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Brief Communication

Tolerance of human embryonic stem cell derived islet progenitor cells to vitrification-relevant solutions ☆

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ABSTRACT

We have previously shown that human embryonic stem cell derived islet progenitors (hESC-IPs), encapsulated inside an immunoprotective device, mature *in vivo* and ameliorate diabetes in mice. The ability to cryopreserve hESC-IPs preloaded in these devices would enhance consistency and portability, but traditional 'slow freezing' methods did not work well for cells encapsulated in the device. Vitrification is an attractive alternative cryopreservation approach. To assess the tolerance of hESC-IPs to vitrification relevant conditions, we here are reporting cell survival following excursions in tonicity, exposure to fifteen 40% v/v combinations of 4 cryoprotectants, and varied methods for addition and elution. We find that 78% survival is achieved using a protocol in which cells are abruptly (in one step) exposed to a solution containing 10% v/v each dimethyl sulfoxide, propylene glycol, ethylene glycol, and glycerol on ice, and eluted step-wise with DPBS + 0.5 M sucrose at 37 °C. Importantly, the hESC-IPs also maintain expression of the critical islet progenitor markers PDX-1, NKX6.1, NGN3 and NEURO-D1. Thus, hESC-IPs exhibit robust tolerance to exposure to vitrification solutions in relevant conditions.

Diabetes affects 287 million people worldwide, and causes nearly 5 million deaths per year, underscoring the need to develop new therapies. Islet replacement is a promising therapeutic approach for diabetes but hampered by limited tissue supplies and the need for chronic post-transplant immunosuppression. Cellular encapsulation has the potential to protect grafted cells from immune rejection, thus mitigating the need for immunosuppressive drugs. We have studied a durable encapsulation device, which not only provides immunoprotection to the graft, but also allows it to be retrieved. We have shown that this bilaminar macroencapsulation (>10^6 cells) device, engineered from polytetrafluoroethylene (PTFE), is immunoprotective in both mice and non-human primates [12,18]. The device is composed of an inner immunoisolating membrane with a pore size of 0.4 μm, a second membrane with 5 μM pore size, an extensive outer polyester mesh, and luminal spaces of 4.5 μL or 20 μL (TheraCyte, Inc.). Unlike microcapsules (approximately 10^3 cells) constructed of semi-solid materials which have an inherent breakage rate [1,3,6,16,20], the PTFE device enables reliable removal of the entire graft.

As a source of human tissue for transplantation, stem cells have the potential to revolutionize regenerative medicine, and they show particular promise for the treatment of diabetes. Recently, we and others have shown that human embryonic stem cell derived islet progenitors (hESC-IPs), encapsulated in the PTFE device matured into insulin producing cells after transplantation and cured diabetes in mice [11,13]. Moreover, we showed that encapsulated cells do not escape from the device into the body. Thus, the fully contained cells remain retrievable, assuaging concerns about potential tumorigenicity of stem cell therapies [11]. Based, in part, upon the finding that macroencapsulated hESC-IPs mature *in vivo* and ameliorate diabetes without cell escape, the

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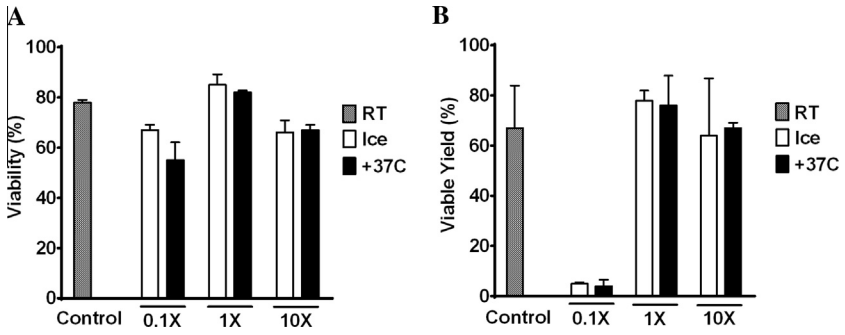

Fig. 1. Osmotic tolerance of hESC-IPs to hyper- and hypo-tonic NaCl solutions. Cells were incubated for 20 min on ice or at 37 °C in isotonic (1×), hypotonic (0.1×), and hypertonic (10×) solutions of NaCl followed by centrifugation and elution of NaCl. Control cells were left at room temperature (RT) without exposure to NaCl and following elution. (A) Cell viability. (B) Viable yield.

Table 1
Cell survival following exposure to 15 vitrificant formulations of permeable vitrificants.

Treatment (combination)	Permeable VFA's, % v/v				Survival, TB⁻	
	P (%)	E (%)	D (%)	G (%)	*M* (%)	s.e.m. (%)
Ctrl*					92	0.6
Iso**					87	0.8
P-E-D-G	10	10	10	10	78	1.8
P-D-G	13.3	13.3	–	13.3	72	1.5
P-E-G	13.3	–	13.3	13.3	71	2.1
D-G	–	–	20	20	69	2.2
P-D	20	–	20	–	67	2.4
P-E	20	20	–	–	65	1.1
G	–	–	–	40	63	1.6
P-G	20	–	–	20	62	1.4
E-D	–	20	20	–	61	1.5
E	–	40	–	–	60	1.9
P-E-D	13.3	13.3	13.3	–	60	2.2
P	40	–	–	–	57	1.8
E-D-G	–	13.3	13.3	13.3	57	2.0
D	–	–	40	–	53	2.3
E-G	–	20	–	20	50	3.1

Cells were exposed to equi-choric (40% v/v total concentration for all experiments) VF solutions. Median % survival (trypan blue exclusion) is shown for: *Ctrl = untreated control; **Iso = negative control for all the same manipulations in the absence of vitrificants, the cells were exposed in an isotonic solution all the time instead; **P** = PG: 1,2-propanediol (propylene glycol); **E** = EG: 1,2-ethanediol (ethylene glycol); **D** = Me_2SO: dimethyl sulfoxide; **G** = GLY: glycerol. The vitrificant solutions were added one-step, the cells were kept for 20 min on ice and then washed in a four-step fashion with DPBS + 0.5 M sucrose at 37 °C. Data shown as average ± s.e.m.

first clinical trial for encapsulated hESC-IPs transplantation for Type I diabetes is underway (https://clinicaltrials.gov/ct2/results?term=viacyte&Search=Search).

Ultimately, commercialization of encapsulated cell therapies will require the implementation of efficient methods for quality control and dissemination of therapeutic units to maximize patient access. While there is significant literature on cryopreservation of cells microencapsulated in semi-solid materials e.g. alginates, there are no existing methods for preserving PTFE devices loaded with cells of any type. We recently investigated traditional "slow freezing" approaches for macroencapsulated cells, and found that cell survival was suboptimal [19]. Our working hypothesis is that the major contributor to poor survival is the exterior fibrous mesh of the device, which may provide numerous nucleation sites for ice crystal formation. On the other hand, we found that the PTFE devices maintained their integrity upon freeze/thaw, a critical piece of information for our future efforts.

Vitrification is an attractive alternative cryopreservation method without ice formation, which has enabled the efficient cryopreservation of challenging cells types: oocytes of human and animal origin and animal embryos are among the most notable [15]. To our knowledge, there are no reports of vitrification of any cells inside a device composed of synthetic fiber mesh. As high concentrations of vitrification (VF) solutions can be damaging, we explored the tolerance of hESC-IPs, to vitrification relevant conditions as a first step toward VF of clinically relevant cells encapsulated in PTFE devices.

The protocol used for differentiation of hESC to islet precursor cells in 2D culture is a modification of Cho et al. [5]. Initially, we established parameters for centrifugation of trypsinized hESC-IPs to maximize yield while avoiding excessive force which can affect cell viability. Following a series of experiments, we chose a relative centrifugal force (RFC) of 500 g for 5 min achieving 98% ± 0.8% total yield with 86% ± 1.4% viability (as measured by trypan blue exclusion). The viable yield (total yield × viability) was 84% ± 2.1%.

During vitrification, cells are usually exposed to highly non-isotonic conditions. Prior to investigating the osmotic tolerance to permeable vitrificants, which largely depends on the ratio of solute-to-water membrane permeability [7], we therefore, have estimated the tolerance, namely, viability (measured as percentage TB-attached cells) and viable yield (product of viability × attachment efficiency) to exposure of cells to an impermeable solute, NaCl of different tonicity. hESC-IPs were exposed to an isotonic (1×), hypertonic (10×) and hypotonic (0.1×) solutions of NaCl, for 20 min on ice or at 37 °C (Fig. 1A and B, cells that did not undergo any additional manipulation after detachment and left on the bench at room temperature are shown as "Control"). Here, cells that were exposed to isotonic NaCl on ice exhibited a viable yield of 76%, which was not statistically significant for the control cells kept at RT. Cell incubation for 20 min in hypertonic 9.0% NaCl on ice was mildly damaging, resulting in a viable yield of 67% for 37 °C. In contrast, exposure to extreme hyposomolarity (0.1× PBS diluted in H_2O) was highly detrimental, as merely 7.5% of cells were recovered, likely due to cell bursting. Thus, while the viability of recovered cells was 55% ± 7.1%, the viable yield was 4% ± 0.3% for 37 °C. Notably, viable yields were not significantly altered by temperature ($p > 0.1$, Student paired *t*-test) in any conditions. Exposure to more moderate hypotonicity in hypotonicity increments, 0.2×, 0.4×, 0.6×, and 0.8× of the iso-value, increased viable yield to 42% ± 14.1%, 36% ± 8.5%, 63% ± 5.7%, and 81% ± 2.8%, respectively (not shown) with similar viability yield values excerpt for 0.2×.

An important consideration for exposure to VF solutions is whether addition and elution are performed in a single step or in multiple steps. In recent studies with cells microencapsulated in alginate beads, Ahmad et al. [2] used a 3-step addition and 4-step elution protocol and the "fixed step molarity" method (FMM) with the most pronounced shrinkage at the first step of addition and at the final step of dilution. However, we have shown that the fixed shrinkage-swelling (FSS) approach with the cell volume excursions are kept equal at all steps, which in the case of lack of exact

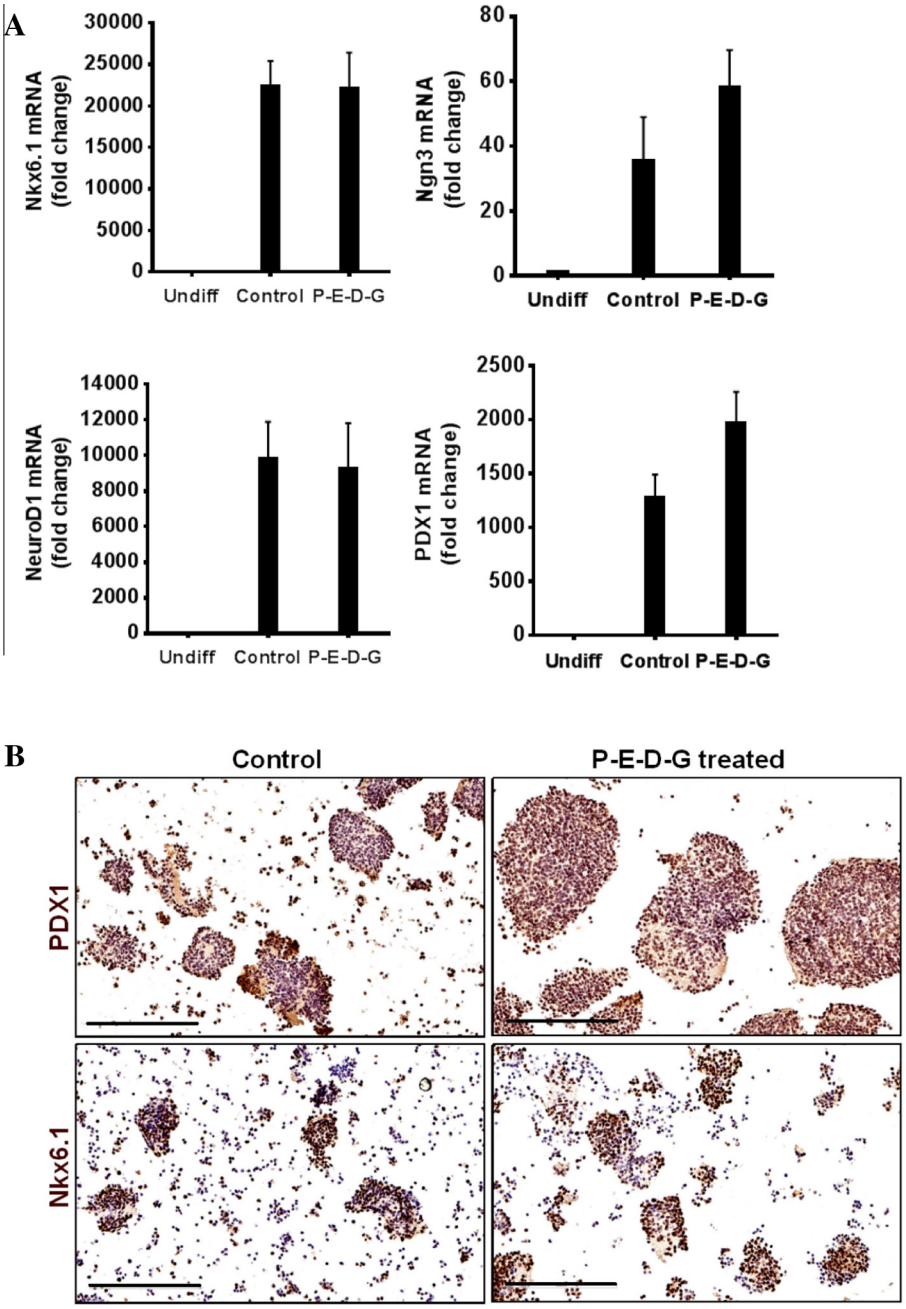

Fig. 2. hESC-IPs maintain lineage markers following exposure to high concentrations of vitrificants. (A) Undifferentiated hES cells (Undiff), hESC-IPs without VF treatment (Control), and hESC-IPs exposed to the 40% v/v vitrificant combination P-E-D-G were analyzed in duplicate by qPCR for 4 markers of islet progenitors: NGN3, NEURO D1, NKX6.1, and PDX-1. Each gene is represented as fold enrichment relative to undifferentiated hESC, which were set at 1. (B) Immunostaining for NKX6.1 (brown) and PDX-1 (brown) in Control and P-E-D-G treated hESC-IPs. Counterstain is eosin, Bar is 200 μ.

permeability data can be approximated as the fixed ratio of the total tonicity at each step, minimizes osmotic damage, [7]. Using a 20% VF solution of equal parts ethylene glycol and glycerol, we found that abrupt addition of VF solutions on ice and 3-step FSS elution (similar to described in [9]) at 37 °C was optimal. Based upon previous studies, 0.5 M sucrose was utilized in elution steps (except the final elution into culture media).

In our experiments, we have tested 40% of final total concentration of permeable vitrificants, the concentration that is widely used for many equilibrium vitrification protocols [15]. Note that we used v/v instead of commonly used w/v for the convenience of experiments, which give even higher molarities, and thus, tonicities as all 4 vitrificants have gravitational density above 1 g/cm^3. The series of 40% v/v (final total volume) solutions were composed of 4 common permeable VF, individually and in all possible equivolume combinations ($N = 2^4 - 1$): (i) (1,2-propanediol (propylene glycol, **P**); (ii) 1,2-ethanediol (**E**); (iii) dimethyl sulfoxide (Me_2SO, **D**); and (iv) glycerol (**G**). The best survival (77% ± 2.8%, compared with 86% ± 1.4% in controls) was achieved with 10% each P-E-D-G (Table 1) while the poorest survival (46% ± 2.0%) occurred from exposure to 20% each E-G. One-way ANOVA showed that the influence of the treatment was statistically significant: the *F*-test gave *p*-values 9.69×10^{-37} (*Ctrl* and *Iso* included) and 1.02×10^{-13} (*Ctrl* and *Iso* excluded). The VF combination yielding the best survival, P-E-D-G, was chosen for further studies.

In addition to preserving the viable yield of hESC-IPs exposed to VFs, it is also critical that the cells maintain their islet progenitor state. Indeed, we previously showed that Me_2SO can interfere with

the differentiation state of human embryonic and induced pluripotent stem cells [8,10]. Therefore, here we measured the expression of critical islet progenitor markers before and after treatment with P-E-D-G, the optimal 40% solution identified above. Importantly, hESC-IPs express little to no insulin *in vitro* and require 4–5 months of *in vivo* maturation to develop glucose responsive insulin secretion. However, the islet progenitor lineage is marked by expression of the transcription factors PDX-1, NKX6.1 and NEURO D1, which are retained in mature pancreatic beta-cells, and also by NGN-3 which is switched off during maturation to the functional beta-cell state. qRT-PCR for the 4 markers demonstrated that there was no significant difference in expression of NKX6.1, NGN3 and NEURO D1 between the Control and VF-exposed populations. Interestingly, P-E-D-G treated cells expressed higher levels of PDX-1 mRNA than controls ($p = 0.006$, Student paired *t*-test). We also performed immunostaining for PDX-1 (Abcam) and NKX6.1 (Developmental Studies Hybridoma Bank) proteins. In agreement with the PCR data, NKX6.1 protein expression in P-E-D-G treated cells was 68%, and indistinguishable from untreated cells (64%), while PDX-1 protein was expressed at 78% in VF treated cells and 59% in controls (Fig. 2). Overall, lineage marker expression in CFA treated hESC-IPs closely mimics that in control untreated cells. Whether the observed increase in PDX-1 expression in P-E-D-G treated cells will have biological relevance *in vivo* is not yet known.

In conclusion, our data demonstrate robust survival and stability of lineage commitment during exposure of hESC-IPs to chemical and osmotic stress in high concentrations of VFs. Our results support the concept that vitrification may be a viable option for cryopreservation of hESC-IPs inside a durable and retrievable cell encapsulation device for clinical use. We expect that development of cryopreservation technology for macroencapsulated stem cells will enhance efficiency, quality control, and dissemination of new therapies for diabetes. In addition, this technology has broad application for regenerative medicine; for diseases ranging from acute liver failure to cancer [4,14,17].

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