/mL GM-CSF.

Mouse FA fibroblasts were isolated from the ear pinnae skin tissues of YG8-800 and WT mice (6-8 weeks old). The cells were cultured in high-glucose Dulbecco's modified Eagle's Medium (Fisher Scientific, 10-569-044) supplemented with 5% FBS and 1% PSG at 37°C in a 5% CO₂ incubator. Human FA fibroblasts were obtained from the Coriell Institute for Medical Research (Camden, NJ, USA) under catalog number GM04078. These cells carry FXN alleles with 541 and 420 repeats at the time of sampling.

Mitochondrial Staining and Function in Co-Culture.

Mitochondrial staining was performed using BioTracker 405 Blue Mitochondria Dye (Sigma Aldrich, SCT135) following the manufacturer's instructions. The dye was added to the respective cell culture media at a final concentration of 100 nM, and cells were incubated at 37°C and 5% CO₂ for 8 hours. After staining, excess dye was removed by washing the cells twice with 1× PBS (Fisher Scientific, 10-010-023). The washed cells were then utilized in co-culture experiments. In co-culture experiments, mouse or human fibroblasts were mixed with DP macrophages at a 1:1 ratio in their respective culture media, according to the experimental conditions. Co-cultures were maintained for 48 to 72 hours to facilitate mitochondrial transfer. To confirm the presence of CD11b+ macrophages in the mouse FA fibroblast and DP macrophage co-culture, CD11b antibody was used for flow cytometry analysis. For the human FA fibroblast and DP macrophages co-culture, CD90, a fibroblast-specific marker, was used.

To assess changes in mitochondrial respiratory function, a Seahorse Mito Stress assay (Agilent, USA) was performed according to the manufacturer's protocol. In brief, 20,000 cells were seeded in 96-well Seahorse plates after the specified co-culture experiments, and the oxygen consumption rate (OCR) was measured and normalized to cell counts by DAPI staining. The culture medium was replaced with Agilent Seahorse XF DMEM Basal Media, supplemented with 2 mM glutamine, 10 nM glucose, and 1 mM sodium pyruvate. Inhibitors, prepared in the same media, were injected during the assay at the following final concentrations: oligomycin (2 µM), FCCP (2 µM), and rotenone and antimycin A (1 µM).

Confocal microscopy of cells

For fluorescence microscopy, cells were cultured on 20 nm coverslips (Cell Treat, 229173) and fixed at room temperature for 15 minutes using 4% paraformaldehyde (Electron Microscopy Sciences). After fixation, cells were washed twice with 1× PBS for 5 minutes each. Nuclear staining was performed by incubating the cells with DAPI/Hoechst 33342 (Thermo Fisher Scientific, 62249) for 5 minutes, followed

by three washes with 1× PBS. The coverslips were mounted on glass slides, and images were acquired using a confocal laser scanning microscope (Zeiss LSM 800). Image analysis and processing were conducted using ZEN software.

Analysis of Co-cultured Cell Populations by Flow Cytometry

For flow cytometry analysis, cell suspensions were prepared at a concentration of 1–5 million cells/ml and incubated with antibodies in the dark for 30 minutes. The following antibodies were used: anti-mouse/human CD11b-APC (clone M1/70, BioLegend), anti-human CD90-APC (clone 5E10, BioLegend). Viability was assessed using eFluor™780 (Thermo Fisher Scientific, 65-0865-18). After incubation, the cells were washed and resuspended in FACS-BL buffer. Stained cells were analyzed or sorted using a BD FACS Aria II cell sorter, and all data were processed using FlowJo software. Following analysis, the sorted cells were counted and prepared for subsequent experiments.

Single-cell RNA sequencing- Sample preparation

Brain cells were isolated, stained, and FACS-sorted as described above. Briefly, brain tissues were harvested from three DP+FA mice and processed using the Adult Brain Dissociation Kit (Miltenyi Biotec, 130-107-677) to obtain single-cell suspensions. Due to the relatively low abundance of live GFP-/mKate2+ cells, which represent FA mouse brain cells that have received mitochondria, tissues from at least three DP+FA mice were pooled to obtain a sufficient number of cells for single-cell RNA sequencing following FACS sorting. This pooling approach ensured an adequate number of cells for downstream transcriptomic analysis. During the FACS analysis, dead cells were stained (Live/dead Fixable Blue Dead Cell Stain Kit; Invitrogen), and gating was performed to exclude dead cells. GFP+ cells were used to separate donor cells from recipient cells. From GFP-recipient cell population, the mKate2+ group (mitochondria-receiving FA recipient brain cells) and the mKate2- group (non-mitochondria-receiving FA recipient brain cells) were classified and sorted. All stained brain cells were acquired and sorted using a BD FACS Aria II cell sorter with BD FACSDiva software. Dissociated single cells were washed with RNase-free PBS containing 0.1% BSA and sorted into ice-cold RNase-free PBS with 0.1% BSA for the viable mKate2+ and mKate2- populations. A minimum of 100,000 cells from each sorted population (mKate2- and mKate2+ recipient FRDA cells) were pooled from the three mice brains and processed for single-cell RNA sequencing by MedGenome Inc. (Foster City, CA, USA) using the Chromium Controller and the Chromium Next GEM Single Cell 3' Reagent Kits (10x Genomics). The libraries were sequenced on a Novaseq 6000 sequencer (Illumina, San Diego, CA) with paired-end 100 base pair (bp) reads.

Single-cell RNA sequencing- Data pre-processing and cell type annotation

The Illumina raw BCL sequencing files were processed through the CellRanger software (10x Genomics) for generating FASTQ files and count matrixes (<https://www.10xgenomics.com/support/software/cell-ranger/latest/analysis/running-pipelines/cr-gex-count>). Feature-barcode matrices obtained from Cellranger count for both samples were processed using the 'Read10X()' function from Seurat package

(v5.1)¹¹³. Next, cell filtering was performed based on nFeature_RNA (> 300), nCount_RNA (> 500) and the percentage of counts from mitochondrial genes (percent mt <10). Normalization and scaling were performed and the top 2000 genes with the highest standardized variance were used to identify significant principal components (PCs). These PCs were utilized to identify clusters using Seurat's FindClusters() function which employs graph-based community method. The resulting clusters were visualized using Uniform Manifold Approximation and Projection (UMAP) Dimension Reduction method (**Figure 4**). To annotate these clusters, we first identified top differentially expressed genes per cluster using the FindAllMarkers() function and then manually annotated them using curated gene lists (**Supplementary Table 1**)

Single-cell RNA sequencing- DGE and pathway analysis

Next, differential gene expression (DEG) analysis was conducted between mKate2+ Recipient FRDA cells and mKate2- Recipient FRDA cells for each of the twelve detected cell types. We used the FindMarkers() function with MAST¹¹⁴ where genes that were expressed in at least 25% of cells in either population were considered (min.pct set to 0.25). Volcano plots were generated via the EnhancedVolcano package in R¹¹⁵. Commonly differentially expressed genes (**Supplementary Table 2**) were identified by extracting the average log2 fold change values of significant differentially expressed genes ($p < 0.05$) in each cell type. The common expression score (CES) for each gene was calculated by subtracting the number of cell types in which the gene was upregulated (average log2FC > 0) from the number of cell types for which the gene was downregulated (average log2FC < 0).

We also conducted gene set enrichment analyses (GSEA) separately for each cell type on the corresponding differentially expressed genes via FGSEA¹¹⁶ (v1.26) on all biological process gene sets from MSIGDBR (v7.5.1). Commonly upregulated and downregulated gene sets were identified by extracting the normalized enrichment score (NES) for significantly enriched gene sets ($p < 0.05$ and $q < 0.25$). The common pathway score (CPS) for each gene set was calculated by subtracting the number of cell types in which the pathway was positively enriched (NES > 0) from the number of cell types in which the pathway was negatively enriched (NES < 0). We also conducted enrichment network analysis on genes with CES > 6 and CES < -6 via Metascape¹¹⁷.

Bulk RNA sequencing

Whole heart tissue was collected from WT and YG8s800 mice at 20- and 22-weeks post-bone marrow transplantation (BMT), following conditioning with Busulfan (BU) and PLX3397. The time points corresponded to an age of 7–8 months for each group. Prior to tissue collection, echocardiography was performed to evaluate cardiac function. Following two washes with cold 1x PBS, the heart tissues were dissected in cold 1x PBS using a sterile knife to facilitate efficient RNA extraction. Total RNA was extracted from the minced heart tissue of each mouse group using the Invitrogen™ PureLink™ RNA Mini Kit (#12-183-018A), following the manufacturer's instructions for RNA purification. Library preparation and sequencing was conducted by MedGenome Inc. (Foster City, CA, USA) using the Illumina Stranded

mRNA Prep kit and Novaseq 6000 sequencer (Illumina, San Diego, CA) with paired-end 100 base pair (bp) reads.

Quality control and read alignment were performed by MedGenome. Data quality control was performed via FastQC (v0.11.9). Low-quality sequence reads were excluded, and adapter sequences were trimmed using fastq-mcf (v1.05) and cutadapt (v4.7). Removal of other unwanted sequences (mitochondrial genome sequences, ribosomal RNA, transfer RNA, adapter sequences, etc.) was performed via Bowtie2 (v2.5.3). Paired-end reads were aligned to the Ensembl Mus musculus genome (GRCm39) via STAR (2.7.11b). Raw read counts were estimated from the aligned reads using HTSeq (v2.0.5). FPKM (Fragments per kilobase per million) expression values for each gene were estimated from the aligned reads using cufflinks (v2.2.1).

Principal component analysis was performed for each of the eight samples using FPKM expression values via the prcomp base function in R¹¹⁵. FPKM expression values for selected genes were log-transformed prior to computing Z-scores for visualizing relative gene expression across samples. Hierarchical clustering and heatmaps were generated using the pheatmap function from pheatmap (v1.0.12) with default settings.

Differential gene expression analysis between DP+FA (n=3) mice and FA+FA (n=3) mice was conducted from the raw count data using DESeq2 (v1.40.2). Gene set enrichment analysis was conducted via FGSEA¹¹⁶ (v1.26) on all biological process gene sets from MSIGDBR (v7.5.1).

Statistical analyses

All the data presented in this manuscript are expressed as either the mean $\pm$ standard deviation of the mean (SD) or mean $\pm$ standard error mean (SE, for behavioral analyses, $n > 10$). The number of samples, denoted as n , refers to the individual mouse for in vivo experiments (where $n=1$ per mouse) or to the number of independent biological replicates for in vitro experiments (where one independent biological replicate corresponds to $n=1$). Statistical analyses were performed using GraphPad Prism 7 (GraphPad Software). Parametric tests were applied to data that followed a normal distribution, as assessed using the Shapiro-Wilk test. The following statistical analyses were as follows: two-tailed unpaired t-test for comparison between two groups, one-way ANOVA with Tukey post hoc or Kruskal-Wallis test with Dunn's correction for comparisons involving more than two groups, or two-way ANOVA with Tukey's or Sidak's post hoc tests for comparisons with multiple variables. The significance threshold for all parametric tests was set at $\alpha = 0.05$, with all tests being two-sided. A p-value of less than 0.05 was considered statistically significant. The specific statistical tests applied to each data set are described in the figure legends. In all figures, significance is denoted as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$. The exact p-values for each comparison are provided in the Source Data file.

Declarations

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Author Contributions

H.C. conducted the study, designed and performed the experiments, carried out the analyses and the interpretation of results, constructed the Figures, and wrote most of the manuscript. R.S. performed the bioinformatic analyses of the scRNA-seq and bulk RNA sequencing data and contributed to preparing the related Figures, Results and Methods section of the manuscript. A.K. contributed to the bioinformatic analyses of the scRNA-seq and bulk RNA sequencing data. P.C. established and characterized the mouse lines and the flow cytometry assays. S.C. performed and interpreted the echocardiography. J.J. performed and interpreted the seahorse studies. J.W. supervised and financed the echocardiography studies. N.G.-O. conceived and directed the study, provided funding, assisted with experimental design, and wrote and revised the manuscript and Figures.

Declaration of Interests

The authors declare no competing interests.

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Figures

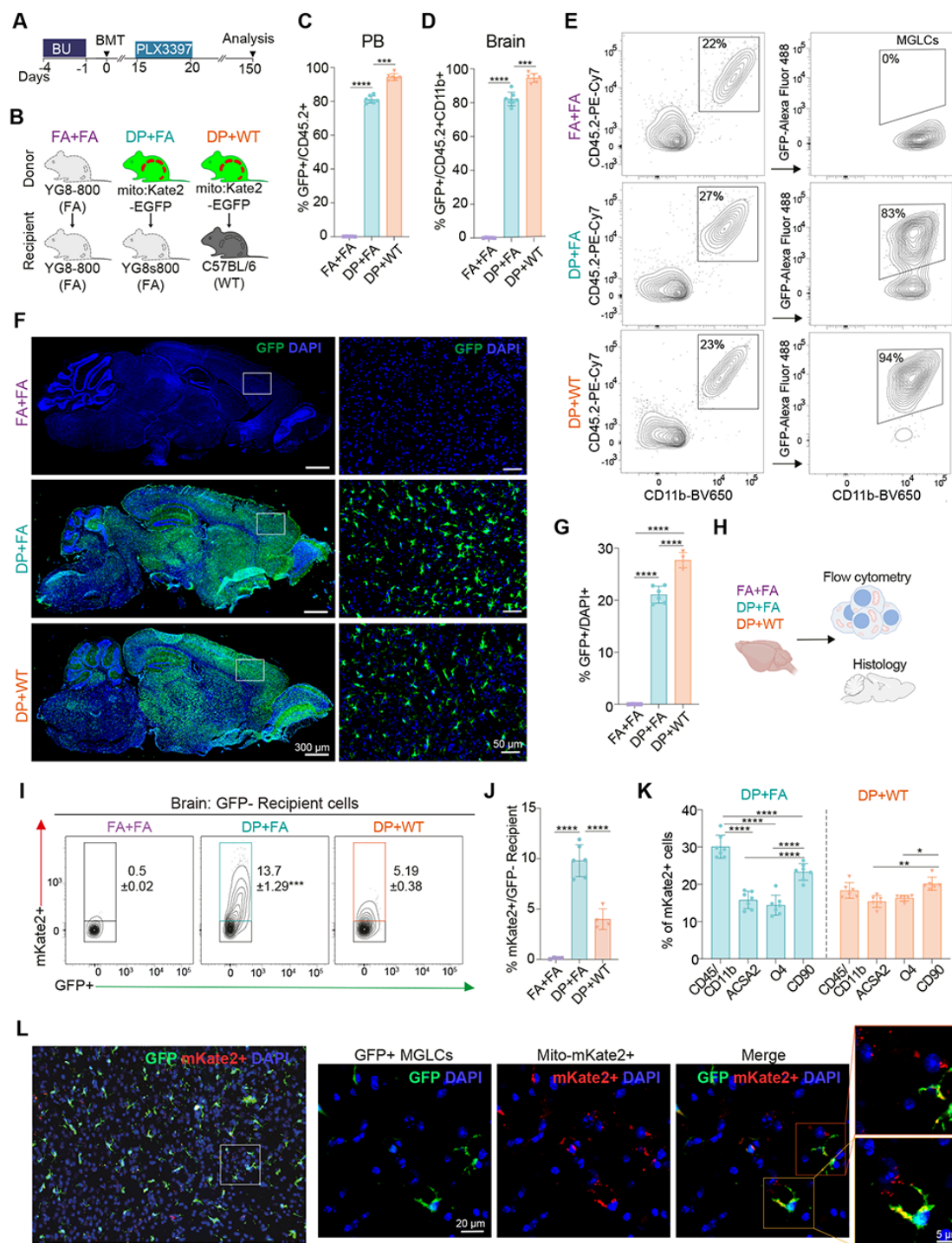


Figure 1

Highly Efficient Microglia Replacement and Mitochondria Transfer to Brain Cells in YG8-800 Mice

- (A) Schematic of microglia replacement protocol using Busulfan (BU, 100 mg/kg, 25 mg/kg/day) and CSF1Ri (PLX3397 600 mg/kg, 100 mg/kg/day) administered via oral gavage starting 15 days after BMT.
- (B) Experimental setup for the study, showing three groups: 1) FA+FA: YG8-800 mice receiving YG8-800 BMT, 2) DP+FA: YG8-800 mice receiving GFP/mKate2 or double-positive (DP) BMT, and 3) DP+WT: WT mice receiving GFP/mKate2 or double positive DP BMT.
- (C) Analysis of the GFP+ CD45.2+ cell population in peripheral blood (PB). See **Extended Data 1** for gating schemes.
- (D) Fraction of GFP+ cells in the CD45+CD11b+ cell population in the brain. See **Extended Data 2** for gating schemes.
- (E) Representative FACS plots showing the proportion of all microglia (CD45+CD11b+) and bone marrow-derived MGLCs (CD45+CD11b+GFP+) in the three groups (FA+FA, DP+FA, DP+WT).
- (F) Representative images of stable engraftment of bone marrow-derived MGLCs (GFP+) in FA+FA, DP+FA and DP+WT groups.
- (G) Quantification of GFP+ cells in the brain, normalized by the number of nuclei.
- (H) Schematic of experimental workflow to examine mitochondrial transfer in the brain.
- (I) Representative FACS plots showing live brain cells containing GFP or mKate2 signals in the FA+FA, DP+FA, and DP+WT mice.
- (J) Quantitative analysis of GFP-/mKate2+ recipient brain cells in the three conditions.
- (K) Distribution mKate2+ signal in different cell types in the brain.
- (L) Representative images showing engraftment of DP cells and mKate2+ signal transfer to FA brain cells in DP+YG8-800 mice, as observed through high-resolution imaging.

All data are presented as mean $\pm$ SEM. Statistical analysis was performed using a one-way ANOVA test with Tukey post-hoc, with significance levels indicated as * $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$, and **** $p < 0.0001$.

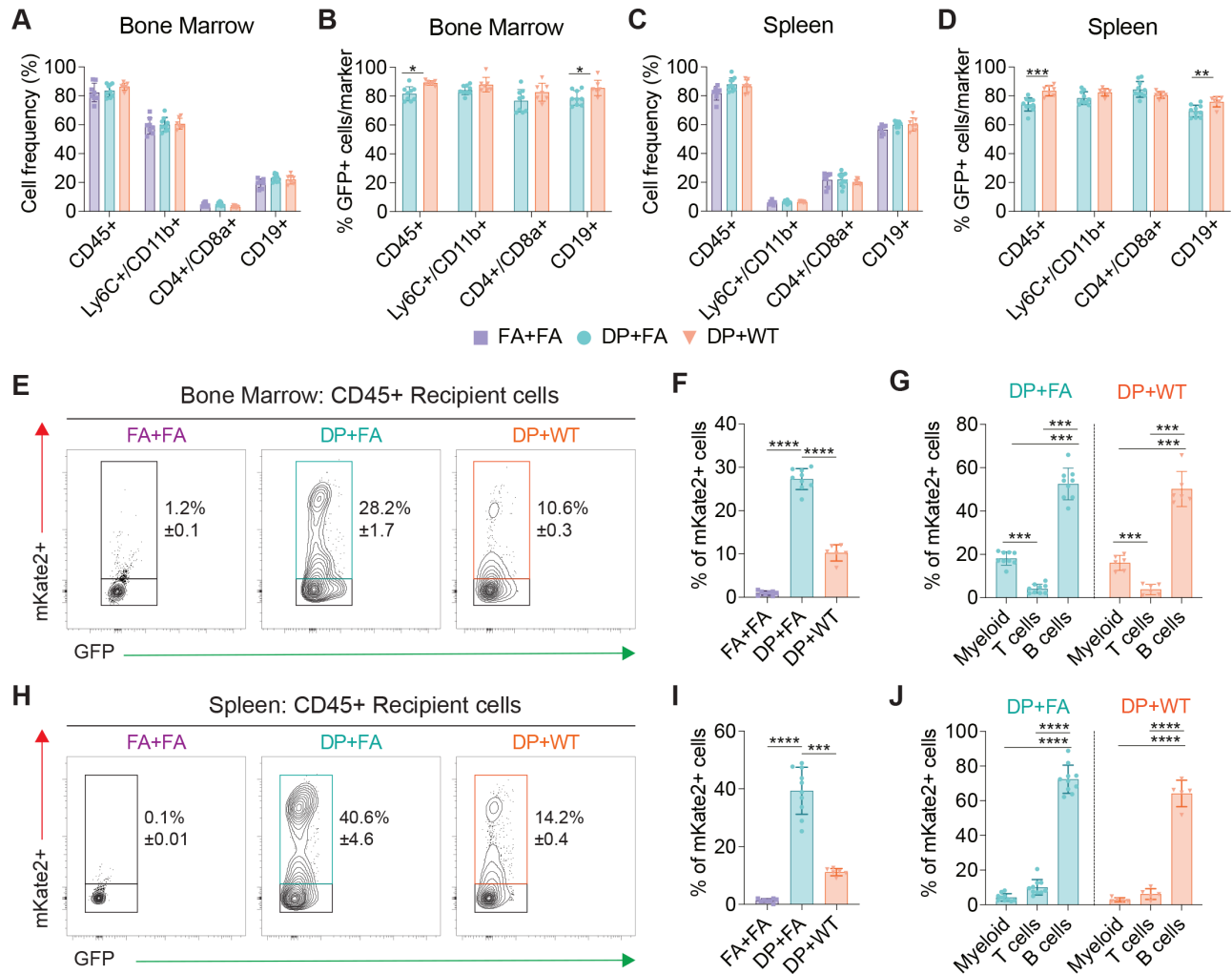


Figure 2

High Hematopoietic Reconstitution and Enhanced Mitochondria Transfer in YG8-800 Mice

Flow cytometric analysis of hematopoietic cell populations and mitochondrial transfer in FA+FA, DP+FA, and DP+WT mice five months post-transplantation. (A-D) Bar graphs display the frequency of various hematopoietic cell types in the bone marrow (A, B) and spleen (C, D) across the FA+FA (purple squares), DP+FA (blue circles), and DP+WT (orange triangles) groups.

(A) Frequency (%) of CD45+, Ly6C+/CD11b+ (myeloid cells), CD4+/CD8a+ (T cells), and CD19+ (B cells) populations in the bone marrow.

(B) Percentage of GFP+ cells expressing the same markers in the bone marrow.

(C, D) Corresponding data for the spleen.

(E-J) Quantification of mKate2+/GFP- cells, representing putative mitochondrial transfer, within the CD45+ recipient cell populations in bone marrow (E-G) and spleen (H-J).

(E) and (H) Representative flow cytometry plots for FA+FA, DP+FA, and DP+WT groups in the bone marrow and spleen, respectively.

(F, I) Percentages of mKate2+/GFP- cells in the bone marrow and spleen, respectively.

(G, J) Percentage of mKate2+/GFP- cells by cell type (myeloid, T cells, B cells) in the bone marrow and spleen, respectively.

All data are presented as mean $\pm$ SEM. Statistical significance for A-D, G and J was determined using two-way ANOVA with Sidak's multiple comparisons test. Statistical significance for F and I was determined using one-way ANOVA test with Tukey post-hoc. Significant differences are indicated by asterisks (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; and **** $p < 0.0001$).

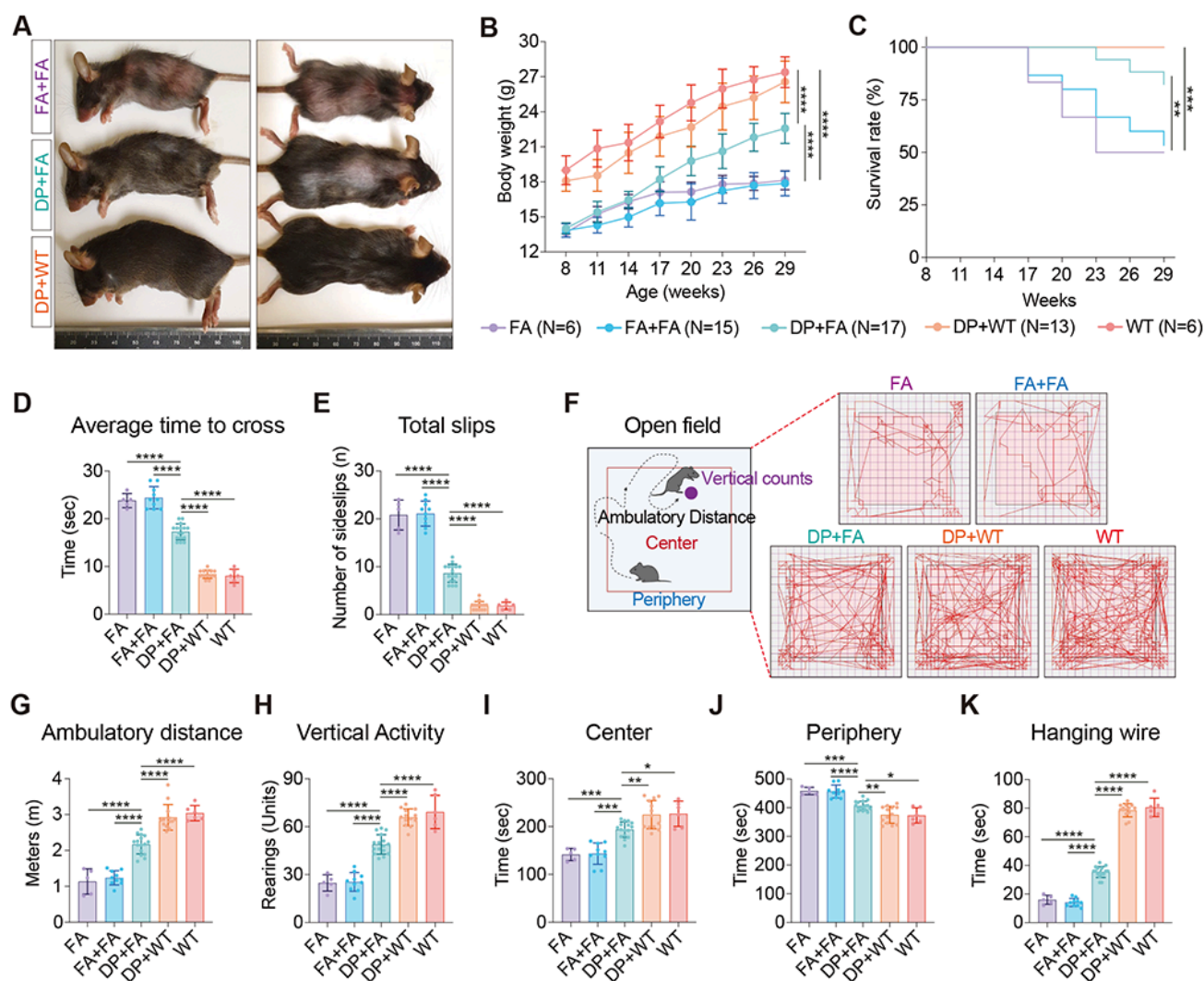


Figure 3

Microglia Replacement Improves Health and Neurobehavior in YG8-800 Mice

- (A) Representative images show the physical differences in size and coat appearance between different experimental groups. FA+FA (top, purple), DP+FA (middle, aqua), and DP+WT (bottom, orange).
- (B-K) Groups: 1) FA (purple, n=6 mice), 2) FA+FA (blue, n=10), 3) DP+FA (aqua, n=17), 4) DP+WT (red, n=13), and 5) WT (orange, n=6).
- (B) Kaplan-Meier survival curve showing the survival rate (%) over time (weeks) for the five groups.
- (C) Body weight (g) over time (weeks) for each experimental group.
- (D) Average time (seconds) to cross a beam in the motor coordination test.
- (E) Total number of slips (errors) while crossing the beam.
- (F) Schematic of the open field test and movements tracked. Representative images of ambulation paths (red lines) during the 10-minute testing period.
- (G) Total ambulatory distance (meters) in the open field test.
- (H) Vertical activity indicated by total rearing events.
- (I) Time spent in the center zone (seconds) during the open field test.
- (J) Time spent in the periphery.
- (K) Hanging wire test results show the time (seconds) mice could hold on to a wire.

All data are presented as mean $\pm$ SEM. Statistical analysis for B was two-way ANOVA with Sidak's multiple comparisons test; for C Log-rank Mantel–Cox survival followed by Bonferroni correction; for D-K one-way ANOVA test with Tukey post-hoc. Significant differences are indicated by asterisks (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; and **** $p < 0.0001$). For D-K only comparisons with respect to DP+FA are shown in the graph.

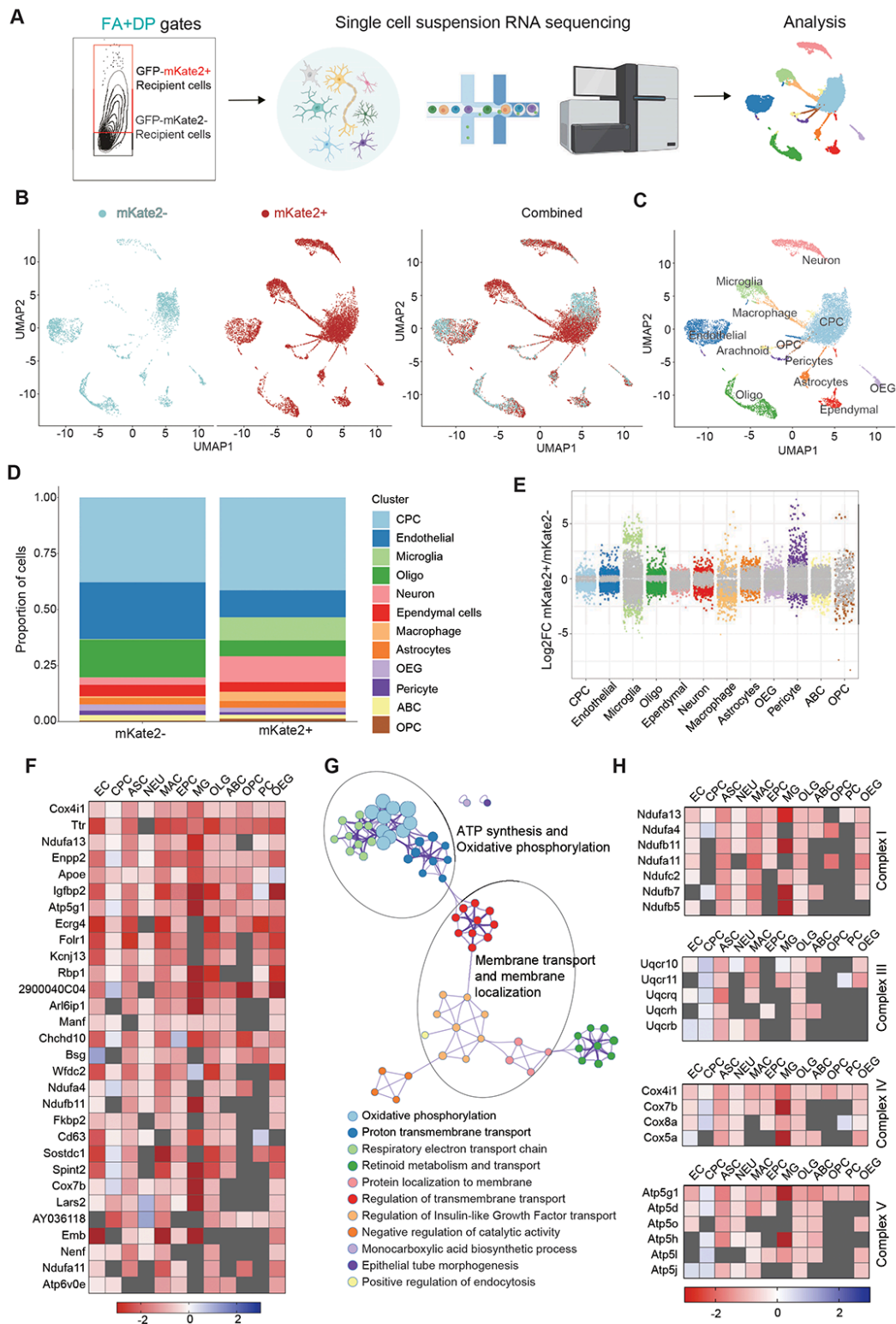


Figure 4

Single-Cell RNA-Seq Analysis Reveals Enhanced ATP Synthesis and Oxidative Phosphorylation After Mitochondrial Uptake.

(A) Workflow for Single Cell RNA Sequencing Sample Preparation to analyze transcriptomic alterations in DP+FA Mouse Brain Cells after mitochondrial transfer.

(B) Uniform Manifold Approximation and Projection (UMAP) visualization of 3,196 GFP-/mKate2- and 9,151 GFP+/mKate2+ recipient cells from the three DP+FA mouse brains.

(C) UMAP visualization of 12 major cell populations, including choroid plexus epithelial cells (CPC), endothelial cells (EC), oligodendrocytes (OLG), microglia (MG), neurons (NEU), ependymal cells (EPC), astrocytes (ASC), macrophages (MAC), olfactory ensheathing glia (OEG), pericytes (PC), arachnoid barrier cells (ABC), and oligodendrocyte precursor cells (OPC).

(D) Distribution of CNS cell types in mKate2+ and mKate2- populations.

(E) Differentially expressed genes detected between mKate2+ and mKate2- cells in 12 cell types ($p < 0.05$ & $FC > 20\%$, detected via MAST)

(F) Heatmap of log2FC depicting genes that are significantly upregulated in mKate2+ compared to mKate2- ($p < 0.05$) with a common expression score > 6 (at least six cell types). Each row represents a gene, and each column represents a different cell type. The color scale indicates fold change, with red shades representing over-expression, blue shades under-expression, and dark gray non-significant data.

(G) Network visualization of enriched biological pathways and processes among genes with a high Common Expression Score ($CES \geq 6$). Nodes represent individual pathways, colored according to functional categories, and the size of each node corresponds to the significance of enrichment ($\log_{10}(P)$). Clusters are formed based on functional similarity.

(H) Heatmap of log2FC of mitochondrial complex subunit expression across CNS Cell Types. Expression levels of various mitochondrial complex subunits (Complex I, III, IV, and V) across different CNS cell types. Each row represents a subunit from one of the mitochondrial complexes, and each column represents a different cell type.

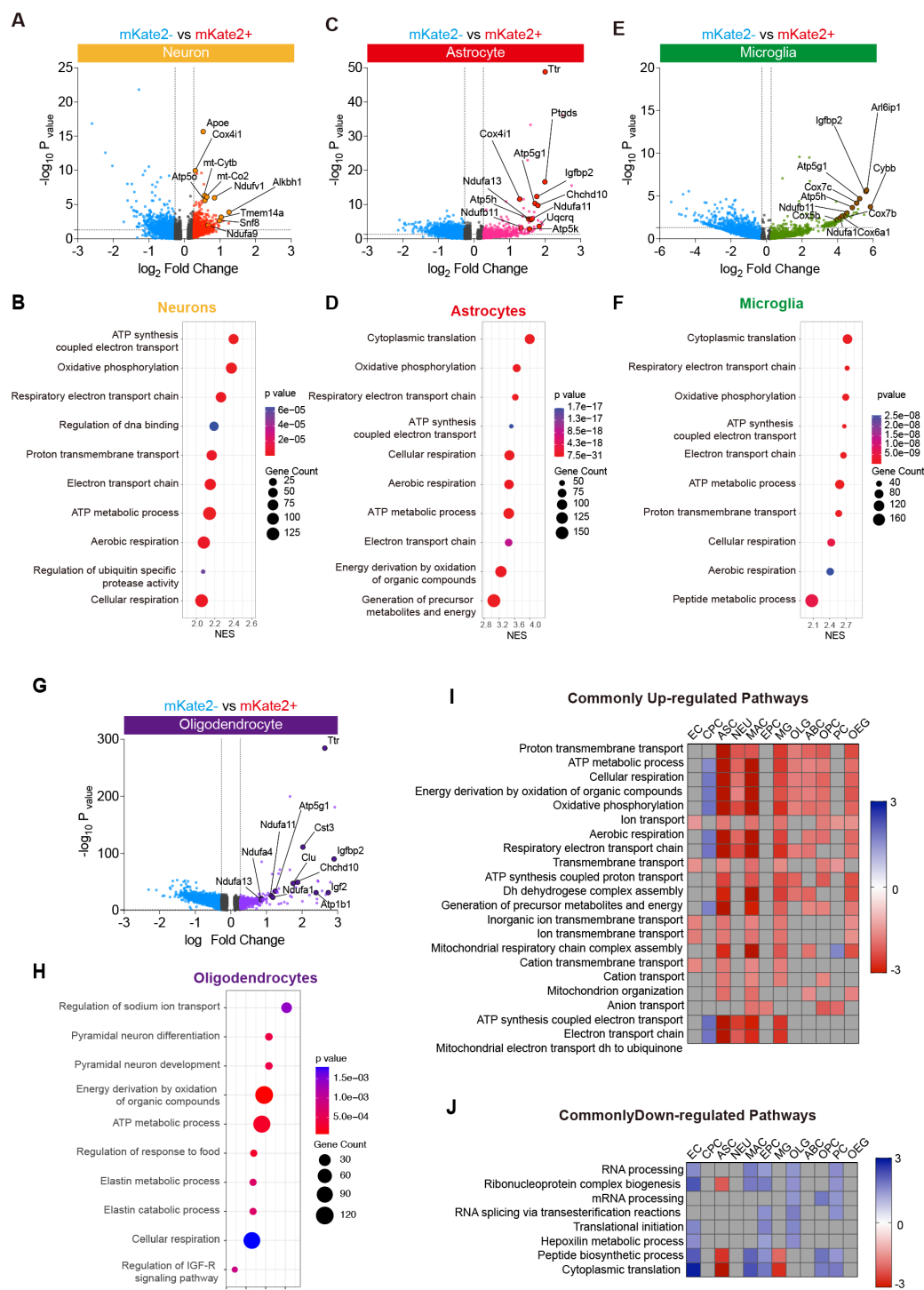


Figure 5

Neurons, Astrocytes, Microglia, and Oligodendrocytes Increase Transcription of Energy Production Genes Following Mitochondrial Uptake

(A) Volcano plot for neurons showing significantly upregulated (red dots) and downregulated (blue dots) genes, with a cutoff of FC ± 0.2 and $P < 0.05$, in mKate2+ cells compared mKate2- cells. Dots highlighted

in orange represent genes related to ATP synthesis, oxidative phosphorylation, and mitochondrial DNA, while gray dots represent genes with no significant change.

(B) Bubble plot of gene set enrichment analysis of the differentially expressed genes between mKate2⁺ and mKate2⁻ neurons. Bubble size reflects the gene count per pathway, color represents the p-values range from blue (less significant) to red (highly significant). Pathways are ordered by the normalized enrichment score (NES) displayed on the X-axis.

(C) Volcano plot for Astrocytes showing significantly upregulated (pink dots) and downregulated (blue dots) genes. Dots highlighted in dark red represent genes related to ATP synthesis, oxidative phosphorylation, and neuroprotection and cellular recovery.

(D) Bubble plot of gene set enrichment analysis of the differentially expressed genes between mKate2⁺ and mKate2⁻ astrocytes.

(E) Volcano plot for Microglia showing significantly upregulated (green dots) and downregulated (blue dots) genes. Dots highlighted in brown represent genes related to ATP synthesis, oxidative phosphorylation, cell survival, and inflammatory responses.

(F) Bubble plot of gene set enrichment analysis of the differentially expressed genes between mKate2⁺ and mKate2⁻ microglia

(G) Volcano plot for oligodendrocytes showing significantly upregulated (green dots) and downregulated (blue dots) genes. Dots highlighted in brown represent genes related to ATP synthesis, oxidative phosphorylation, cell survival, and inflammatory responses.

(H) Bubble plot of gene set enrichment analysis of the differentially expressed genes between mKate2⁺ and mKate2⁻ oligodendrocytes.

(I) Heatmap of NES displaying commonly up-regulated pathways ($p < 0.05$ and $q < 0.25$) across different CNS cell types, with common pathway score (CPS) values between 3 and 8.

(J) Heatmap of NES displaying commonly down-regulated pathways ($p < 0.05$ and $q < 0.25$) across different CNS cell types, with common pathway score (CPS) values between -3 and -5.

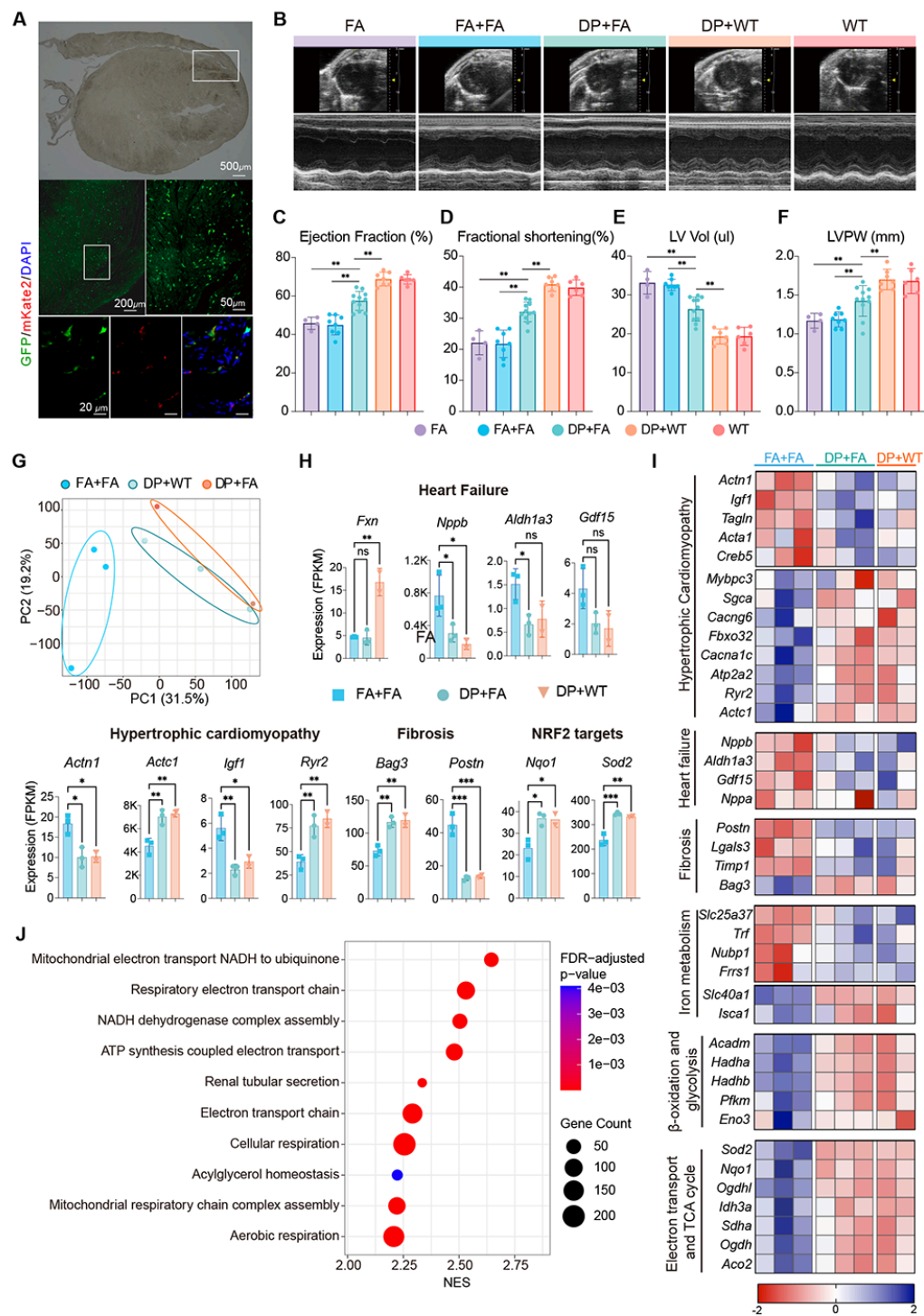


Figure 6

Macrophage replacement restores cardiac function in YG8-800 mouse hearts

(A) Representative histological images of YG8-800 mouse hearts displaying robust engraftment of donor-derived macrophages. GFP+/mKate2+ cells (green) are distributed throughout the heart, with mKate2 signals (red) also observed without GFP co-localization. Nuclei are stained with DAPI (blue).

Insets show magnified areas of interest. Scale bars: top panel = 500 μm , middle panel left = 200 μm , middle panel right = 50 μm , bottom panels = 20 μm .

(B) Representative short-axis M-mode echocardiographic images illustrating left ventricular structure and function across experimental groups: FA (unmanipulated control), FA+FA (sham control), DP+FA (treated group), DP+WT (healthy transplant control), and WT (unmanipulated healthy control).

(C-F) Quantitative analyses of echocardiographic parameters, including left ventricular ejection fraction (LVEF) (C), fractional shortening (FS) (D), left ventricular end-diastolic volume (LV-vol-s) (E), and left ventricular posterior wall thickness at end-diastole (LVPWd) (F). Data are shown as mean $\pm$ SD. Statistical analysis was performed using two-way ANOVA with Tukey's multiple comparisons test (**p < 0.01).

(G) Principal Component Analysis (PCA) of heart transcriptomic profiles comparing FA+FA, DP+FA, and DP+WT groups. DP+WT samples cluster distinctly from FA+FA, reflecting differential gene expression, while DP+FA samples cluster closer to DP+WT, indicating partial normalization of gene expression following treatment. PC1 and PC2 account for 31.5% and 19.2% of the variance, respectively.

(H) Bar plots of gene expression (FPKM) for markers associated with heart failure (*Aldh1a3*, *Gdf15*, *Nppb*), hypertrophic cardiomyopathy (*Actn1*, *Ryr2*, *Igf1*, *Actc1*), fibrosis (*Postn*, *Bag3*), and NRF2 targets (*Nqo1*, *Sod2*). Data are presented as mean $\pm$ SD. Statistical analysis was performed using one-way ANOVA with Sidak's multiple comparisons test compared to FA+FA (*p < 0.05; **p < 0.01; ***p < 0.001).

(I) Heatmap of Z-scores of FPKM expression for 39 out of 52 marker genes associated with cardiac phenotypes in FA murine and cellular models (For a list and analysis of all 52 genes see **Supplementary Table 6 and Extended Data 10**). Each row represents a gene, and each column represents a mouse sample. The color scale indicates relative expression levels (red = up and blue = down).

(J) Bubble plot of functional enrichment analysis of gene expression data. Bubble size reflects the gene count per pathway, color represents the FDR-adjusted p-values range from blue (less significant) to red (highly significant). Pathways are ordered by the normalized enrichment score (NES) displayed on the X-axis.

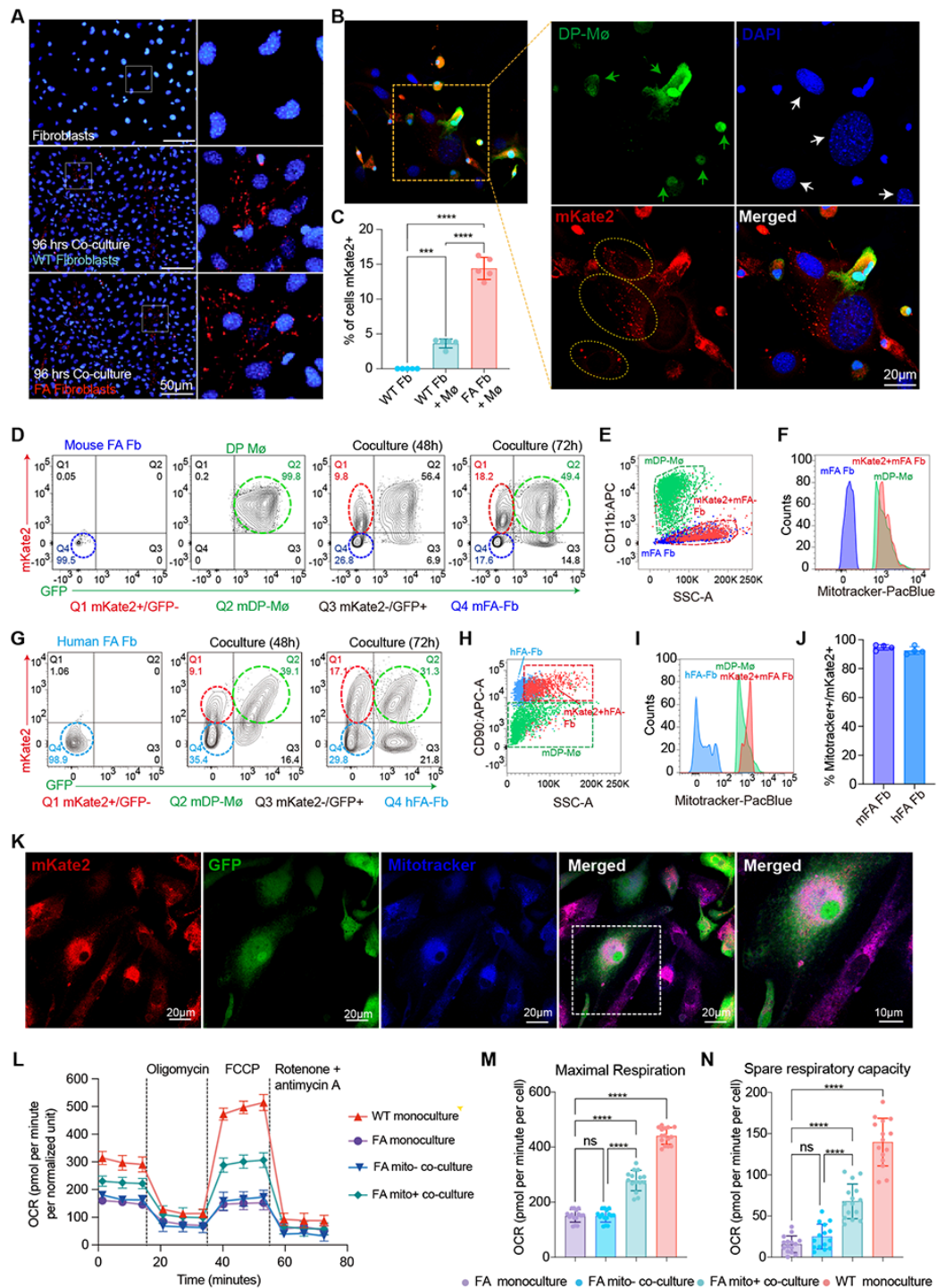


Figure 7

Intercellular Mitochondrial Transfer Restores Metabolic Function in FA Cells

(A) Representative images of FA fibroblasts after co-culture with wild-type GFP+/mKate2+ cells macrophages (which have been removed). Nuclei are stained with DAPI (blue) and red represent mKate2 signal from mitochondria. Insets show magnified areas of interest. Scale bar: 50 μ m.

(B) Representative images of double-positive macrophages (DP-Mø, green arrows) showing mitochondrial transfer in FA fibroblasts (Fb) co-cultured with wild-type macrophages (WT Mø). Images include GFP (green), mKate2 (red), DAPI-stained nuclei (blue), and merged channels. The enlarged area (dashed yellow box) highlights mKate2+ mitochondria in recipient FA fibroblasts, indicated by white arrows. Scale bar = 20 µm.

(C) Quantification of mKate2+ cells in the WT and FA fibroblast (Fb) co-culture with DP macrophages. Scale bar = 20 µm.

(D) Representative plots of flow cytometry analysis of co-culture experiments between mouse FA Fb (mFA fb) and double-positive macrophages (mDP-Mø). Gating strategies identify mKate2+/GFP- (Q1), mKate2+/GFP+ (Q2), mKate2+/GFP- (Q3), and mKate2-/GFP- populations.

(E) Flow cytometry plot showing CD11b (Mø marker) vs. SSC of Mø, mKate2+ mFA Fb and all mFA Fb.

(F) The histogram of different cell populations using Mitotracker-PacBlue fluorescence. Unlabeled mFA Fb (blue), mKate2+ mFA fibroblasts (red), and double-positive macrophages (mDP-Mø, green).

(G) Representative plots of flow cytometry analysis of co-culture experiments between human FA Fb (hFA fb) and double-positive macrophages (mDP-Mø).

(H) Flow cytometry plot showing CD90 (human Fb marker) vs. SSC of Mø, mKate2+ hFA Fb, and all hFA Fb.

(I) The histogram of different cell populations using Mitotracker-PacBlue fluorescence. Unlabeled hFA Fb (light blue), mKate2+ hFA fibroblasts (red), and double-positive macrophages (mDP-Mø, green).

(J) Fraction of total mKate2+ events that are both mitotracker and mKate2+ in mouse and human FA Fb.

(K) Representative images of co-cultured Mitotracker-loaded double-positive macrophages (DP-Mø) with human Friedreich's ataxia fibroblasts. The images show mKate2 (red), GFP (green), Mitotracker (blue), and the merged channels. The enlarged region (dashed white box) highlights a GFP-negative fibroblast that is positive for mKate2 and Mitotracker. See also **Extended Data 11**

(L) Oxygen consumption rate (OCR) over time in WT monoculture (red), FA mito+ co-culture (cyan), FA mito- co-culture (purple), and FA monoculture (blue). Treatments with oligomycin, FCCP, and rotenone + antimycin A are indicated.

(M) Quantification of maximal respiration in all four conditions.

(N) Spare respiratory capacity in all four conditions.

All data are presented as mean ± SD. Statistical analysis for C, M and N was one-way ANOVA with Sidak's multiple comparisons test. Significant differences are indicated by asterisks (**p < 0.01; ***p < 0.001; and

*** $p < 0.0001$).

Supplementary Files

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