Culturing with modified EGM2 medium enhances porcine neonatal islet-like cell clusters resistance to apoptosis in islet xenotransplantation

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Abstract

Background: Neonatal pig islet-like cell clusters (NICC) are an attractive source of insulin-producing tissue for potential transplantation treatment of type 1 diabetic patients. However, a considerable loss of NICC after their transplantation due to apoptosis resulted from islet isolation and instant blood-mediated inflammatory reaction remains to be overcome.

Methods: EGM2 medium depleted with hydrocortisone and supplemented with 50 mmol/L isobutylmethylxanthine, 10 mmol/L nicotinamide, and 10 mmol/L glucose was used to culture NICC at day 1, the day after isolation and changed every other day. NICC cultured with EGM2 or control Ham’s F-10 medium were collected at day 7 of culture for the following assays. The viability of NICC was evaluated by AO/EB staining and FACS. Static assay and oxygen consumption rate analysis were performed to assess the function of NICC. Insulin and glucagon gene expression were measured by real-time PCR. Tubing loops model and TUNEL assay were performed to confirm the apoptosis-resistant ability of NICC cultured with modified EGM2 medium. Serum starvation and hypoxia treatment were used to test the tolerant capability of NICC in the microenvironment of hypoxia/nutrient deficiency in vitro. The molecules involved in apoptosis pathways in NICC were analyzed by Western blotting.

Results: Compared with Ham’s F-10 medium, culturing NICC with EGM2 medium led to increased number and viability of NICC with higher stimulation index, upregulated gene expression of both insulin and glucagon, and enhanced mitochondria function. Furthermore, fewer modified EGM2 medium cultured NICC were found under apoptosis when evaluated in an in vitro tubing loop model of IBMIR. Moreover, EGM2 medium cultured NICC demonstrated much less apoptotic cells under either serum starvation or hypoxia/nutrient deficiency conditions.

Abbreviations: AO/EB, acridine orange/ethidium bromide; ATP, adenosine triphosphate; Bcl-2, B-cell lymphoma 2; Bcl-xL, B-cell lymphoma-extra large; cDNA, complementary DNA; DAB, diaminobenzidine; DNA, deoxyribonucleic acid; DFP, designed pathogen free; ECs, endothelial cells; EGM2, endothelial cell growth medium-2; ELISA, enzyme-linked immunosorbent assay; EPCs, endothelial progenitor cells; FACS, fluorescence-activated cell sorter; FCCP, carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone; FGF-b, basic fibroblast growth factor-b; HBSS/HAS, Hank’s balanced salt solution/human serum albumin; H&E, hematoxylin and eosin; HIF, human epidermal growth factor; HIF-1α, hypoxia-inducible factor; HIF-1α, hypoxia-inducible factor-1α; HMGB1, high-mobility group box 1; HRP, horseradish peroxidase; iBMX, isobutylmethylxanthine; iEQ, ietl equivalent quantity; Mc1-1, myeloid cell leukemia-1; NICC, neonatal islet cell clusters; OCR, oxygen consumption rate; PBS, phosphate buffer saline; PI, propidium iodide; PI3K/Akt, phosphatidylinositol 3-kinase/protein kinase; PVC, polyvinyl chloride; qPCR, quantitative polymerase chain reaction; RNA, ribonucleic acid; RNase-IGF-1, insulin-like growth factor-1; SD, standard deviation; SPSS, Statistic Package for Social Science; TAT, thrombin-antithrombin; 7-AAD, 7-aminoactinomycin D; TMRE, tetramethylrhodamine ethyl ester perchlorate; VEGF, vascular endothelial growth factor; WBC, white blood cells.
**1 | INTRODUCTION**

Islet transplantation is an effective therapy for severe diabetes. Nevertheless, the short supply of donor pancreases constitutes a formidable obstacle to its extensive clinical application. NICC which are capable of hormonal maturation and reversing hyperglycemia in animals and non-human primates have been accepted as a potential source of beta cells for transplantation. However, the degree of loss in NICC number and function caused by apoptosis was unacceptably high and remains a barrier to clinical application for islet xenotransplantation. Islets apoptosis occurs following their isolation as well as in the early stage after transplantation. Studies have shown that during the isolation, islets are exposed to variety of cellular stresses which disrupts the cell-matrix relationship and causes loss of vasculature and eventual hypoxia, thereby leading to apoptosis. Poor oxygenation in the immediate post-islet transplantation period is also recognized as a possible reason for islet loss and dysfunction, and up to greater than 50% of islets could be lost in this period. Furthermore, when transplanted intrahepatically, islet oxygenation is susceptible to being affected by thrombus formation mediated by instant blood-mediated inflammatory reaction (IBMIR), which therefore results in hypoxia and/or nutrient deficiency that in turn to induce both apoptosis and necrosis of islet cells, especially β cells. The demise of β cells not only contributes to insufficient β-cell graft, but also triggers infiltration and activation of macrophages in the islets by releasing high-mobility group box 1 (HMGB1) and cyclophilin A, which, in turn, enhances inflammation and accelerates attack by immune cells. Thus improving the ability of NICC to resist apoptosis is essential to achieve effective islet xenotransplantation.

EGM2 medium is originally used for stem cell or endothelial progenitor cell differentiating into endothelial cells. EGM2 medium supplement set contains such components of FGF-b, VEGF, R3-IGF-1, ascorbic acid, hEGF, heparin that are not included in conventional NICC culture medium Ham’s F-10. It has been shown FGF-b is responsible for the endothelial differentiation. VEGF can increase the islet integrity and survival, EGF can stimulate the beta-cell regeneration, and IGF-1 can help cell differentiation and repair. As such, we hypothesized that selection of EGM2 medium for culturing NICC may improve their survival and/or function in tough environments during in vitro culture and post-transplantation.

In this study, we present a simple manipulation of NICC by changing conventional culture medium to modified EGM2 as a means of effectively improving their yield, viability, in vitro maturation and function and more importantly, their ability to survival in hypoxia and nutrient deficiency environments.

**2 | MATERIALS AND METHODS**

**2.1 | Animals**

Three- to five-day-old newborn landrace pigs of either sex were purchased from local farms and were used for the preparation of porcine islets. The animal study was approved by the Animal Ethics Committee of the Third Xiangya Hospital.

**2.2 | Preparation of porcine islet-like cell clusters**

NICC were isolated from donor pancreas as described previously. Modified EGM2 medium was prepared with the EGM2 supplement set (Lonza, Basel, Switzerland) consisting of 7 separated individual components of FGF-b, VEGF, R3-IGF-1, ascorbic acid, hEGF, heparin and hydrocortisone according to the manufacturer’s instructions, but without adding hydrocortisone in order to avoid its cytotoxicity to neonatal β-cells. The resulting modified EGM2 was named EGM2 thereafter throughout the text. Isolated NICC were cultured at 37°C, 5% CO2 in EGM2 medium or conventional Ham’s F-10 medium (Gibco™, ThermoFisher Scientific, Waltham, Massachusetts, USA) for 7 days. Both media were supplemented with 50 mmol/L isobutylmethyloxanthine, 10 mmol/L nicotinamide, 10 mmol/L glucose (all from Sigma-Aldrich, St. Louis, Missouri, USA), and 10% porcine serum (Gibco™ Molecular Probes™ Porcine Serum). The day after isolation was counted as day 1. Modified EGM2 medium was used at day 1 and then changed every second day thereafter. Seven days after culture, NICC were collected for the following assays.

**2.3 | AO/EB staining**

NICC samples were taken at different time points after culture for AO/EB staining (Perlemiker, Shanghai, China) according to the manufacturer’s instructions. Images of AO/EB staining were taken by AMG.
2.4 | In vitro test of NICC function

At the day 7, glucose-stimulated insulin release was measured and expressed as the stimulation index, calculated as the ratio of insulin release in high (25.0 mmol/L) with 1 mmol/L IBMX to low (2.5 mmol/L) glucose during 60 minutes of static incubation in Krebs’ Ringer bicarbonate buffer. Insulin levels were determined with an insulin immunoradiometric assay kit (Biosource, Nivelles, Belgium).

2.5 | Oxygen consumption rate (OCR) measurement

OCR of NICC cultured with EGM2 or Ham’s F-10 was measured with XF24 respirometer platform (Seahorse Bioscience XF24 Extracellular Flux Analyzer, Agilent, Santa Clara, California, USA). A total of 200 NICCs/well (XF24 islet capture microplates, Seahorse Bioscience) were washed twice with HBSS/HSA, and rested in modified XF media (XF media from Seahorse Bioscience, Agilent) were supplemented with 0.1% fetal calf serum and 3 mmol/L glucose for 1 hour at 37°C without CO2. The following compounds were added to test cellular function (Figure 3 and Table 1): 20 mmol/L glucose was added to determine XF response to high glucose; 5 μmol/L oligomycin was added to inhibit ATP synthase and measure coupling efficiency; 2 μmol/L of carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) was added to uncouple the mitochondrial oxidative phosphorylation and measure both maximum respiration and spare capacity; 5 μmol/L rotenone and 5 μmol/L antimycin D were added to inhibit the respiratory chain and measure non-mitochondrial respiration (all from Seahorse Bioscience, Agilent). After XF24 respirometer platform recording, NICC were collected for DNA content analysis using Quant-it PicoGreen dsDNA Assay (Life Technologies, Waltham, MA, USA). All the data were analyzed with the software wave desktop according to the manufacturer’s instructions.

2.6 | Flow cytometry

Accutase-dispersed (Sigma-Aldrich, St. Louis, MO, USA) single NICC cells were stained with TMRE (tetramethylrhodamine ethyl ester perchlorate, Invitrogen, Waltham, MA, USA) and 7-AAD (7-aminomitoctinomycin D, Invitrogen) to determine NICC viability, and with Annexin V and PI using Alexa Fluor® 488 Annexin V/Dead Cell Apoptosis Kit (Invitrogen) to examine NICC apoptosis. CD31 (CD31-PE, BD Pharmingen, San Diego, CA, USA) and Newport Green™ PDX (Invitrogen) were used to detect the endothelial cells and beta cells in NICC cultured with either medium for 7 days, respectively. The proportion of endothelial cells or β cells in NICC was calculated by Flow Jo software.

2.7 | Real-time PCR

Total RNA was isolated from NICC with the TRizol reagent (Invitrogen) according to the manufacturer’s instructions, followed by cDNA synthesis using the SuperScript™ IRT (Invitrogen). Real-time PCR was performed with Stratagene Mx3000P (Agilent Technologies, Redwood City, CA, USA) using SYBR Green qPCR Superscript-UDG universal PCR Master Mix (Invitrogen). cDNA was subjected to PCR with PCR primers specific for porcine insulin (sense: 5′-GGAGCCTCTGTATGAGAAGTCT-3′ and anti-sense: 5′-CGAGGCTTAGAAATGTT-3′), glucagon (sense: 5′-ACATTGGCAACCGTCAGATG-3′ and anti-sense: 5′-GGCTCTCCTCGGCCTTTCA-3′), and somatostatin (sense: 5′-CCAACCCAGCCGGAATGAT-3′ and anti-sense: 5′-CCATAGCGGGGTTTGAATTATGATA-3′). The reaction was amplified as follows: 40 cycles of 95°C for 15 seconds, 61°C for 20 seconds, and 72°C for 10 seconds followed by an extension at 72°C for 10 minutes. PCR reaction with porcine β-actin primers (sense: 5′-CACGGCATCTCGTGTTGAGTTA-3′ and anti-sense: 5′-AGCACCCTGTTGCGTATGG-3′) was used as an internal control.

2.8 | Western blotting

Western blotting was performed as previously described. Briefly, after preparation of NICC samples, blotting membranes were incubated at 4°C for overnight with anti-VEGF (A-20) and anti-HIF-1α (SC-53546) antibodies (Santa Cruz Biotechnology, Santa Cruz, California, USA), Pro-Survival Bcl-2 and Pro-Apoptosis Bcl-2 Family Antibody Sampler Kits (Cell Signaling Technology, Danvers, MA, USA), respectively. Anti-β-actin antibody was used as an internal control for Western blotting.

2.9 | Tubing loop model of blood-islet interactions

This study was approved by the Human Research and Ethics Committee of the Third Xiangya Hospital. A tubing loop model of

### TABLE 1 Oxygen consumption rate analyses of NICC cultured with Ham’s F-10 or EGM2

<table>
<thead>
<tr>
<th></th>
<th>Ham’s F-10 pmol/min/ug DNA</th>
<th>EGM2 pmol/min/ug DNA</th>
<th>P</th>
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<tr>
<td>Glucose response</td>
<td>18.539 ± 3.361</td>
<td>32.859 ± 14.705</td>
<td>.0273</td>
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<td>Coupling efficiency</td>
<td>80.551 ± 19.064</td>
<td>93.501 ± 14.811</td>
<td>.1813</td>
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<tr>
<td>Proton leak</td>
<td>25.925 ± 6.633</td>
<td>52.130 ± 19.975</td>
<td>.0064</td>
</tr>
<tr>
<td>Max capacity</td>
<td>158.031 ± 26.173</td>
<td>228.611 ± 54.375</td>
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<tr>
<td>Spare capacity</td>
<td>77.117 ± 12.321</td>
<td>57.291 ± 37.452</td>
<td>.2081</td>
</tr>
<tr>
<td>ATP production</td>
<td>59.447 ± 16.945</td>
<td>119.837 ± 25.862</td>
<td>.0007</td>
</tr>
</tbody>
</table>

Data represent mean ± SD of 3 independent experiments, each performed in 7 replicates.
blood-islet interactions was performed as described previously. Three pig donors were used for each of 9 independent loop experiments. At day 7 of culturing, 1000 NICC IEQ was suspended in 100 uL of PBS and transferred to a heparin-bonded PVC tube (inner diameter 6.3 mm, length 450 mm) (Medtronic Inc., Minneapolis, Minnesota, USA), followed by addition of 7 mL of fresh human blood to each loop containing control medium cultured NICCs or EGM2 medium cultured NICCs. The loops were then placed in a shaking incubator at 37°C for 60 minutes prior to collecting samples for the subsequent assessments. Blood clots were measured for weight, and remaining blood was collected for hematological analysis using Coulter ACT-diff analyzer (Beckman Coulter, Miami, Florida, USA) and coagulation activation (thrombin-antithrombin (TAT) using commercially available EIA kits and assays of C3a complement activation using ELISA assay kits (BD Biosciences, San Jose, CA, USA).

2.10 | Immunohistochemistry

For analyzing the apoptosis of NICCs in blood clots, 4- to 6-um tissue sections of paraffin-embedded blood clots were used for tunnel staining using in situ Apoptosis Detection Kit (Abcam,Cambridge, UK) according to the manufacturer's instructions. And sections (5 μm) of NICC samples were also incubated with guinea pig anti-porcine insulin antibody (Invitrogen) overnight at 4°C. After washing, the sections were incubated with a corresponding HRP-conjugated secondary antibody dilution (goat anti-guinea pig, Santa Cruz Biotechnology) for 60 minutes. The reaction was then developed using diaminobenzene (DAB) (Agilent) as substrate.

2.11 | Statistical analysis

Comparisons involving 2 groups were evaluated using the Student's t test, and those involving multiple groups were evaluated using ANOVA with Tukey's multiple comparison test (JMP, version 4.02 software, SAC, USA) and were presented as mean ± SD. P < .05 was considered significant.

3 | RESULTS

3.1 | Culturing with EGM2 medium increased NICC number and viability

To evaluate the effect of EGM2 medium on the yield and viability of NICC in culture, an equal 15 000 NICC were cultured with either conventional Ham's F-10 or EGM2 medium for 9 days. NICC number was counted every other day from day 1 to day 9 of culture. The results showed a gradual decrease in NICC number in both culture media from day 1 to day 9 (Figure 1A), showing that the number NICC cultured with Ham's F-10 was down to 7950 ± 650 and 3825 ± 875, at day 7 and 9, respectively. However, compared to their Ham's F-10 cultured counterparts, EGM-2 cultured NICC demonstrated much less reduction in their number at the same
time points after culture (10480 ± 1075 at day 7 and 6780 ± 785 at day 9, respectively) (Figure 1A). As NICC at day 7 of culture have been shown in good form for transplantation, we next examined NICC viability by flow cytometry on day 7 of culture. Much more viable cells (TMRE single-positive staining cells) (82.82% ± 2.85% vs 68.98% ± 3.29%, \( P < .05 \)) were detected in NICC cultured with EGM2 medium than that in Ham's F-10 medium (Figure 1B). Consistently, more NICC cultured in EGM2 medium at day 7 were stained in green (viable cells) by AO/EB staining with very few stained in red (dead cells) when compared to their counterparts cultured in Ham's F-10 medium (Figure 1C). Taken together, these results indicate increased number and viability of NICC by culturing them in EGM2 medium.

3.2 EGM2 medium improved NICC in vitro function and upregulated endocrine hormone genes expression

To evaluate insulin release function of NICC, the static insulin secretion was measured using 200 IEQs of NICC from day 7 of culture. Considering that an immature characterization of neonatal porcine islets with much lower or undetectable insulin secretion compared to their adult counterparts in response to glucose alone stimulation, we included IBMX, 1 of cAMP increase agents in glucose stimulation to enhance insulin secretion by NICC, thereby achieving detectable insulin secretion after glucose stimulation. Results are presented in Figure 2, showing a significant higher insulin level secreted under a high glucose with IBMX condition by NICC from the culture in EGM2 medium when compared to their counterparts cultured in Ham's F-10 medium, although a similar insulin release by NICC from both media was detected upon a low-glucose stimulation (Figure 2A). Consistently, EGM2 cultured NICC demonstrated a higher stimulation index than that shown by NICC cultured in Ham's F-10 medium (Figure 2B). To investigate whether culturing with EGM2 increased \( \beta \) cells in NICC, the proportion of \( \beta \) cells from either medium was analyzed by FACS using Newport Green to specifically stain zinc-positive \( \beta \) cells, showing no difference in \( \beta \)-cell proportion of NICC regardless of being cultured in which medium (Figure 2C). The results indicate that EGM2 medium cultured NICC did not contain more \( \beta \) cells but were more sensitive or more mature to the change in glucose concentration, thereby suggesting a better in vitro function than their control counterparts.

**FIGURE 2** NICC function on day 7 of culture. A, Insulin release of NICC in different culture medium. B, Static assay of insulin secretion by NICC. Insulin stimulation index was calculated as the ratio of insulin release in high (25.0 mmol/L) glucose in the presence of 1 mmol/L 3-isobutyl-1-methylxanthine (IBMX) to low (2.5 mmol/L) glucose. C, Newport Green staining for beta cells of NICC. D, Insulin, glucagon, and somatostatin expression of NICC on day 7 of culture. All data are presented as mean ± SD of 3 independent experiments with NICC from 3 individual donor pigs. *\( P < .05 \), compared with cultured in Ham's F-10 medium.
The effect of EGM2 culture medium on islet endocrine gene expression was performed by real-time PCR showing markedly upregulated expression of both insulin and glucagon genes detected in EGM2 medium cultured NICC when compared to their counterparts from Ham’s F-10 medium, whereas no significant difference in somatostatin gene expression observed (Figure 2D). Collectively, these results suggested a positive impact of EGM2 culture medium on the integrated function of NICC in islet xenotransplantation.

### 3.3 EGM2 medium improved NICC metabolic function in vitro

To determine the effect of EGM2 medium on NICC viability, the metabolic function of NICC was measured on an XF analyzer using several bio-energetic parameters associated with cellular functionality. OCR assay was performed on NICC cultured with Ham’s F-10 or EGM2 medium. The overall respiration rate of NICC cultured with EGM2 medium was increased compared to Ham’s F-10 cultured NICC (Figure 3A), and in particular, the basal OCR/DNA of EGM2 cultured NICC was significantly higher than that of their Ham’s F-10 cultured counterparts (171.320 pmol/min/μg ± 48.095 SD vs 80.913 pmol/min/μg DNA ± 19.579 SD, P < .001). Moreover, culturing with EGM2 medium improved basal NICC function by about 52.77% (Figure 3B), which was consistent with the findings of AO/EB staining and FACS analysis (Figure 1B&C). Analyses of the cumulative XF data from 3 independent experiments demonstrated that compared to Ham’s F-10 cultured NICC, EGM2 medium cultured NICC exhibited substantially increased maximum capacity (P = .0093), and a significant improvement in proton leak (P = .0064), glucose response (P = .0273), and ATP production (P = .0007) (Table 1). However, there was no noticeable difference in coupling efficiency and spare capacity in NICC cultured with EGM2 or Ham’s F-10 medium.

![FIGURE 3](image)

**FIGURE 3** Oxygen consumption rate analyses demonstrated metabolic function improvement of NICC in EGM2 cultured medium. A. Intracellular levels of OCR for the NICC after cultured with EGM2 medium (red line) compared with Ham’s F-10 medium cultured group (black line) in response to various stimuli as indicated A.20 mmol/L glucose, B. Oligomycin, C. FCCP, D. rotenone. B. Cumulative average mean baseline OCR of EGM2 medium cultured NICC vs Hamster F-10 medium cultured NICC. Data represent mean ± SD of 3 independent experiments, each performed in 7 replicates. Whiskers represent 5% to 95% spread of the data. ***P < .001

### 3.4 Modified EGM2 medium could enhance NICCs’ ability to resist apoptosis

Next, we used tubing loops assay, a simple model to mimic IBMIR in vitro, to test whether NICC cultured with different medium have different ability to overcome IBMIR which is a hypoxia environment during transplantation procedure. To assess the viability of NICB after IBMIR, blood clots collected from loops were examined by immunohistochemically staining with TUNEL. From H&E and insulin staining, islets were found in the clots from loops with NICCs cultured by Ham’s F-10 or EGM2 (Figure 4A-D). More TUNEL-positive staining cells were found in entrapped islets collected from loops cultured with Ham’s F-10 compared to the modified EGM2 cultured ones (12 ± 4/NICCs vs 4 ± 2/NICCs, P < .05) (Figure 4G), suggesting there was more apoptosis cells in Ham’s F-10 cultured NICCs. And also, we tested the platelets, white blood cells, monocytes, and neutrophils (Table 2). The t tests showed that the consumption of platelets (12 ± 3 in the loops with EGM2 cultured NICCs vs 9 ± 2 in the loops with Ham’s F-10 cultured NICCs, P > .05), white blood cells (WBC) (1.38 ± 0.19 in the loops with EGM2 cultured NICCs vs 1.22 ± 0.14 in the loops with Ham’s F-10 cultured NICCs, P > .05), monocytes (0.02 ± 0.01 in the loops with EGM2 cultured NICCs vs 0.18 ± 0.04 in the loops with Ham’s F-10 cultured NICCs, P > .05), and neutrophils (0.25 ± 0.02 in the loops with EGM2 cultured NICCs vs 0.31 ± 0.04 in the loops with Ham’s F-10 cultured NICCs, P > .05) has almost no difference between the EGM2 cultured NICCs and Ham’s F-10 cultured NICCs incubated with human blood. Consistent with these findings, significantly increased activation of TAT (5328 ± 113 ug/L) and complement C3a (79.7 ± 8.7 ng/mL) was also detected in the loops with EGM2 cultured NICCs and Ham’s F-10 cultured NICCs (4969 ± 134 ug/L; 87.2 ± 7.2 ng/mL). All the data suggested that EGM2 cultured NICC did not reduce the severity IBMIR but had enhanced capability to resist apoptosis.

### 3.5 Culturing with EGM2 medium improved the ability of NICC to resist hypoxia-induced apoptosis in vitro

Since that more than 7 days is required for islet revascularization after transplantation, coping with hypoxia becomes 1 of key factors to improve NICC survival and function in islet xenotransplantation. To assess whether application of EGM2 medium for NICC culture was able to improve their survival subjected to hypoxia,
NICC treated with 150μm CoCl₂ which mimics a hypoxia environment were collected at 24 hours and 48 hours, respectively, for analysis of hypoxia-inducible factor-1α (HIF-1α) protein expression to measure cellular responses to hypoxia induction. CoCl₂ treatment increased HIF protein expression in NICC in cultured either medium at a time-dependent manner compared to that without CoCl₂ treatment (Figure 5A), indicating a successful hypoxia induction. But there was no significant difference between Ham’s F-10 cultured and EGM2 cultured NICC. We further investigated there was a correlation of HIF protein expression with NICC apoptosis by FACS analysis of NICC viability, showing that after 24 hours CoCl₂ treatment, no significantly increased apoptosis was detected in both EGM2 and Ham’s F-10 medium cultured NICC (Figure 5B). This may be due to a natural resistance of neonatal porcine islets.

**FIGURE 4** TUNEL staining showed apoptosis cells in NICCs entrapped by blood clot. A and B, H&E staining showed NICCs cultured by control medium or EGM2 medium were entrapped in the thrombosis. C and D, Insulin staining for β cell in NICCs cultured by control medium or EGM2 medium. E and F, TUNEL staining showed apoptosis cells in entrapped NICCs cultured by control medium or EGM2 medium. G, Average apoptosis cell in entrapped islets collected from loops cultured with control medium compared to the EGM2 cultured ones. Data represent mean ± SD of 3 independent experiments.
However, when increased time for CoCl$_2$ treatment to 48 hours, the number of Ham’s F-10 medium cultured NICC undergoing necrosis or late apoptosis was significantly increased compared to their EGM2 medium cultured counterparts (15.67% ± 1.29% vs 2.61% ± 0.081%, $P < .05$). In addition to induction of increased necrosis and late apoptosis in Ham’s F-10 medium cultured NICC, 48 hours treatment with CoCl$_2$ resulted in a significant loss of their living cells compared with that shown by EGM2 medium cultured NICCs (81.21% ± 1.40% vs 96.72% ± 0.081%, $P < .05$). Thus, our data clearly demonstrated the ability of EGM2 medium cultured NICC to cope with hypoxia environment for survival.

As it has been shown that increase in VEGF production by β cells modulated intra-islet vasculature and promoted β-cell regeneration and expansion, we then investigated whether a hypoxia environment had any impact on the expression of VEGF in EGM2 medium cultured NICC. As a result, whereas no VEGF expression was detected in NICC cultured with Ham’s F-10 before and after CoCl$_2$ treatment, EGM2 medium cultured NICC demonstrated upregulated

**TABLE 2** Blood cell counts, complement, and coagulation analysis

<table>
<thead>
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<th>Ham’s F-10 culture NICCs</th>
<th>EGM2 cultured NICCs</th>
<th>No NICCs</th>
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<tbody>
<tr>
<td>Platelets (×10$^9$/L)</td>
<td>9 ± 2</td>
<td>12 ± 3</td>
<td>174 ± 10</td>
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<tr>
<td>WBC (×10$^9$/L)</td>
<td>1.22 ± 0.14</td>
<td>1.38 ± 0.09</td>
<td>4.85 ± 0.15</td>
</tr>
<tr>
<td>Monocytes (×10$^9$/L)</td>
<td>0.18 ± 0.04</td>
<td>0.02 ± 0.01</td>
<td>0.43 ± 0.04</td>
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<tr>
<td>Neutrophils (×10$^9$/L)</td>
<td>0.31 ± 0.04</td>
<td>0.25 ± 0.02</td>
<td>2.4 ± 0.32</td>
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<tr>
<td>TAT (ug/L)</td>
<td>4969 ± 134</td>
<td>5328 ± 113</td>
<td>260 ± 65</td>
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<tr>
<td>C3a (ng/mL)</td>
<td>87.3 ± 9.2</td>
<td>79.7 ± 8.7</td>
<td>36.4 ± 5.8</td>
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</table>

Data represent mean ± SD of 3 independent experiments.
level of VEGF expression at 48 hours after CoCl₂ treatment compared to an undetectable expression level of NICC in Ham’s F-10 medium (Figure 5C), suggesting that upregulated VEGF protein expression in EGM2 medium cultured NICC may promote their survival from injury by hypoxia in vivo.

3.6 | EGM2 medium cultured NICC were endurable to serum starvation

As both islet viability and function are severely impaired when they are exposed to a nutrient-deficient environment immediate after transplantation, we tested whether culturing in EGM2 medium could improve NICC survival under serum starvation condition. After 7 days in original EGM2 or Ham’s F-10 cultures with 10% porcine serum, NICC were collected for a further culture in fresh EGM2 and Ham’s F-10 media, respectively, with only 1% porcine serum. NICC were harvested at 24 hours or 48 hours after serum starvation for staining with Annexin V and PI prior to analysis of apoptosis by FACS. The results showed that 24 hours culturing with serum starvation had no significant impact on early apoptosis in EGM2 medium cultured NICC when compared to that without under the starvation condition (Figure 6A). By contrast, a considerable number of cells from NICC cultured in Ham’s F-10 medium underwent early apoptosis at the same time point after serum starvation. Increase in time under serum starvation condition to 48 hours led to more cells dying in Ham’s F-10 medium cultured NICC from early apoptosis than NICC cultured in EGM2 medium (61.61% ± 1.86% vs 36.85% ± 3.36%, P < .05) (Figure 6A). EGM2 medium cultured NICC demonstrated their endurance in a nutrient-deficient environment. This led us to further investigate the possible mechanism involved by Western blotting analysis of protein expression of anti-apoptotic members of Bcl-2 family, including Bcl-2, Bcl-xl, Mcl-1 in NICC after serum starvation. Whereas culturing with either medium did not affect Bcl-2 protein expression significantly in NICC regardless of being under serum starvation condition or not, both Mcl-1 and Bcl-xl protein expression were upregulated in EGM2 medium cultured NICC before starvation compared to their Ham’s F-10 medium cultured counterparts (Figure 6B). The Mcl-1 protein expression in EGM2 cultured NICC was further increased under serum starvation in a time-dependent fashion, as evidenced by a further higher Mcl-1 expression level detected at 48 hours than that at 24 hours after serum starvation (Figure 6B). However, expression of pro-apoptotic members including bim, bak, and bad remained unchanged in NICC cultured in either medium under serum starvation condition (data not shown). Taken together, these results demonstrated that the enhanced endurance of NICC in a nutrient-deficient situation by culturing with EGM2 medium was associated with upregulated expression of anti-apoptotic Bcl-2 family member Mcl-1 in NICC.

4 | DISCUSSION

Pig-to-human islet cell xenotransplantation is currently progressing closer to clinical evaluation.²³ NICC have several advantages over adult pig islets as the preferred source of β cells for transplantation. However, a major drawback of their use is their immature state at the time of isolation, which required culturing for several days to promote their maturation. During culture days although the exocrine cells were eliminated due to apoptosis, a proportion of NICC could also die from loss of vasculature due to hypoxia.¹⁴ Moreover, in vivo, islet mass loss occurs immediately after intraportal transplantation subjected to hypoxia caused by IBMIR.⁶ As our study showed that culturing with EGM2 medium enhanced NICC resistance to apoptosis in despite of suffering from IBMIR with the similar severity to Ham’s F-10 medium cultured NICC detected in the tubing loops assay, it suggests that culturing with EGM2 medium may improve NICC survival in an IBMIR environment after transplantation. Despite the fact that transplantation of encapsulated islets intraportally or transplantation of islets to alternative sites, such as greater omentum or intramuscularly,²⁵ may reduce islet injury caused directly by IBMIR, a hypoxia and/or nutrient-deficient environment during the first days post-transplantation is still a major contributor to loss of islets due to apoptosis.²⁶ Therefore, considering that sufficient viable pig islets are required for successful islet xenotransplantation, improving of yield, viability, maturation, and survival of NICC by enhancing their resistance to apoptosis under the environment of hypoxia during culture and transplantation may be a feasible and cost-efficient approach to achieve better transplantation outcome.

Several strategies have been investigated for the improvement of the viability of islets or the acceleration of islet proliferation by the addition of various growth factors and compounds,²⁷ including human albumin, insulin, sericin, and glutamine compounds into the conventional islet cell culture medium. However, their effects on NICC survival in a hypoxia/nutrient-deficient environment are yet to be determined. In this study, the effect of EGM2 medium on NICC in vitro survival was examined. As islet OCR measurement is a sensitive assay of their viability²⁸, our findings of greater mitochondrial capacity and better viability detected by OCR assessment in EGM2 medium cultured NICC than that observed in NICC cultured with Ham’s F-10 medium further confirmed the enhanced NICC capacity to survive during culture shown by both the AO/EB staining and FACS analyses in this study. Compared to that cultured in conventional Ham’s F-10 medium, NICC cultured in modified EGM2 medium exhibited increased number, enhanced viability and in vitro insulin secretion, upregulated expression of endocrine hormone genes, and more impressed, enhanced capability to resist apoptosis resulted from hypoxia and/or nutrient deficiency. Thus, these findings suggested a potential effective approach to improving outcome in islet xenotransplantation. The effect of EGM2 culture medium on characteristics of NICC was most likely attributed to the combined contributions from their components, including FGF-1, VEGF, R3-IGF-1, ascorbic acid, hEGF, heparin that Ham’s F-10 medium does not contain. Among those components, growth factors VEGF, IGF-1, and EGF are known to be beneficial collectively for islet cell integrity, survival, regeneration, and differentiation.¹⁰¹¹ However, the accurate mechanism(s) for all these factors, in a combination or in isolation, to improve NICC function and enhance
their resistance to apoptosis need be further explored and eventually, evaluated in an islet xenotransplantation animal model.

As both fetal and neonatal pancreas contain predominantly pancreatic precursor cells that require some months of maturation in vivo before, they develop into mature beta cells and provide adequate glucose control. NICC xenotransplantation outcome may also be improved by optimizing the isolation and culture conditions to minimize the time required for their maturation in vivo. A recent study has shown that extension of the time of NICC in Ham’s F-10 media from standard 6-day culture to 19 days in culture results in increased ATP production by NICC, an indicator of islet health and maturation over time, and which, consequently, led to shorten the time required for normalizing blood glucose levels from 63 days to 32 days when transplanted to diabetic mice compared to day-6 NICC. This
study suggests the importance of NICC maturation in the improved efficiency in transplantation. Our findings of increased insulin release upon glucose stimulation in high concentration, upregulated levels of insulin gene expression and increased ATP production by EGM2 medium cultured NICC with unchanged proportion of insulin secreting β-cells indicate that culturing with EGM2 medium for only 6 days can also promote NICC maturation, but not the differentiation of pancreatic precursor cells within NICC into β-cells in vitro, suggesting a possible improvement in NICC function in vivo after transplantation. However, which particular components in EGM2 medium by which mechanisms act in promoting NICC maturation remain to be determined.

EGM2 but not Ham’s F-10 medium cultured NICC expressed upregulated level of VEGF protein under the hypoxia condition, suggesting the involvement of VEGF in EGM2 medium enhanced endurance of NICC in hypoxia, due to, at least in part the maintenance of intact vasculature in NICC promoted by VEGF. This hypothesis is supported by studies that when co-transplantation of pancreatic islets with endothelial progenitor cells (EPCs) or endothelial cells (ECs) into diabetic recipient mice, the revascularization of islets occurred more rapidly than transplantation of porcine islets alone. The rapid revascularization was mainly by stimulating VEGF production from porcine islet grafts rather than the direct incorporation of islet with EPCs or ECs. Collectively, these studies suggest that VEGF contained in EGM2 medium plays an important role in improving the ability of NICC to resist hypoxia during in vitro culture by maintaining or promoting the vascularization of NICC and their production of VEGF, and in turn, NICC-produced VEGF can further enhance the endurance of NICC in a hypoxia environment, including in vivo in the immediate period after transplantation. Indeed, we detected an increased number of CD31-positive endothelial cells within NICC 7 days after culture in EGM2 but not control medium (8.10% ± 0.22% vs 3.92% ± 0.30%, P < .05) (data not shown), which may help enhancing the revascularization of NICC for them to better survive in any tough environment.

Pancreatic islets have a dense capillary network, while islet isolation destroyed pancreatic islet blood vessels, which compromises the survival of cells due to insufficient supply of oxygen and nutrient. A period of 10-14 days post-transplantation is needed for islet revascularization, which leads to a poor nutrient and hypoxia microenvironment for islets to survive. Hence, improving the ability of NICC to survive in a nutrient-deficient situation is essential to ensure their application in islet xenotransplantation. Our data showed that much less apoptosis was induced under the serum starvation condition in EGM2 but not Ham’s F-10 medium cultured NICC, and this increased endurance in the nutrient-deficient environment correlated with upregulated gene expression of anti-apoptotic Bcl-2 family members Mcl-1. Unlike other Bcl-2 family members, Mcl-1 is rapidly transcribed via a PI3K/Akt-dependent pathway, resulting in its increased expression during cytotoxic stimuli and cytokine stimulation. Thus, the finding of remarkably high levels of Mcl-1 protein only detected in EGM2 medium cultured NICC before and 24 hours after the serum starvation suggests that some growth factors contained in EGM2 medium may stimulate Mcl-1 expression in NICC through PI3K/Akt pathway to increase their resistant to nutrient deficiency-mediated apoptosis as reported in other studies. However, this hypothesis needs to be tested in a separate study.

In summary, our study supports the hypothesis that changing NICC culture medium from conventional Ham’s F-10 to EGM2 is a simple and effective approach to promote NICC maturation to reduce the time required for their maturation in vivo and to improve their capability to resist apoptosis induced by hypoxia and nutrient deficiency during culture and transplantation, thereby holding the potential to be developed into a clinically applicable strategy in islet xenotransplantation.

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AUTHOR CONTRIBUTION

Ma X, Yi S, and Wang W contributed to concept design. Ma X, Yang C, Zhang J, Wang J, Li W, Xu C, Jiang J, and Wu M performed experiments. Rong P and Ye B contributed to data acquisition and statistical analysis. Ma X and Wang J contributed to drafting of the manuscript. Yi S contributed to final reviewing of the manuscript. All authors read and approved the final manuscript.

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