Hypoxia and iPSC: A Low Oxygen Atmosphere Supports the Xeno-free Generation, Expansion, and Differentiation of Human Induced Pluripotent Stem Cells

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Abstract

We previously demonstrated that using low oxygen tension (4 %) increased the efficiency of reprogramming human somatic cells to pluripotency (Figure 1). In this study, we extend our findings and further increased the development of this culture paradigm using different coating substrates. We are able to observe that post-electroporated fibroblasts cultivated at 4 % O_2 grow on synthetic and biological surfaces. Further, we observed normal spreading of iPSC and a high level of purity in early iPSC passages. The cells were successfully differentiated into motor neuron and cardiomyocyte lineages emphasizing the efficacy of low oxygen levels during cell cultivation.

4 % O_2 17 % O_2
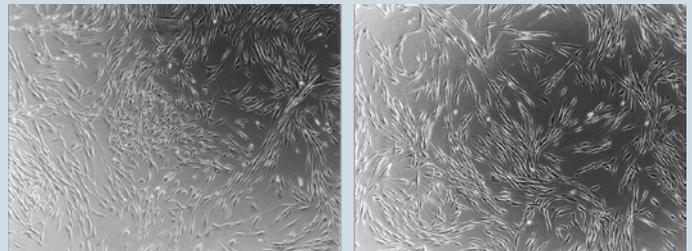
Figure 1: Generation of iPSCs under varying oxygen concentrations. After 12 days, early colony formation is seen in low O_2 cultures (left), whereas this is not observed as early in norm-oxy cultures (right).
Read more in Application Note No. 338: Low Oxygen Levels Enhance the Efficiency of Reprogramming Human Somatic Cells to Pluripotency

Introduction

Reprogramming human somatic cells into the pluripotent state is a subject of thousands of publications since their discovery in 2007 [1, 2]. Originally these studies were pioneered with a set of four genes found to be expressed in native human embryonic stem cells; Oct4, Sox2, KLF4, and c-Myc (Lin28, and Nanog), delivered using genetically modifying methods such as retrovirus, and cultured on non-defined matrices. In the 13 years since these seminal studies many improvements were made such as (1) replacing genetically modifying methods with non-genome altering safer alternatives; (2) replacement of c-Myc oncogene with non-transforming family member, L-Myc; (3) inclusion of small molecules to boost efficiency of reprogramming; (4) optimizing culture conditions which includes the use of low O_2 tension (4-5 %) and; (5) use of clinically relevant defined medias and matrices [3-11]. In this novel study, we combined many of the improvements to demonstrate the successful reprogramming of human foreskin fibroblasts with low O_2 conditions in the CellXpert® C170i CO_2 Incubator and an improved upon single fluorescent and selectable polycistronic Episomal vector as described in Application Note 338. We observed that the tested growth substrates offered stable cell adhesion and spreading following electroporation of fibroblasts, which in turn led to a robust production of karyotypically normal iPSC colonies capable of robust expansion in a defined media, and ability to differentiate into neural and cardiac lineages.

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Materials and Methods

The reagents and procedures used in this Application Note are similar to that described in Application Note 338. Notable differences or updates are summarized below, and more in-depth details are seen in Appendix 1. We have improved the pERCv1 plasmid as previously described by including a PGK promoter upstream from the EBNA-1 gene cassette (pERCv2, Figure 2, [12]). When indicated, culture vessels were coated with 5 ug/ml of Vitronectin as per manufacturer instructions (PeproTech®) and used in comparison with a synthetic substrate (Eppendorf FN1 motifs CCCadvanced® cultureware).

Once iPSC colonies appeared in Reprogramming Media, they were transitioned into Animal Free Low Protein hESC media for expansion (PeproTech, alternatively prepare as detailed in Chen et al., 2011 [13] or in Kuo et al., 2020 [14]). All iPSC expansion and early steps in neuronal differentiation was carried out with 6 well format dishes, whereas cells analyzed for immunostaining or terminally differentiated to motor neurons or cardiomyocytes were spin seeded (300 x g, 5 min at RT), onto 24 well format culture plates. Neurons were developed as described by Bianchi et al. 2018 [15] using the same base medias as described by Francis et al. 2019 [16]. Cardiomyocytes were generated using protocols as described by Burridge and Coworkers [17, 18].

Figure 2: The plasmid pERC-V2, is a novel all-in-one episomal based reagent for cellular reprogramming. This plasmid expresses 5 genes connected by 4 canonical "2A" self-splicing motifs: Oct4, Sox2, KLF4, L-Myc, and a Red fluorescence (RFP)/Blasticidin resistance (Blast) fusion protein, under the control of the chimeric CMV early enhancer/chicken beta actin (CAG) promoter. The RFP/Blast fusion protein allows for easy visualization and selective enrichment of positively transfected cells. This plasmid differs from that described in Application note 388 by the addition of the PGK promoter upstream from EBNA-1 region to upregulate its expression.

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Results and Discussion

Reprogramming of Fibroblasts
Twenty four hours after electroporation, the fibroblast cultures plated onto biological and synthetic coatings were observed for red fluorescence (Figure 3). Even though a smaller number of cells (0.75×10^6 vs. 1×10^6) were plated on the synthetic coating, it was clear that the cultures survived, and spread more robustly. This led to a failure to observe reprogramming in many of the Vitronectin plated cultures. We were able to observe reprogramming events as early as

11 days post modification (Figure 4). The normally evenly distributed spindle shaped fibroblasts adopted a cuboidal appearance and organized into rudimentary colonies in both electroporated (Fig 4 A/B) and lentiviral transduced (Fig. 4 c-d) cultures. After 11 days only a minor amount of RFP was observed in both types of reprogramming paradigms, indicating possible loss of plasmid (episomal), or epigenetic inactivation (lentiviral).
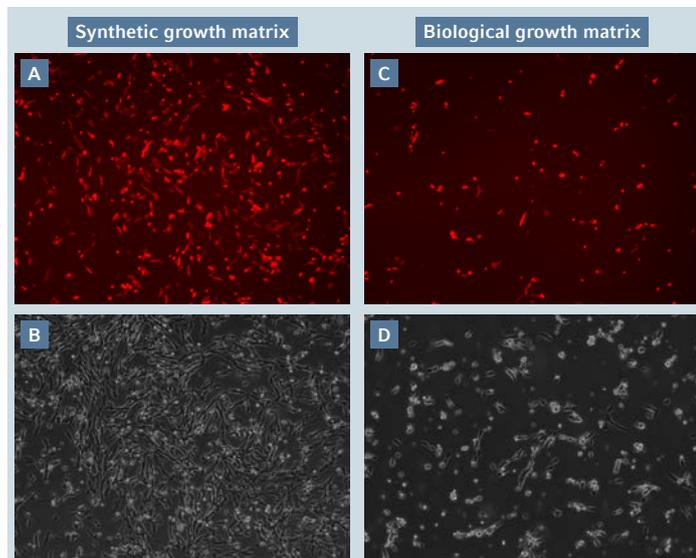
Figure 3: RFP-expression 24h post electroporation. Fibroblasts were removed from 10 cm uncoated TC grade dishes using TrypLE, electroporated, and plated onto 6-well plates coated with synthetic (0.75×10^6 cells in A/B), or biological (1×10^6 cells in C/D) substrates.

Figure 4: Appearance of presumptive reprogrammed colonies 11 days post electroporation (A/B) or lentiviral transduction (C/D). Fibroblasts (1×10^6) were electroporated as described or transduced with lentivirus containing the identical poly-cistronic supergene as seen in the Episomal plasmid. Cells under both conditions were cultured in a similar fashion. While RFP signal was significantly downregulated in both conditions (B/D), the 4 factors presumably reached a threshold for successful reprogramming at least by day 11 as seen in the appearance of small clusters (A/C). These small clusters went on to form mature looking colonies within 3 weeks.

Expansion and Characterization of iPSCs under hypoxic conditions
The rudimentary colonies shown in figure 4 gave rise to more mature colonies at day 21 to day 30 post modification and were passaged using gentle non-enzymatic methods (Figure 5). After 3 routine passages, we observed that the colonies derived and cultured on the synthetic matrix

(A/B vs. C/D) appeared purer than on Vitronectin, with less appearance of non-reprogrammed fibroblasts. In order to purify the culture of Vitronectin derived cells, manual picking of colonies was used, whereas iPSCs grown on synthetic substrates outgrew any minor number of fibroblasts that were dislodged during the non-enzymatic passaging.

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After 7 passages, the Episomal plasmid reprogrammed line from the synthetic coating was analyzed for karyotype and found to be normal (Figure 6). Similar cultures were spin seeded onto 24 well dishes and analyzed for routine pluripotency or differentiation markers. (Figure 7). At passage 8, the cultures were found to lack any appreciable expression of SSEA1, a marker of differentiation, and robustly expressed both the SSEA4 surface marker, and Oct4 transcription factor. Likewise, these cell co-expressed Lin28 cytoplasmic RNA binding protein, Nanog transcription factor and the Tra-1-60 cell surface marker.

Synthetic growth matrix   Vitronectin growth matrix
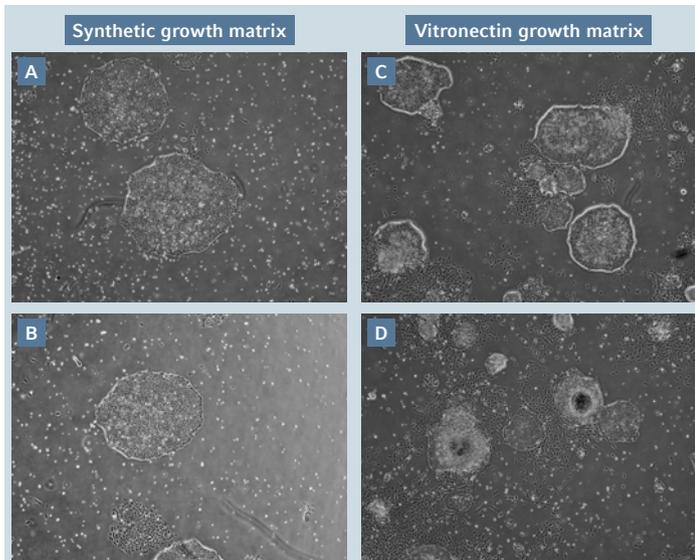
Figure 5: Standard passaging techniques with iPSCs grown on different substrates. iPSCs grown on synthetic (A/B) or Vitronectin (C/D) matrices were developed and passaged using described techniques. After 3 routine passages using PBS-EGTA, the colonies developed on Vitronectin appeared to differentiate around the edges more readily and carry more non-reprogrammed or spontaneously differentiating fibroblasts into the serial cultures.
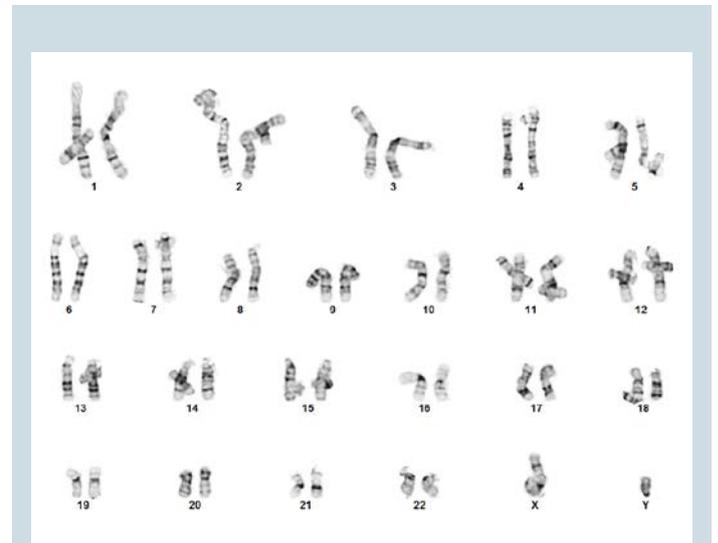
Figure 6: Karyotype analysis of PM1Ep5-ERCv2p7 iPS Cells. Cytogenetic analysis was performed on 20 G-banded metaphase cells and demonstrated normal male karyotype.
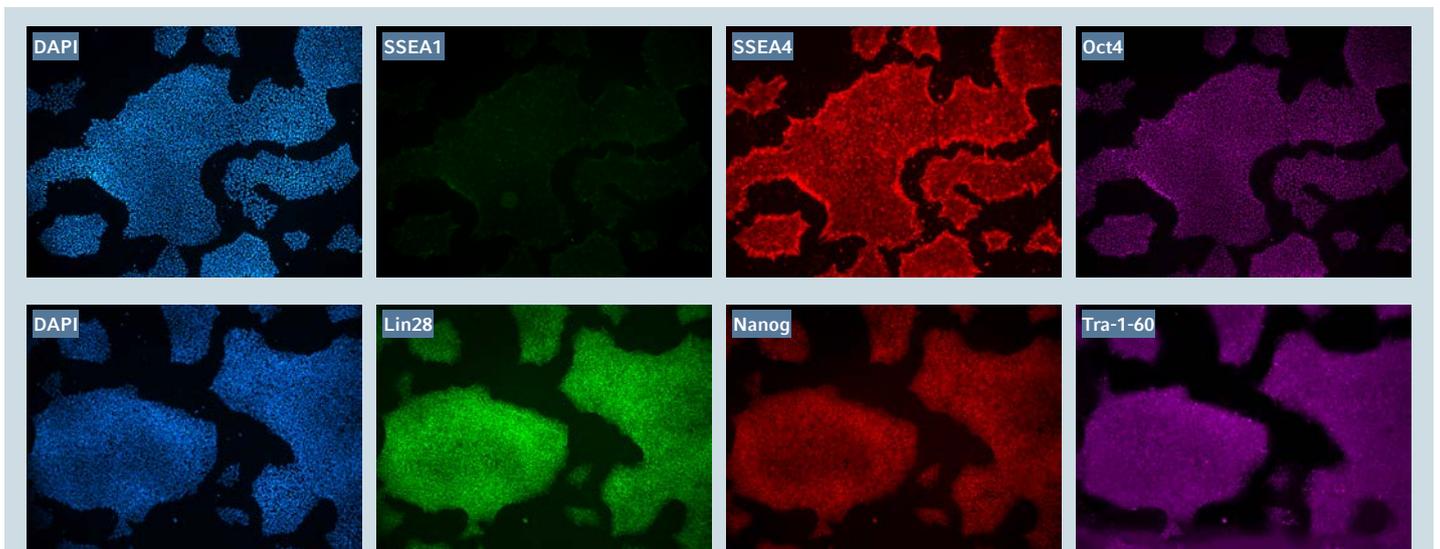
Figure 7: Immunofluorescence staining of markers for pluripotency and differentiation in low passage iPS cells

Differentiation of iPSCs to neuronal and cardiomyocyte lineages

Using standard published protocols, the cells were first differentiated into neuroepithelial cells (not shown) and then secondly into neural stem cells (Figure 8). After 4 passages in neural stem cell media, the cells appeared to differentiate into a uniform mat (8A), with repeating patterns of rosettes (8C), characteristic of neural stem cell stage. Many of these cells were OTX2+, with a greater number expressing both Pax6, and Nestin.
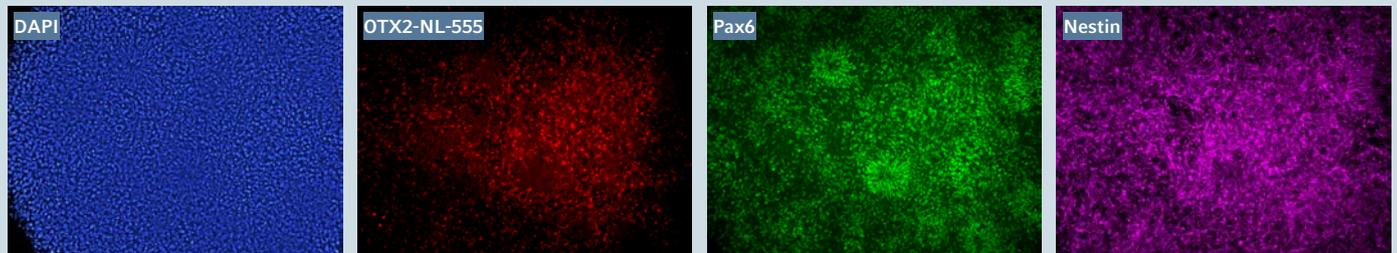
Figure 8: Differentiation of iPSC into neural stem cells in low oxygen environment; iPSCs were differentiated using protocol as described by Bianchi and coworkers. Cells at second stage (MN2) were expanded using FGF-2 and EGF and are described as neural stem cells (NSC).

Since this was the second step out of four to derived motor neurons, we continued to differentiate the stem cells into presumptive motor neuron precursors. The small molecules and growth factors were altered to continue the differentiation protocol (Figure 9, upper panel), and some of the cells continued to express OTX2 and Nestin, while many began to express Olig2, a marker of motor neuron lineage. After one week under these conditions, the cells were finally exposed to a set of cytokines and small molecules that induced the final differentiation into presumptive motor neurons (Figure 9, lower panel). After 7 days in the final media cocktail the cells organized a fine network of process which were robustly Neurofilament H+, with some remaining TUJ1+ appearance and few GFAP+ glial cells. Many of these cells expressing TUJ1, also expressed canonical markers of motor neurons, such as HB9, and Islet-1 (data not shown). Together these results clearly showed that hypoxic incubation can be used to derive cells of neuroectodermal lineage.
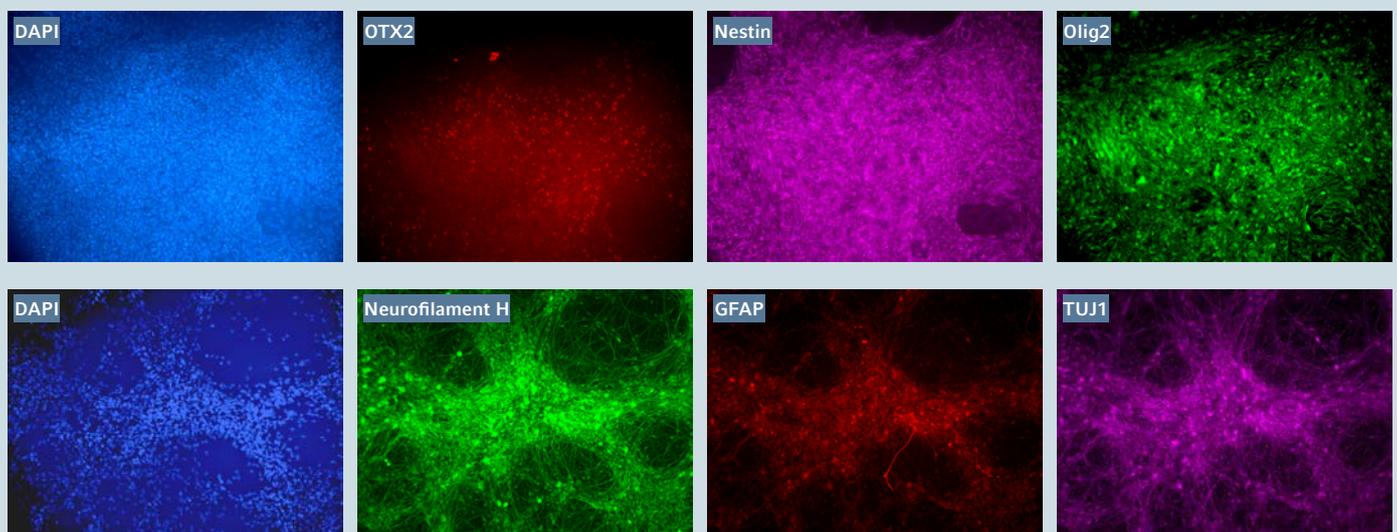
Figure 9: Differentiation into presumptive motor neuron precursor cells in low oxygen environment. Immunostaining of PM1Ep5-ERCv2p8 iPSC cells using multi-step motor neuron protocol.

In addition, we were able to first differentiate the cells into definitive endoderm using Wnt pathway activator CHIR99021 using an optimized two step procedure (data not shown), and then finally develop them into cardiomyocytes after Wnt inhibition using wntC-59 (Figure 10). After 10-11 days in culture, while many cells died from the lack of insulin, the surviving cells organized into small patches and clumps. These "nodes" were highly three dimensional and expressed Troponin T, Nkx2.5 and Smooth muscle actin (SMA). Further, we also observed robust expression of Troponin T along with GATA4 and MHCv. Together this indicates the successful development of the cardiomyocyte lineage in an hypoxic environment.
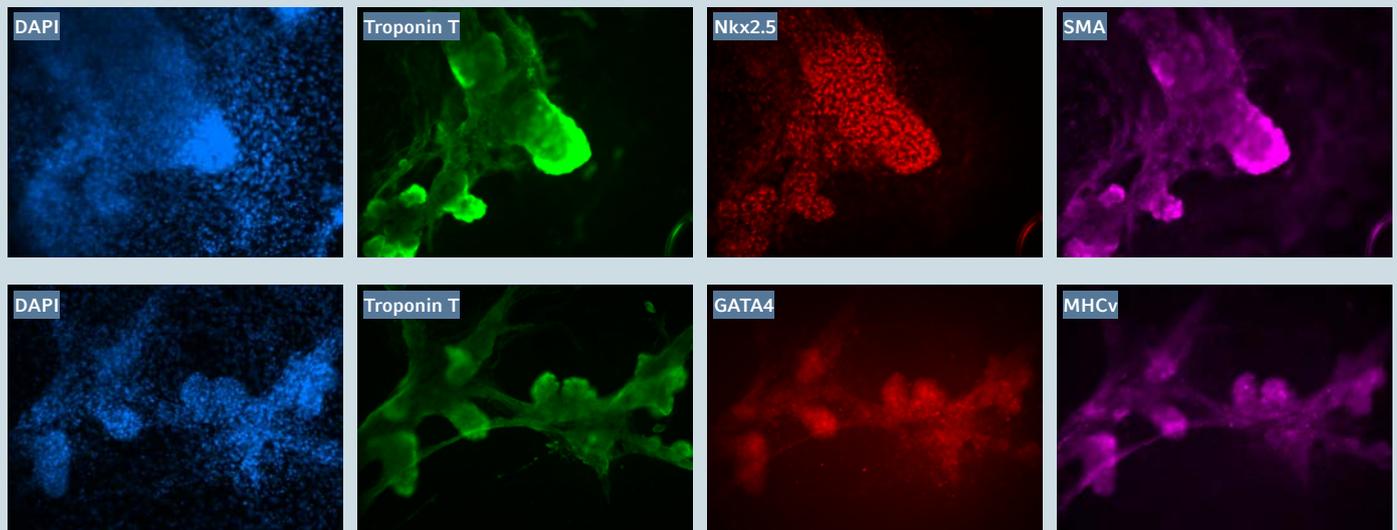
Figure 10: Differentiation into cardiomyocytes in low oxygen environment. Immunostaining of PM1Ep5-ERCv2p9 iPSC cells grown and differentiated using two step cardiomyocyte generation protocol.

Conclusion

Using the techniques described herein we successfully demonstrated the robust reprogramming of human foreskin fibroblasts in low oxygen tension (5 %) in the CellXpert C170i CO_2 incubator (Figure 11). The "PM1eP5-ERCv2" iPSC line was characterized as karyotypically normal and found to express expected markers of pluripotency Oct4+/ SSEA4+ (but not SSEA1-) and Nanog+/Lin28+/Tra-1-60+. Further, the low O_2 environment supported, the cells were able to reliable differentiation of iPS cells into various stages of ectoderm (not shown), neuronal stem cells, presumptive motor neuron precursors, and finally motor neurons. As well, the hypoxic atmosphere inside the incubator supported the differentiation of the same iPSC line into cardiomyocytes, an endodermal derivative, using standard protocols ([15, 16]).

Figure 11: The CellXpert C170i CO_2 Incubator—Learn more.

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[32] Appendix 1: Materials and Methods. For supplementary information, contact your local Eppendorf partner.