

Mitigating Ischemic Injury of Stem Cell-Derived Insulin-Producing Cells after Transplant

Gaetano Faleo,^{1,4} Holger A. Russ,^{2,3,4} Steven Wisel,¹ Audrey V. Parent,² Vinh Nguyen,¹ Gopika G. Nair,² Jonathan E. Freise,¹ Karina E. Villanueva,² Gregory L. Szot,¹ Matthias Hebrok,² and Qizhi Tang^{1,2,*}

¹Department of Surgery, University of California San Francisco, San Francisco, CA 94143, USA

²UCSF Diabetes Center, University of California San Francisco, San Francisco, CA 94143, USA

³Barbara Davis Center for Diabetes, School of Medicine, University of Colorado Denver, Aurora, CO 80045, USA

⁴Co-first author

*Correspondence: qizhi.tang@ucsf.edu

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SUMMARY

The advent of large-scale *in vitro* differentiation of human stem cell-derived insulin-producing cells (SCIPC) has brought us closer to treating diabetes using stem cell technology. However, decades of experiences from islet transplantation show that ischemia-induced islet cell death after transplant severely limits the efficacy of the therapy. It is unclear to what extent human SCIPC are susceptible to ischemia. In this study, we show that more than half of SCIPC die shortly after transplantation. Nutrient deprivation and hypoxia acted synergistically to kill SCIPC *in vitro*. Amino acid supplementation rescued SCIPC from nutrient deprivation, likely by providing cellular energy. Generating SCIPC under physiological oxygen tension of 5% conferred hypoxia resistance without affecting their differentiation or function. A two-pronged strategy of physiological oxygen acclimatization during differentiation and amino acid supplementation during transplantation significantly improved SCIPC survival after transplant.

INTRODUCTION

Islet transplantation can be a minimally invasive curative therapy for type 1 diabetes (T1D) (Barton et al., 2012). Although rates of insulin independence after islet transplantation have been steadily improving, wide application of this therapy is limited by the scarcity of eligible deceased donors and the frequent need of more than one donor per recipient (Moassesfar et al., 2016; Wisel et al., 2016). Because islets are detached from the vasculature during isolation, return of blood supply to transplanted islets is delayed relative to whole organ transplants, resulting in prolonged ischemia and reduced graft viability that hinders the efficacy of the therapy.

The high metabolic demand and reliance on aerobic metabolism make β cells highly sensitive to proper oxygenation. Oxygen tension in the native pancreas is estimated to be 5% O₂ (Carlsson et al., 1998), but drops below 1% O₂ in islet grafts shortly after transplant (Carlsson et al., 2001). Hypoxia induces ER stress (Bensellam et al., 2016) and production of reactive oxygen species in β cells (Clanton, 2007), both of which can lead to β cell death. β cells that survive hypoxia switch from aerobic glucose metabolism to anaerobic glycolysis that impairs β cell function (Cantley et al., 2010, 2013; Puri et al., 2013). Many strategies are being evaluated to minimize hypoxia-induced β cell death and metabolic reprogramming, including oxygen supplementation, hypoxia preconditioning, promoting neovascularization, and cytoprotection using antioxidants and pro-survival genes (Barkai et al., 2013; Ema-

maullee et al., 2005a, 2005b; Faleo et al., 2012; Grey et al., 2003; Hogan et al., 2012; Pedraza et al., 2012; Plesner et al., 2005; Yin et al., 2006). However, ischemia also results in nutrient deprivation that can affect cell viability and function. The extent to which nutrient deprivation affects β cell survival, alone or in combination with hypoxia, is unknown.

Recent advances in the generation of functional human stem cell-derived insulin-producing cells (SCIPC) have raised the possibility of providing a renewable source of β cells for transplantation, overcoming the shortage of human donors (Pagliuca et al., 2014; Rezanian et al., 2014; Russ et al., 2015; Yoshihara et al., 2016). Currently a clinical trial is ongoing to evaluate the safety and efficacy of human embryonic stem cell-derived pancreatic progenitor cells (NCT02239354). However, how human SCIPC respond to hypoxia and nutrient deprivation has not been investigated. SCIPC are generated in artificial culture media under atmospheric 21% oxygen and are relatively immature when compared with islets isolated from pancreata. Thus, their requirements for nutrients and oxygen may be distinct from those for mature islets. In addition, SCIPC produced using current protocols, while being glucose responsive, are not fully mature β cells, and exposure to ischemia may alter their differentiation state. In this trial, pancreatic progenitor cells are encapsulated to provide an immune-protective barrier and prevent the escape of potentially undifferentiated cells. While encapsulation provides a much-needed safety assurance in early clinical trials using stem cell-derived cellular products, it further

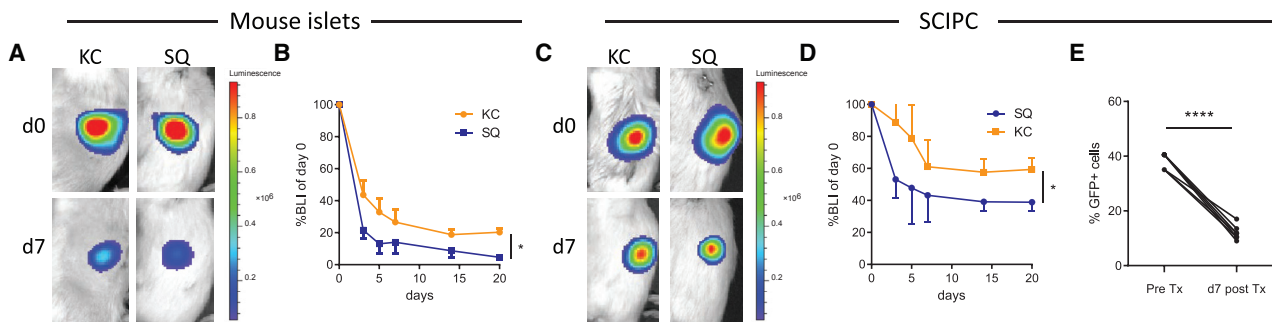


Figure 1. Survival of Mature Mouse Islets and Human SCIPC after Transplant

(A) Representative bioluminescence images of B6.MIP.Luc islets transplanted into non-diabetic B6 albino mice under the kidney capsule (KC, $n = 10$) or subcutaneously (SQ, $n = 3$).

(B) Bioluminescent intensity (BLI) of islet grafts over time is shown as percentage of day 0. The data are a compilation of results from three independent experiments.

(C) Representative bioluminescence images of SCIPC.LUC transplanted into non-diabetic NSG mice under KC ($n = 8$) or SQ ($n = 10$).

(D) BLI of SCIPC grafts over time are shown as percentage of day 0. The data are a compilation of results from five independent experiments. For (B) and (D), a Wilcoxon signed-rank test is used to determine the statistical significance between each post-transplant data point with a theoretical value of 100%. A t test is used to compare KC and SQ grafts at each time point.

(E) SCIPC grafts were harvested 7 days after transplant, and percentage of GFP⁺ human cells were quantified using flow cytometry. Data shown are a summary of three independent experiments ($n = 10$). A paired t test was used to compare the two groups.

For (B), (D), and (E), data are expressed as mean \pm SEM. * $p < 0.05$; **** $p < 0.0001$.

exacerbates ischemia and graft failure (Weir, 2013). Thus, there is a pressing need to determine SCIPC response to ischemia to better realize the therapeutic potential of stem cell technology. In this study, we compared the effects of hypoxia and nutrient deprivation on human SCIPC and mature islets. Importantly, we offer strategies to minimize damage by these two insults to optimize engraftment of SCIPC.

RESULTS

Attrition of SCIPC and Mature Islet Grafts after Transplant

To enable quantitative measurement of SCIPC survival after transplant, we generated a human embryonic stem cell line that expresses a firefly luciferase gene under the control of the constitutive endogenous AAVS1 loci using TALEN technology. For mature islets, we used islets isolated from mice that express a firefly luciferase transgene under the control of the endogenous mouse insulin promoter (B6.MIP-Luc) (Park and Bell, 2009). We transplanted luciferase-expressing SCIPC clusters into NSG mice and B6.MIP-Luc islets into B6 albino mice under the kidney capsules (KC) or in the subcutaneous (SQ) space. We monitored graft survival over time using bioluminescent imaging. Graft mass of both SCIPC and mouse islets dropped sharply within the first 3 days and stabilized by day 7 after transplant (Figure 1). SCIPC grafts survived better than mature islets, and the highly vascularized KC site afforded

better graft survival when compared with the SQ site. Nonetheless, approximately 50% of SCIPC graft mass was lost within 1 week after transplant.

SCIPC clusters consist of a mix of β cells and pancreatic progenitor cells at different differentiation stages (Russ et al., 2015). To specifically monitor the fate of insulin-producing β cells, we use the Mel1^{INS-GFP/WT} cell line (Micallef et al., 2012) that has a GFP transgene knocked into one allele of the insulin locus, thus specifically marking insulin-producing cells with GFP. We generated SCIPC^{INS-GFP} and determined the percentages of GFP⁺ β cells before and after transplantation under the KC, a site that showed 60% overall SCIPC survival. The fraction of cells expressing GFP among viable cells significantly decreased by 67% (Figure 1E). Thus, although SCIPC as a whole population are more resistant than mature islets to post-transplant graft loss, the more differentiated insulin-producing cells are selectively lost when compared with pancreatic progenitors within the heterogeneous SCIPC population.

Impact of Nutrient Deprivation on Viability and Function of SCIPC and Islets

Ischemia leads to both hypoxia and nutrient deprivation. It has been well documented that hypoxia is detrimental to mouse (Bensellam et al., 2016) and human islets (Moritz et al., 2002). However, the effect of nutrient deprivation on islets is less clear. To study the impact of nutrient deprivation without the confounding contribution of hypoxia and to provide a system for mechanistic studies and



screening for interventions, we developed an *in vitro* assay of nutrient deprivation for SCIPC and islets. We created an *in vitro* nutrient-limiting environment by increasing the density of SCIPC or islets cultured in complete medium while keeping oxygen levels constant (Figure S1). We cultured mouse islets expressing GFP under the mouse insulin promoter at various densities and monitored GFP fluorescence over time using time-lapse video (Figures S2A and S2B). The islet-associated GFP signal dropped sharply within 6 hr under high density, particularly in the center of islets (Figures S2A and S2C).

These results are consistent with the interpretation that islets cultured under high density suffer from nutrient deprivation. A hallmark of cellular nutrient deprivation is the activation of autophagy (Fujimoto et al., 2009), which can be detected by the formation of autophagosomes. We thus examined autophagosome formation by using islets from mice expressing an autophagosome reporter transgene, light chain 3-GFP fusion protein (Martino et al., 2012). Microscopic imaging and flow cytometry revealed a significant increase of GFP^{bright} autophagosomes in cells cultured at high density (Figures S3A and S3B), consistent with cells undergoing nutrient deprivation.

To quantitate cell death and allow analysis of non-transgenic cells, we stained islets and SCIPC clusters with the viability dye propidium iodide (PI) and determined the percentage of PI⁺ dead cells using flow cytometry. SCIPC, human islets, and mouse islets displayed a density-dependent increase in cell death after 6 hr of incubation (Figure 2A). However, SCIPC were more resilient to increases in cell density, as a 3-fold higher density was necessary to induce similar levels of cell death seen with mature human or mouse islets.

High-density culture may kill cells in ways other than depletion of nutrients, such as localized hypoxia and or accumulation of metabolic waste. Therefore, we simulated nutrient deprivation alternatively by diluting culture media with PBS while keeping the culture density constant. SCIPC and mouse islets showed a significant increase in cell death with media dilution (Figure 2B) comparable with high-density cultures. Lastly, we assessed islet function after exposure to high-density conditions by measuring *in vitro* glucose-stimulated insulin secretion (GSIS). Mouse islets maintained in low-density conditions had a stimulation index of more than 20, whereas islets cultured at high density showed no increase (Figures 2C and 2D). Together, these results show that short exposure to nutrient deprivation can independently kill mature islets and SCIPC without overt hypoxia.

Impact of Hypoxia on SCIPC and Islet Viability

To assess the effect of hypoxia on cell survival *in vitro*, we cultured SCIPC, human islets, and mouse islets at 1%

oxygen to simulate hypoxic conditions they may encounter post transplant. More than 50% of human and mouse islets died, whereas SCIPC showed less than 20% death (Figure 2E).

We then compared the rate of cell death when SCIPC, human islets, and mouse islets were challenged with hypoxia and nutrient deprivation individually or in combination. While human and mouse islets were vulnerable to either insult alone, and exposure to both resulted in an additive effect resulting in >90% cell death (Figure 2F), SCIPC were more resistant to individual challenges, but the combination of both acted synergistically, killing most SCIPC (Figure 2F). Together, these results suggest that SCIPC are more resilient than mature islets when exposed to individual components of ischemia, but are similarly vulnerable to their combined actions. Thus, optimal therapeutic outcomes using SCIPC will require strategies to mitigate the impacts of nutrient deprivation and hypoxia.

Mitigating Nutrient Deprivation-Induced Cell Death *In Vitro*

We next set out to identify the limiting nutrients in high-density cultures by adding individual nutrients. Addition of glucose to high-density SCIPC and mouse islet cultures had no effect on cell survival (Figure 3A). Next, we supplemented cultures with amino acids. We selected alanine and glutamine because they are the most abundant amino acids in the interstitial fluid (Curi et al., 2005); cysteine for its cytoprotective effects on islets (Rasilainen et al., 2002), and leucine, tryptophan, and methionine for their ability to inhibit starvation-induced autophagy in hepatocytes (Kanazawa et al., 2004). Supplementation of alanine and tryptophan greatly enhanced mouse islet survival in high-density cultures, glutamine, cysteine, and leucine offered moderate protection, whereas methionine had no effect (Figure 3B). Similarly, alanine and glutamine supplementation preserved SCIPC and human islet survival under high-density conditions (Figure 3C). These results show that addition of single amino acids can efficiently protect islets and SCIPC from nutrient deprivation.

Mechanisms of Amino Acid Protection against Nutrient Deprivation

Amino acids have a multitude of cellular effects including serving as precursors of protein synthesis, regulating gene expression, providing energy, and stimulating insulin secretion in β cells (Cunningham et al., 2005; Curi et al., 2005; Dixon et al., 2003; Eto et al., 1999). Amino acids activate protein synthesis via mammalian target of rapamycin (mTOR) (Gulati et al., 2008), which is inhibited under nutrient-limiting conditions (Yu et al., 2010). Thus, we investigated the role of mTOR in amino acid-mediated protection of SCIPC and islets against nutrient deprivation. We

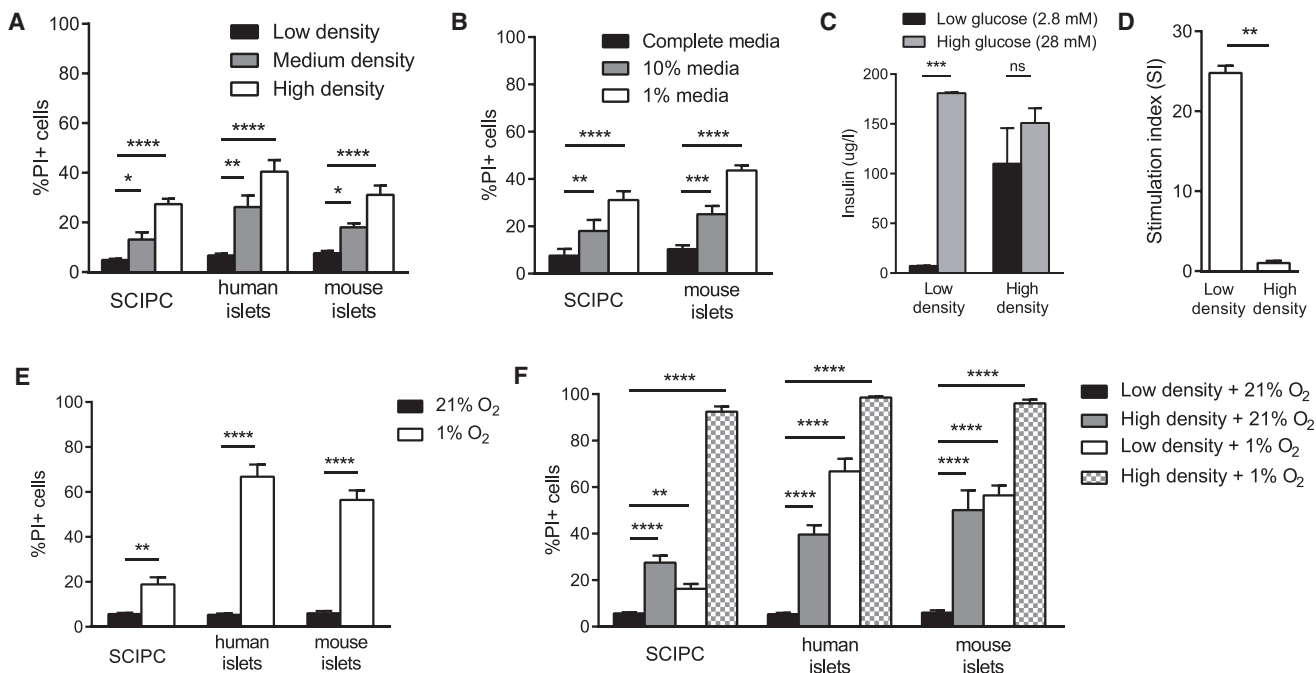


Figure 2. Impact of Hypoxia and Nutrient Deprivation on Islets and SCIPC Cell Death

(A) SCIPC, human islets, and mouse islets were cultured at different densities for 6 hr as described in [Experimental Procedures](#). Percentages of PI⁺ dead cells were quantified using flow cytometry. Data are a compilation of results from five independent experiments (SCIPC: n = 12, 10, 6; human islets: n = 6 at each density; mouse islets: n = 9 at each density).

(B) SCIPC and mouse islets were cultured in complete or diluted RPMI medium for 6 hr. Cell viability was quantified as described in (A). Data are a compilation of results from four independent experiments (SCIPC: n = 9 per condition; mouse islets: n = 4 per condition). For (A) and (B), statistical significance of the differences among each cell type at different densities is determined using one-way ANOVA with Holm-Sidak multiple comparisons.

(C) GSIS was measured using mouse islets after 6-hr low-density and high-density cultures. Data are a compilation of results from three independent experiments with paired low- and high-density cultured islets (n = 3 per group). Statistical difference was calculated using two-way ANOVA with Sidak’s multiple comparisons test.

(D) Stimulation index of mouse islets shown in (C). A two-tailed paired t test was used to determine statistical significance of the difference (p = 0.0020).

(E) SCIPC, human islets, and mouse islets were cultured for 24 hr in the presence of 21% or 1% oxygen. At the end of the experiment cell viability was measured as described in (A). Data are a compilation of results from three independent experiments (n = 6–7 per condition for each cell type). Statistical significance of the difference between 21% and 1% oxygen for each cell type was calculated using an unpaired t test with Welch’s correction.

(F) SCIPC, human islets, and mouse islets cultured for 24 hr at various densities with 1% or 21% oxygen. At the end of the experiment cell viability was measured as described in (A). Data are a compilation of results from three independent experiments (n = 6 per condition for each cell type). Statistical difference was calculated using one-way ANOVA with Holm-Sidak multiple comparisons.

All data are expressed as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001; ns, not significant.

found a significantly lower percentage of SCIPC and islet cells with phosphorylated S6 (pS6), a downstream target of mTOR, under nutrient-limiting conditions (Figure 4A), correlating with reduced cell survival (Figure 4B). Alanine supplementation restored pS6 levels in SCIPC and mouse islets cultured at high density and prevented cell death. Addition of rapamycin, an inhibitor of mTOR, reduced pS6 below the levels seen in high-density cultures with or without alanine supplementation, but did not abrogate the protective effect of alanine on SCIPC and islet survival.

Moreover, rapamycin alone promoted SCIPC and mouse islet survival in high-density cultures (Figure 4B). These results show that protection by alanine is independent of mTOR- and pS6-regulated cellular processes in SCIPC and mouse islets.

In addition to serving as cellular building blocks, amino acids can help to sustain cellular metabolism via anaplerosis, serving as substrates for replenishing tricarboxylic acid (TCA) cycle intermediates. For example, alanine, tryptophan, and cysteine can be converted into pyruvate and

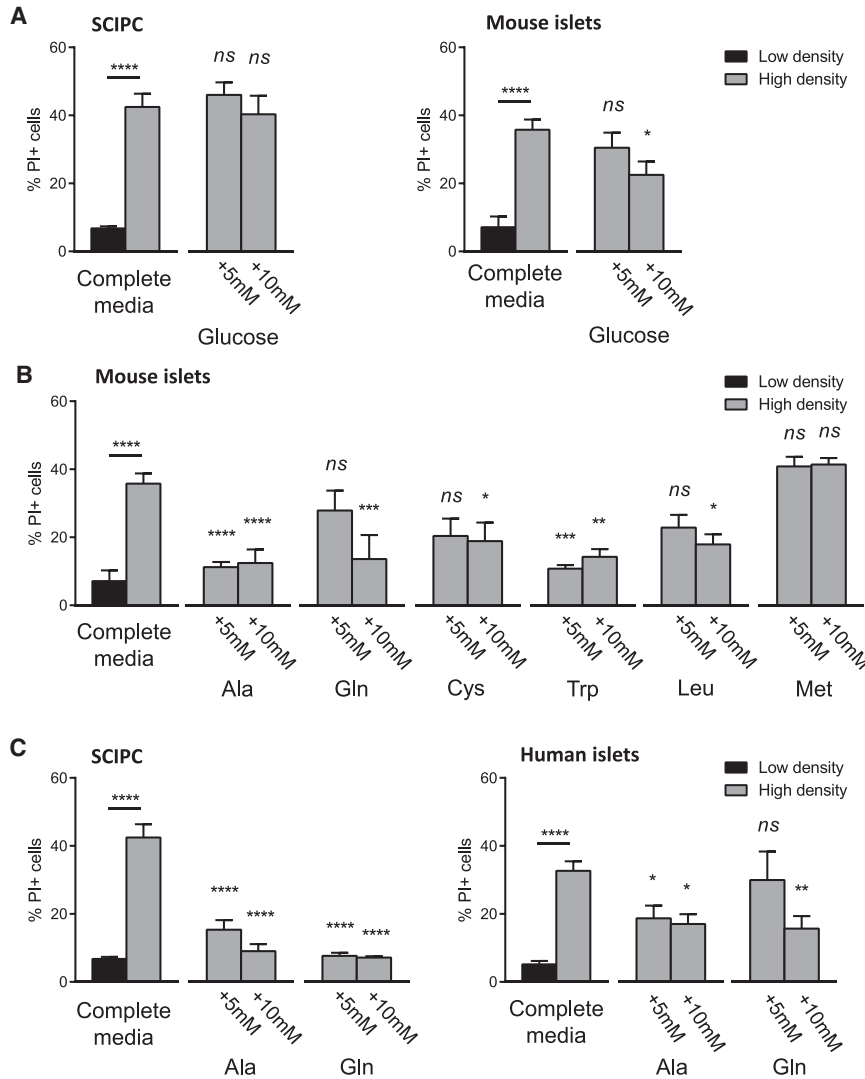


Figure 3. Effect of Nutrient Supplementation on Islet Cell Death *In Vitro*

(A) SCIPC and mouse islets were cultured at various densities in complete RPMI with or without addition of glucose. Cell viability was quantified using PI staining via flow cytometry. Data are a compilation of results from three independent experiments (SCIPC: $n = 9$ per group; mouse: $n = 12$ per group).

(B) Mouse islet survival in high-density cultures supplemented with selected amino acids. Ala, alanine; Gln, glutamine; Cys, cysteine; Trp, tryptophan; Leu, leucine; Met, methionine. Data are a compilation of results from three independent experiments ($n = 9$ per condition).

(C) SCIPC and human islet survival in high-density cultures (SCIPC: 3,000 clusters/mL; human islets: 1,000 islets/mL) supplemented with selected amino acids. Cell viability was quantified as described in (A). Data are a compilation of results from three independent experiments ($n = 9$ per condition).

For all panels, statistical significance of differences from the high-density condition of the same cell type was determined using one-way ANOVA with Holm-Sidak multiple comparisons. All data are expressed as mean \pm SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$; ns, not significant.

then to the TCA cycle intermediate oxaloacetate. To test whether enhancing anaplerosis can protect islets and SCIPC against nutrient deprivation, we supplemented high-density cultures with pyruvate. We found that pyruvate efficiently rescued SCIPC and mouse islets against cell death, supporting the notion that sustaining the TCA cycle may be essential for β cell survival under nutrient-limiting conditions (Figure 5).

Mitigating Hypoxia-Induced Cell Death *In Vitro*

Upon transplantation, grafted cells experience a sudden decrease in oxygen availability, from atmospheric oxygen concentration of 21% to less than 1% in the grafts (Carlsson et al., 2001). Oxygen levels in the native pancreas are estimated to be 5% (Carlsson et al., 1998). We hypothesize that adjusting to physiological oxygen levels of 5% during SCIPC differentiation may reduce the impact of

hypoxia after transplantation. We therefore transitioned differentiating cultures from 21% to 5% oxygen on day 10, before the induction of endocrine differentiation (Figure 6A) (Russ et al., 2015). Percentages of pancreatic and duodenal homeobox 1 (PDX1)⁺ C-peptide⁺ and Homeobox protein 6.1 (NKX6.1)⁺ C-peptide⁺ cells were comparable between SCIPC differentiated at 21% oxygen throughout (SCIPC^{21%}), and SCIPC differentiated with 5% oxygen from day 10 onward (SCIPC^{5%}) (Figure 6B), indicating that transition to 5% oxygen at the progenitor stage of direct differentiation did not affect the differentiation efficiency of SCIPC.

We next compared the metabolic profiles SCIPC^{5%}, SCIPC^{21%}, human islets, and mouse islets. We quantified oxygen consumption rate (OCR), a measurement of mitochondrial respiration, and extracellular acidification rate (ECAR), a measurement of glycolysis. SCIPC^{21%} exhibited

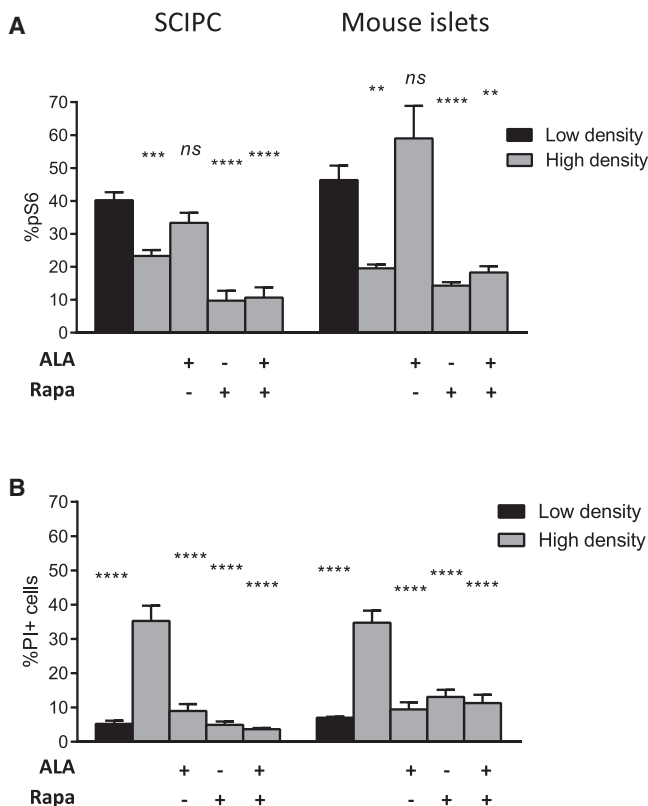


Figure 4. Effect of mTOR on Amino Acid Rescue of Islet Survival under Nutrient Deprivation

(A) Percentage of cells with phosphorylated S6 (pS6) in SCIPC and mouse islets cultured in various conditions were determined using intracellular staining and flow cytometry (n = 6 per condition for SCIPC; n = 9 per condition for mouse islets).

(B) SCIPC and mouse islet viability in various conditions were measured using PI staining and flow cytometry.

In both panels, data are a compilation of results from three independent experiments (n = 6 per condition for SCIPC; n = 9 per condition for mouse islets). One-way ANOVA with Holm-Sidak multiple comparisons was used to determine statistical significance of differences from the low-density condition (A) or high-density condition (B). All data are expressed as mean ± SEM. **p < 0.01, ***p < 0.001, ****p < 0.0001; ns, not significant.

significantly elevated basal OCR and ECAR when compared with mouse and human islets. SCIPC^{5%} showed reduced OCR and ECAR when compared with SCIPC^{21%} (Figure 6C). These results show that the metabolic state of SCIPC^{5%} more closely resembles that of mature islets when compared with SCIPC^{21%}. Moreover, SCIPC^{5%} were significantly more resistant to challenges with 1% oxygen, high-density cultures, and the combination of the two (Figure 6D). These results suggest that SCIPC^{5%} may survive better *in vivo* after transplantation.

Extreme hypoxia induces permanent changes in glucose sensing of β cells by altering expression of genes involved in

glucose metabolism, leading to decoupling of glucose exposure and insulin secretion (Cantley et al., 2013). Thus, we examined the expression of hypoxia-induced genes in SCIPC^{5%} and SCIPC^{21%} after exposure to 1% oxygen for 18 hr. Consistent with previous reports, SCIPC^{21%} exposed to 1% oxygen showed dramatic increase of hypoxia-induced gene transcripts *GLUT1*, *HK2*, *PDK1*, *VEGF-A*, *LDHA*, and *MCT4* (Figure 6C). In comparison, SCIPC^{5%} exhibited a significantly blunted response to 1% oxygen challenge, indicating an increased resistance to hypoxic reprogramming. *In vitro* functional assessments showed similar GSIS by SCIPC^{21%} and SCIPC^{5%} before hypoxic exposure (Figure 6E). However, after 18 hr of hypoxia, SCIPC^{21%} failed to respond to glucose stimulation, whereas functionality of SCIPC^{5%} was preserved (Figure 6E). Taken together, these results indicate that differentiation of SCIPC at a physiological oxygen concentration of 5% results in a protective adaptation without impairing differentiation or function.

Promoting SCIPC Survival after Transplant

Our *in vitro* results thus far suggest that generation of SCIPC at 5% oxygen alone or in combination with amino acid supplementation may protect against ischemia after transplantation. Hence, we transplanted SCIPC^{5%} or SCIPC^{21%} with or without amino acid supplementation in SQ space and monitored graft survival using bioluminescent imaging (Figure 7A). In these experiments, we used a stringent criterion of maintenance of more than 80% of the initial cell mass to define graft survival. Most of the control SCIPC^{21%} without amino acid supplementation rapidly dropped below this threshold. SCIPC^{5%} without amino acid supplementation showed improved survival early after transplant, but the protective effect faded by day 10, resulting in overall survival similar to that of the SCIPC^{21%} control (Figure 7B). Glutamine and alanine supplementation to SCIPC^{21%} also showed improvement early after transplant, but this protective effect did not persist. Combining SCIPC^{5%} with amino acid supplementation significantly improved graft survival, with 75% of grafts surviving to day 20 (Figure 7B). The SCIPC^{5%} + amino acid group not only have better graft mass but also better preservation of insulin-producing cells when compared with the other groups (Figure 7C). Taken together, these results suggest that hypoxia and nutrient deprivation are two major contributors of SCIPC graft loss after transplantation and mitigating both factors is needed to preserve SCIPC graft mass after transplant.

DISCUSSION

Graft survival is a major barrier to islet transplantation, and its impact on stem cell-derived β cell replacement therapy

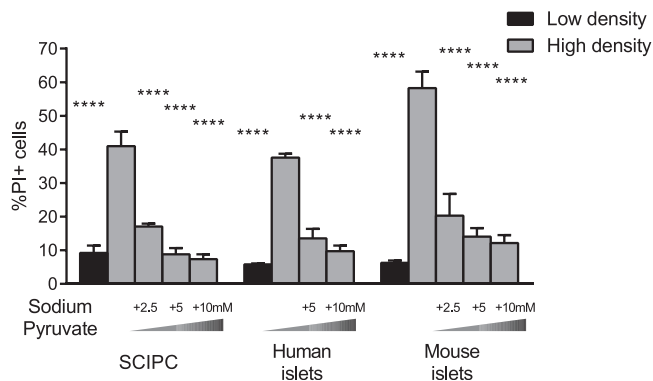


Figure 5. Effect of Pyruvate on Islet and SCIPC Cell Death during Nutrient Deprivation

Various concentrations of sodium pyruvate were added to high-density SCIPC, human islet, and mouse islet cultures, and viability was measured using PI staining and flow cytometry. Data are expressed as mean \pm SEM and are a compilation of results from three independent experiments (SCIPC: $n = 9$; human islets: $n = 9-12$; mouse islets: $n = 6-9$). One-way ANOVA with Holm-Sidak multiple comparisons was used to determine the statistical significance of the differences from the high-density condition. **** $p < 0.0001$.

is unclear. In this study, we demonstrate a significant decline of SCIPC graft mass shortly after transplant with a preferential loss of more mature insulin-producing cells. Although SCIPC are more resistant to hypoxia and nutrient deprivation individually when compared with mature islets, they are vulnerable to their combined action. We have developed strategies for mitigating the impact of ischemia to achieve greater retention of SCIPC graft mass after transplant. Our results suggest that effective therapeutic applications of SCIPC should incorporate strategies to mitigate the impact of hypoxia and nutrient deprivation for promoting graft survival after transplant. Importantly, the concepts and the approaches presented in this study may be applicable to other stem cell-based therapies.

It is well known that β cells in mature islets are sensitive to ischemia and that the majority of them die shortly after transplantation (Emamaullee and Shapiro, 2007; Potter et al., 2014; Rickels et al., 2005). Previous studies comparing adult islets and fetal islet-like cells have shown better survival of fetal cells due to their ability to withstand ischemia and to replenish graft mass by proliferation (Beattie et al., 1997; Hayek and Beattie, 1997). These findings have kindled the hope that SCIPC would be similarly resistant to ischemia, thus providing a solution to the islet graft survival problem. Indeed, survival and continued differentiation of pancreatic progenitor cells after transplantation has been demonstrated (Bruin et al., 2013; Kroon et al., 2008; Vegas et al., 2016). However, some experimental evidence suggests that consistent engraftment of pancreatic progenitor cells is challenging to achieve (Courtney et al.,

2010; Matveyenko et al., 2010). Our side-by-side quantitative comparisons showed that SCIPC indeed survived better than mature islets after transplant, although substantial graft mass was still lost. Moreover, the more mature insulin-producing cells among SCIPC preparations were preferentially lost after transplant. The increased vulnerability of more mature insulin-producing cells may be due to preferential death or de-differentiation of more mature cells under ischemia. Previous studies have shown that hypoxia can induce de-differentiation (Puri et al., 2015). The combined effect of cell death and de-differentiation may explain the inconsistent results with SCIPC grafts and the long duration needed to achieve graft function reported previously (Kroon et al., 2008; Matveyenko et al., 2010).

There are currently two general experimental approaches to the control of hypoxia-induced death of mature β cells from islets: preconditioning before transplant or provision of oxygen after transplant. Preconditioning is based on the concept that cells can adapt to survive under low oxygen tensions if the change is introduced gradually (Abaci et al., 2010; Hals et al., 2015). Moreover, transient hypoxia induces hypoxia-induced factor (HIF) that promotes angiogenesis (Pugh and Ratcliffe, 2003), which benefits long-term graft function. Artificial induction of HIF-1 α improves transplant outcomes (Faleo et al., 2008; Stokes et al., 2013). The current process of islet transplantation extracts islets from native pancreas with 5% O₂ and rests them at atmospheric 21% O₂ for a few days before infusing them into the portal vein with 1–2% O₂. The abruptness of going from suprphysiological oxygen tension to hypoxia deprives the cells time to adapt. Preconditioning the islets using pulsatile cycles of 5–21% oxygen levels for 1 hr helps to maintain islet function during later exposure to hypoxia *in vitro* (Lo et al., 2012). These results argue that exposure to hypoxia can be beneficial for long-term survival and function of islet and SCIPC grafts. However, dropping oxygen tension too low for too long may kill islets and change the metabolic program of surviving cells, leading to long-term dysfunction (Cantley et al., 2013; Smith et al., 2017). Thus, the challenge is to define the proper conditions under which to prepare the cells to withstand hypoxia without metabolic reprogramming.

Current stem cell differentiation protocols are carried out at suprphysiological oxygen levels of 21% instead of the physiological oxygen tension in the naive pancreas of 5%. We reasoned that acclimatizing SCIPC to physiological 5% oxygen tension will provide protection from hypoxia-induced cell death upon transplantation. Encouragingly, transitioning cultures to 5% oxygen after 10 days of differentiation did not negatively affect SCIPC generation, and adjusted their metabolic profile to those observed in mature islets. Lower metabolic demand of SCIPC^{5%} could be responsible for their higher resilience under

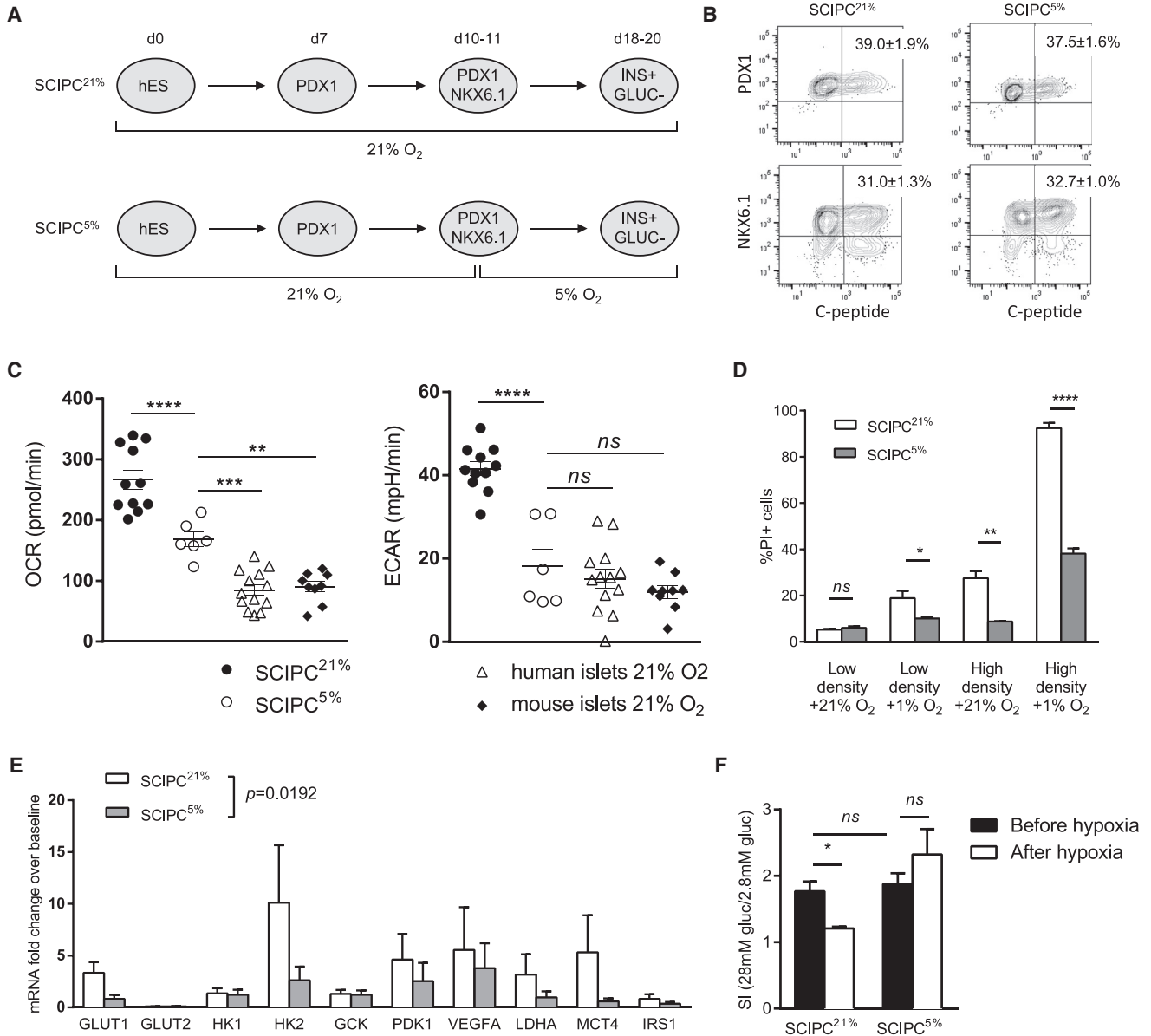


Figure 6. Generation of SCIPC at 5% Oxygen

(A) Schematic for the differentiation of SCIPC with 21% or 5% oxygen (referred to as SCIPC^{21%} and SCIPC^{5%}, respectively).

(B) Representative flow plots of SCIPC^{21%} and SCIPC^{5%} from three independent experiments are shown, and mean ± SEM of percentages of cells are listed in the upper right quadrants (n = 6 per condition).

(C) OCR and ECAR of SCIPC^{21%} (n = 11), SCIPC^{5%} (n = 6), human islet (n = 13), and mouse islet (n = 9) cultures were measured at 21% oxygen level. Data are a summary of three independent experiments with individual data points, mean, and SEM shown. Statistical significance of the differences of the means was determined using one-way ANOVA Holm-Sidak multiple comparisons test.

(D) SCIPC^{21%} and SCIPC^{5%} were cultured in high- or low-density (20 and 3,000 clusters/mL) with 21% or 1% oxygen for 24 hr. Cell viability was measured using PI staining and flow cytometry. Data are expressed as mean ± SEM and are a compilation of results from three independent experiments (SCIPC^{21%}: n = 6–10; SCIPC^{5%}: n = 10–16). An unpaired t test with Welch's correction was used to compare the two groups.

(E) qPCR analysis of expression of hypoxia-induced gene transcripts in SCIPC^{21%} or SCIPC^{5%} after 18-hr exposure to 1% oxygen. Results are expressed as fold change over pre-exposure baseline after normalization to the housekeeping gene GAPDH. Values are mean ± SEM with n = 3 for each cell type. Statistical significance of the difference between the two cell types across all 10 genes was determined using a paired t test (p = 0.0192).

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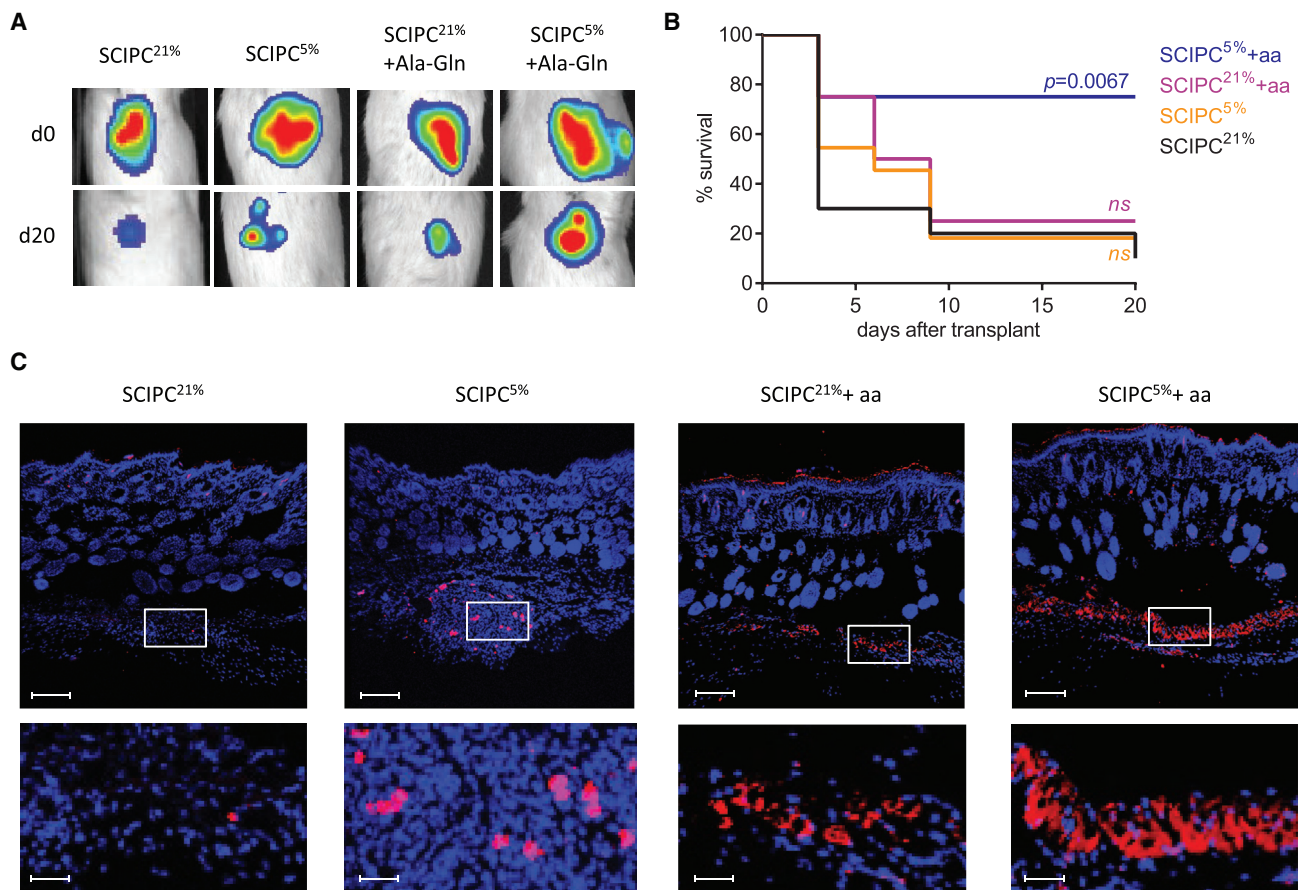


Figure 7. Effect of Amino Acid Supplementation and Oxygen Acclimatization on SCIPC Survival after Transplantation

(A) Representative bioluminescence images of SCIPC transplanted subcutaneously into non-diabetic NSG mice at days 0 and 20.

(B) Graft survival over time after transplantation of SCIPC^{21%} (n = 10), SCIPC^{5%} (n = 11), SCIPC^{21%} + amino acid (aa) (n = 8), and SCIPC^{5%} + aa (n = 8). Grafts were considered “failed” when the BLI dropped below 80% of day 0. Significance of differences in survival from SCIPC^{21%} without supplement was determined for each experimental condition using a log-rank (Mantel-Cox) test.

(C) Top: representative images of insulin staining of SCIPC grafts in (B) collected on day 20. Scale bars, 200 μ m. Bottom: magnified views of the insets in the larger images. Scale bars, 50 μ m. DAPI (blue), insulin (red).

conditions of hypoxia and nutrient deprivation. Importantly, SCIPC^{5%} showed much-attenuated response to later encounter of extreme hypoxia of 1% oxygen and, most importantly, SCIPC^{5%} remain functional after hypoxic exposure while SCIPC^{21%} do not. Thus, generating SCIPC at physiological oxygen tension appears to achieve a balance of inducing protection against hypoxia without impairing the SCIPC developmentally or functionally.

One other major conclusion from this study is that nutrient deprivation, another consequence of ischemia, can also kill islets and SCIPC and contribute to poor outcomes after transplant. In searching for a strategy to

mitigate the impact of nutrient deprivation on islet and SCIPC survival, we found some amino acids to be effective. It is important to note that this newly identified role of amino acids in sustaining β cell viability under nutrient deprivation is distinct from the previously reported effect of amino acids in potentiating β cell insulin secretion upon glucose stimulation (Newsholme et al., 2007; Prentki et al., 2013). Two features of amino acid-mediated rescue are important: single amino acid supplementation can be sufficient and not all amino acids work. Furthermore, our study suggests that amino acids likely protect β cells by sustaining mitochondria oxidation via anaplerosis instead of

(F) GSIS of SCIPC^{21%} and SCIPC^{5%} before and after 18-hr exposure to 1% oxygen. Stimulation indexes are shown (n = 3 per group). Statistical significance of the differences among all conditions was determined using one-way ANOVA with Holm-Sidak multiple comparisons test.

All data are expressed as mean \pm SEM. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001; ns, not significant.



mTOR-dependent anabolism. However, most amino acids can enter the TCA cycle, but not all equally protect islets and SCIPC against nutrient deprivation. In addition, glucose also has anaplerotic effects, but did not confer any protection at a wide range of concentrations tested. Entrance to cells and mitochondria and activities of specific anaplerotic enzymes may impose limitations on some nutrients to become TCA intermediates. Definitive demonstration of anaplerosis and/or amino acid oxidation in islets and SCIPC will require comprehensive metabolic profiling that is currently ongoing in our laboratories. These efforts may shed light on the mechanisms of amino acid rescue of islets and SCIPC under nutrient deprivation and help to guide optimized strategy to support islet and SCIPC survival after transplant.

The surprising finding that rapamycin alone sustained SCIPC and islet survival under nutrient deprivation suggests that limiting protein synthesis under nutrient deprivation may be protective by reserving the cellular pool of amino acids. In this regard, short 2-hr rapamycin exposure has been shown to improve cell viability of human liver carcinoma HepG2 cells and human embryonic kidney HEK293 cells via induction of autophagy (Kapuy et al., 2014). Autophagy is a cell-intrinsic adaptation strategy to starvation by breaking down proteins to fuel basal survival needs (Mizushima and Komatsu, 2011). However, longer 24- to 48-hr treatment of rapamycin impairs islet survival despite induction of autophagy (Tanemura et al., 2012). Thus, although islets have the ability to adapt to nutrient deprivation by autophagy, this catabolic survival mechanism is likely unsustainable. Supplementation of critical nutrients will likely be a more effective approach to sustain islets and SCIPC survival after transplant until revascularization.

Glutamine and alanine are the two most abundant amino acids in plasma (Curi et al., 2005). The highest levels of alanine have been found in the endocrine system, including islets (Ota et al., 2014). Islets normally have a high demand for glutamine and alanine (Dixon et al., 2003). During whole-body starvation, muscle breakdown provides a source of glutamine and alanine to support cellular functions (Chang and Goldberg, 1978). Therefore, supplementation of glutamine and alanine to transplanted islets and SCIPC may mimic the physiological response to endure nutrient deprivation.

Our *in vivo* results of greatly improved SCIPC survival by combining physiological oxygen acclimatization and amino acid supplementation suggest that hypoxia and nutrient deprivation are likely the two most important contributors to SCIPC death after transplant. These experiments mainly focused on cell viability, because cell survival is a prerequisite to function and needs to be addressed first. It is encouraging that this approach not only sustained cell viability but also insulin expression, suggesting functional

preservation. Future studies are needed to evaluate this strategy in a diabetes model. Overall, our study demonstrates that by addressing individual contributors of ischemia-induced SCIPC death, dramatic improvement of SCIPC engraftment can be achieved. The general strategy of physiological oxygen acclimatization and nutrient supplementation may be applicable to other stem cell-derived grafts to improve their survival and therapeutic efficacy.

EXPERIMENTAL PROCEDURES

All animal procedures were performed under approved protocols and in accordance with ethical guidelines by the Institutional Animal Care and Use Committee (IACUC) at the University of California, San Francisco.

In Vitro Ischemia Simulations

SCIPC, human islets, and mouse islets were cultured at increasing densities in RPMI media with 5% fetal calf serum (R5) for 6–24 hr to simulate decreasing nutrients. SCIPC were cultured in concentrations of 200 clusters/mL for low density (LD), 1,500 clusters/mL for medium density (MD), and 3,000 clusters/mL for high density (HD). Mouse and human islets were cultured at concentration of 200 islets/mL for LD, 500 islets/mL for MD, and 1,000 islets/mL for HD. Alternatively, SCIPC, human islets, and mouse islets were cultured with 1% and 10% R5 diluted in PBS for 6–24 hr to simulate nutrient deprivation. For the hypoxia challenges, SCIPC, human islets, and mouse islets were cultured in R5 with 1% oxygen in incubators with adjustable oxygen levels (Heracell 240, Thermo Fisher Scientific, Waltham, MA).

Islet and SCIPC Transplantation

Transplantation of mouse islets and SCIPC into renal capsules or the subcutaneous space was performed as reported previously (Halberstadt et al., 2005; Szot et al., 2007). For amino acid supplementation, additional 10 mM alanine and 10 mM glutamine were supplemented to SCIPC culture medium 2 hr before the cells were collected for transplant. SCIPC were transplanted subcutaneously together with 50 μ L of the alanine- and glutamine-supplemented media.

Other experimental procedures are described in [Supplemental Experimental Procedures](#).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, three figures, and one table and can be found with this article online at <http://dx.doi.org/10.1016/j.stemcr.2017.07.012>.

AUTHOR CONTRIBUTIONS

Conceptualization, Q.T.; Methodology, G.F., H.A.R., and Q.T.; Writing – Original Draft, G.F. and Q.T.; Investigation, G.F., H.A.R., V.N., S.W., A.V.P., G.G.N., J.E.F., K.E.V., and G.L.S.; Writing – Review & Editing, G.F., H.A.R., M.H., and Q.T.; Funding Acquisition and Supervision, M.H. and Q.T.



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Stem Cell Reports, Volume 9

Supplemental Information

**Mitigating Ischemic Injury of Stem Cell-Derived Insulin-Producing Cells
after Transplant**

Gaetano Faleo, Holger A. Russ, Steven Wisel, Audrey V. Parent, Vinh Nguyen, Gopika G. Nair, Jonathan E. Freise, Karina E. Villanueva, Gregory L. Szot, Matthias Hebrok, and Qizhi Tang

Supplementary Data:

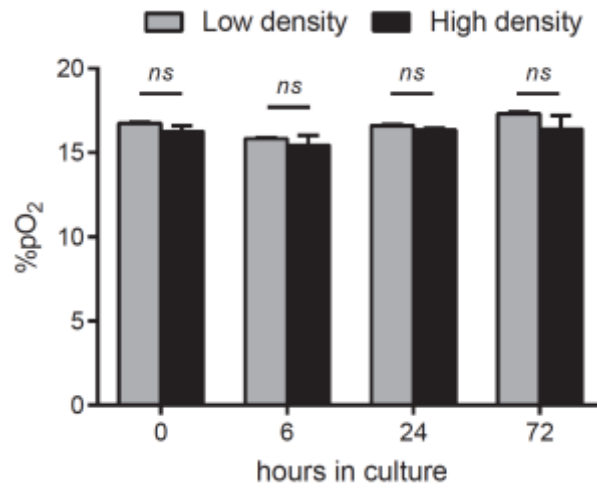


Figure S1. Oxygen tension in low density and high density cultures. Oxygen tensions were measured in low density and high density cultures over time. Data are expressed as mean \pm SEM and are a compilation of results from 3 independent experiments (n= 14 per condition). Statistical significance of the difference between low density and high density group was calculated using multiple t test.

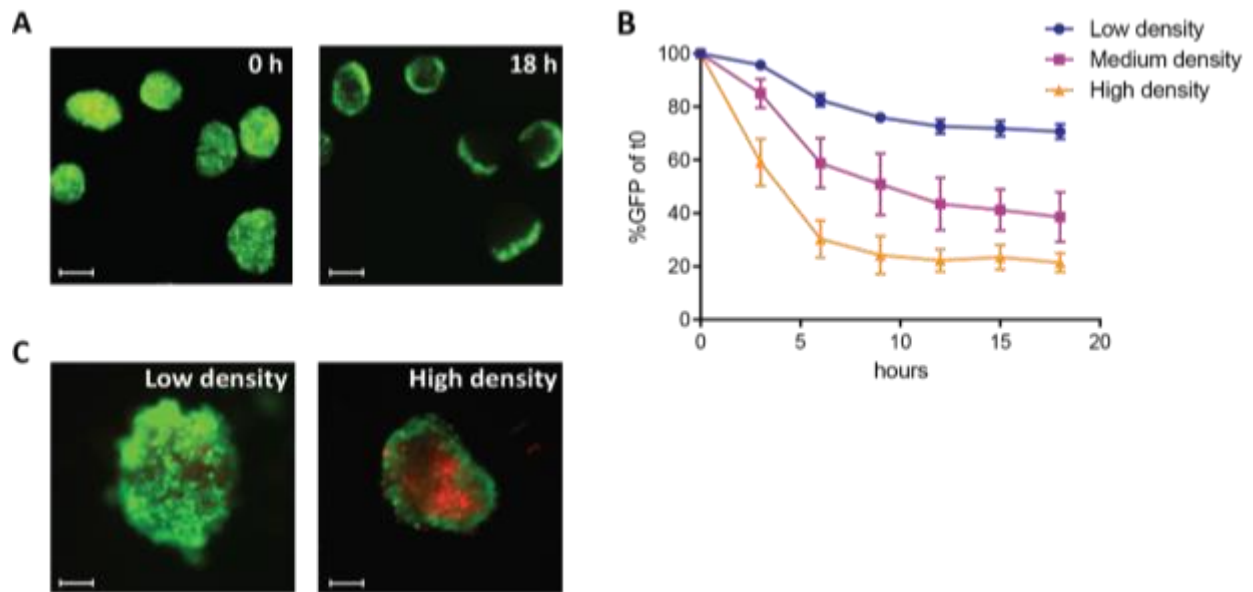


Figure S2. Nutrient-dependent cell death *in vitro*. (A) GFP-expressing mouse islets were cultured at high density (1000 islets/mL) and cell death resulted in loss of membrane integrity and GFP signal. Scale bar 100 μ m. (B) Kinetics of cell death of various culture densities were monitored in real time for 18 hours. Data shown as mean \pm SEM are a summary of 3 independent experiments (n=3 per condition). (C) MIP.GFP islets cultured in low- and high-density cultures stained for Propidium Iodide (red) after 6 hours. Images are representative of 3 independent experiments. Scale bar 40 μ m.

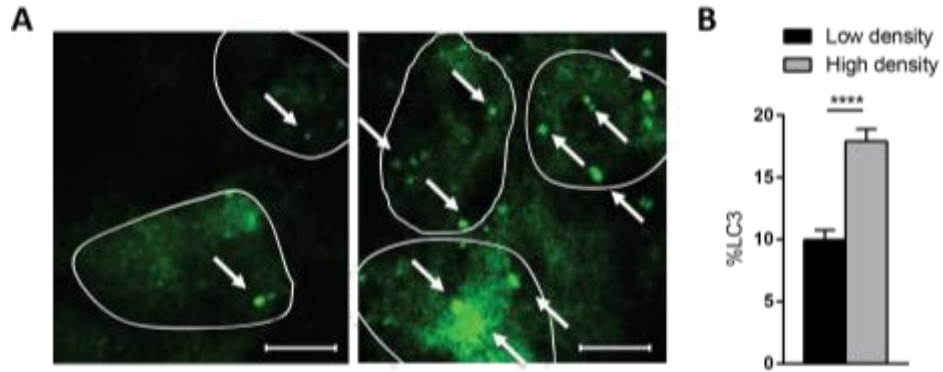


Figure S3. Nutrient-dependent cell death in vitro. (A) GFP-labeled LC3 autophagosome formations (arrows) in cells (outlined) in low-density (left), and high-density conditions (right) for 3 hours. Magnification 200x. Pictures shown are representative of 3 independent experiments (n=3 per group). Scale bar 5 μ m. (B) Islets are dissociated to single cells and percentage of LC3+ cells are quantified through flow cytometry (****p= 0.0007). Data shown as mean \pm SEM are a summary of 3 independent experiments. Statistical significance of the difference between low-density and high-density group was calculated using paired t test.

Table S1. Primers used for RTqPCR analysis of human SCIPC.

Gene symbols	Forward primers	Reverse primers
<i>SLC2A1 (GLUT1)</i>	5'-GTCCCTCTCAGTGGCCATCTTT	5'-GGCCAAAGCGGTAAACGAAA
<i>SLC2A2 (GLUT2)</i>	5'-TGGAATTGACAGGACTCCCAAC	5'-TCCAGTGGAACACCCAAAACA
<i>HK1</i>	5'-AATTCTGCGGGTCAAGTGA	5'-ACTGCCGTGCACGATGTTCT
<i>HK2</i>	5'-CAAAGTGACAGTGGGTGTGG	5'-GCCAGGTCCTTCACTGTCTC
<i>GCK</i>	5'-GGAGCGTGAAGACCAAACAC	5'-GTAGTCGAAGAGCATCTCAGC
<i>PDK1</i>	5'-AGGGTTACGGGACAGATGCAG	5'-TCAGCCTCGTGGTTGGTGT
<i>LDHA</i>	5'-GCACCCAGTTTCCACCATGA	5'-TCTTCAAACGGGCCTCTTCC
<i>VEGFA</i>	5'-GCTACTGCCATCCAATCGAG	5'-ACACAGGATGGCTTGAAGATG
<i>PDX1</i>	5'-TGGAGCTGGCTGTCATGTTG	5'-GCGCTTCTTGTCCTCCTCT
<i>MCT4 (SLC16A3)</i>	5'-TCATCACTGGCTTCTCCTACGC	5'-AGCCGATCCCAAACCTCCTGT
<i>IRS1</i>	5'-CCACTCGGAAAACCTTCTTTCAT	5'-AGAGTCATCCACCTGCATCCA
<i>GADPH</i>	5'-TTGCCATCAATGACCCCTTCA	5'-CGCCCCACTTGATTTTGGGA

SLC2A1 (GLUT1): solute carrier family 2 (facilitated glucose transporter), member 1 (glucose transporter 1);

SLC2A2 (GLUT2): solute carrier family 2 (facilitated glucose transporter), member 2 (glucose transporter 2);

HK1, hexokinase 1;

HK2, hexokinase 2;

GCK, glucokinase (HK4);

PDK1, pyruvate dehydrogenase kinase 1;

LDHA, lactate dehydrogenase A;

VEGFA, vascular endothelial growth factor A;

PDX1, pancreatic and duodenal homeobox 1;

MCT4 (SLC16A3), monocarboxylic acid transporter 4 (solute carrier family 16, member 3);

IRS1, insulin receptor substrate 1;

GADPH, glyceraldehyde 3-phosphate dehydrogenase.

Supplemental Experimental Procedures

Mice

C57BL/6 (B6), B6.MIP-Luc, B6.MIP-GFP, B6.LC3-GFP, and NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ (NSG) mice were housed and bred at the University of California, San Francisco Animal Barrier Facility. All animal procedures were performed under approved protocols and in accordance with ethical guidelines by the Institutional Animal Care and Use Committee (IACUC) at the University of California, San Francisco.

Islet isolation

Mouse islets were isolated as previously (Szot et al., 2007). Purified islets were rested in RPMI medium overnight and healthy islets were handpicked for experiments. Human research islets provided by the UCSF DRC Islet Production Core following current standard protocols on research consented pancreas donors (Szot et al., 2009). All human studies were approved by local ethics committees. Pancreata were procured from multiorgan cadaveric donors using cold perfusion with University of Wisconsin solution (ViaSpan, DuPont Pharmaceuticals Ltd.).

Derivation of constitutive luciferase-expressing MEL1 ^{INS^{GFP/wt}} cell line

DNA encoding a firefly luciferase gene was targeted to the AAVS1 locus of the MEL1 ^{INS^{GFP/wt}} cells using a published TALEN pair and a targeting vector containing homology sequences and a Puromycin selection (Addgene plasmid #58409, #59025, and #59026), which was modified by inserting a firefly luciferase gene via a T2A cleavage site after the Puromycin resistance gene. Human ES cells were targeted by electroporation of 5ug of each TALEN vector with 20ug of the Luciferase containing targeting vector using a Biorad GenePulser (Hercules, CA) using an exponential decay and 250V and 500uF settings. Cells were plated on DR4 resistant MEFs containing plates and selected after 24-48 hours with 0.5ug/ml Puromycin for 3-4 days. Successfully targeted cells were identified using bioluminescent followed by PCR of genomic DNA to verify site directed integration of the targeting vector. Bioluminescence imaging demonstrates a cell dose-dependent increase of bioluminescence signal (not shown).

SCIPC differentiation

Human embryonic stem cell line containing a GFP gene knocked into the endogenous insulin locus, MEL1 ^{INS^{GFP/wt}} (Micallef et al., 2012), was maintained as described (Russ et al., 2015). Suspension-based differentiations were carried out as described previously (Russ et al., 2015) with modification of the last differentiation stages. Cultures were incubated in DMEM (Gibco) containing 25 mM glucose, 1:100 B27 (StemCell Technologies), 50 ng/ml EGF (RnD Systems), and 50 ng/ml KGF (RnD Systems) for days 9 and 10. Thereafter, clusters were incubated in DMEM (Gibco) containing 25mM Glucose supplemented with 1:100 B27 (StemCell Technologies), 1:100 non-essential amino acids (Gibco), 1mM N-Acetyl-L-Cysteine (Sigma), 10µg/ml Heparin Sodium Sulfate (Sigma), 10µM Zinc Sulfate heptahydrate (Sigma), 10µM ALK inhibitor 2 (Axxora), 2µM T3 (3,3',5-Triiodo-L-thyronine sodium salt) (Sigma), 500nM LDN-193189 (Stemgent), 1µM Gamma-Secretase inhibitor (XXi) (Millipore), 2µM Bay K 8644 (Tocris), and 0.5mM 2-Phospho-L-ascorbic acid (Sigma). Medium was changed daily by aspirating approximately 5ml followed by addition of 5ml fresh media.

Islet metabolic measurement

Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were quantified as previously described (Wikstrom et al., 2012). XF24 plates that are specifically designed to assess islets and similar clusters were used. 20 islets or SCIPC clusters were deposited at the bottom of individual wells and restrained by a mesh to prevent clusters dispersion. Cells were incubated for 1 hour at 37°C after which respiration was measured. Samples were analyzed using Seahorse XF24 Extracellular Flux Analyzer (Seahorse, North Billerica, MA).

Bioluminescence Imaging

Luciferase-expressing islet or SCIPC graft mass was monitored using bioluminescence imaging as described previously (Faleo et al., 2016).

Islet live imaging

MIP-GFP islets were used to visualize islet survival *in vitro* in real time. Isolated islets were plated into a 96-well plate at various concentration and placed on a stage of a microscope that was encased in an environmentally controlled chamber to maintain 37°C and 5% CO₂ throughout the experiment. Green fluorescent signal and bright field images were captured every 3 hours for an 18-hour follow-up period. The pictures were then analyzed using NIS Elements

software (Nikon) to quantify GFP signals.

Oxygen tension measurements

Oxygen tensions of islet cultures were measured using the FOSPOR-PI600-2M Oxygen Sensor probe (Ocean Optics, Dunedin, FL). The probe was immersed deep into the media of each well. For each sample, 14 measurements were performed at each time point and the mean \pm SEM was calculated.

Flow cytometry

To measure cell viability after *in vitro* culture, SCIPC clusters or whole islets were stained with 25 μ g/mL Propidium Iodide (PI, Sigma) for 15 minutes. The clusters were then washed in PBS 3 times before dissociation into single cells using Accumax (Innovative cell technologies, San Diego, CA). The cells were washed before analysis on an Accuri flow cytometer (BD Bioscience, San Jose, CA). To quantify cells expressing GFP after transplant, grafts were harvested from the renal capsule at day 7 and the dissociated cells were stained with PI and anti-HLA-ABC-PE-Cy5 (W6/32, A18548, Invitrogen Antibodies, Carlsbad, CA) before analysis on Accuri. Flow cytometric analyses of expression of β cell maturation markers by SCIPC were performed as previously described. Data were analyzed with FlowJo software (FLOWJO LLC, Ashland, Oregon).

GSIS assay

GSIS was measured as described previously for islets (Kloster-Jensen et al., 2016). Insulin concentrations in supernatant were measured using enzyme-linked immunosorbent assays (ELISA) for mouse insulin (Merckodia, Uppsala, Sweden) and human C-peptide (ALPCO, Salem, NH). Stimulation index is calculated using the following formula: $SI = \frac{[\text{Insulin}]_{\text{high glucose}}}{[\text{Insulin}]_{\text{low glucose}}}$.

RTqPCR Analysis

Total RNA was isolated from SCIPC using the RNeasy MicroKit (Qiagen, Hilden, Germany) first-strand cDNA was prepared using iScript cDNA Kit (Bio-Rad, Hercules, CA), and quantitative RT-PCR was performed on a CFX Connect Real-Time System (Bio-Rad) with FastStart Universal SYBR Green Master Mix (Roche, Basel, Switzerland). Relative changes in gene to *GADPH* ratio between hypoxic and normoxic conditions were computed using the $2^{-\Delta\Delta C_t}$ method. See Table 1 in supplementary methods for primer sequences.

Immunohistochemistry

Tissue-bearing SCIPC grafts were fixed in paraformaldehyde (Sigma), incubated overnight in 30% sucrose (Sigma), before being embedded in tissue-Tek[®] OCT (Sakura Finetek, Torrance, CA). A polyclonal guinea pig anti-insulin antibody (1:100, A0564, Dako, Glostrup, Denmark) was applied overnight and Alexa Fluor[®] 568 goat anti-guinea pig IgG (1:200, A11075, Invitrogen) was incubated for 1 hour. Images were taken using Leica SP5 upright confocal microscope (Leica Microsystems, Buffalo Grove, IL) and processed with Imaris software (Bitplane, Concord, MA).

Statistical Analysis

Statistical analyses were performed with the aid of GraphPad software (GraphPad Prism, La Jolla, CA).

Supplemental References

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