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Hypoxic Cell Culture in the New Brunswick™ Galaxy® 170R Incubator: Normal Growth, Morphological Changes

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Abstract

New Brunswick Galaxy 170 series incubators have a wide array of options that allow for not only CO_2 and temperature control, but also for O_2 concentration control which can be used to create a hypoxic internal environment. Cancer cells are known to be resistant to the toxicity of O_2 deprivation. To demonstrate the low O_2 capability of this system, we grew prostate cancer (LNCaP) cells in 2 % O_2 and observed their growth and morphology over time. In contrast to the effects of hypoxia on normal cells, LNCaP cells were able to grow normally and displayed morphological changes.

Introduction

Oxygen is a critical regulator of cellular homeostasis and as such, oxygen deprivation is lethal to normal cells. In contrast to normal tissues, solid tumors often have regions of significantly reduced oxygenation due to an inconsistent and disorganized blood supply at the center of the tumor. Tumor O_2 deprivation, or hypoxia, can result in chemotherapeutic drug resistance and gene expression changes in cancer cells. Decades of cancer research have established that tumor cell growth, survival, motility, the recruitment of blood vessels (angiogenesis), energy metabolism, and cellular differentiation are affected by hypoxia[3]. Patients with tumors displaying high levels of hypoxia often have poor prognoses and negative clinical outcomes[4]. This effect has been documented for many deep tissue tumors such as breast, prostate, ovarian and uterine cancer[2].

A number of human tumor cell lines have been used to model the effects of hypoxia in an effort to target these drug-resistant cells with new therapeutics. One such cell line, LNCaP, was derived from a bone metastasis from a terminal prostate cancer patient. These cells have been widely used in prostate cancer drug discovery and are uniquely sensitive to androgen (e.g. testosterone and dihydrotestosterone) levels. Danza and colleagues have shown that LNCaP cells previously stimulated with androgens before growth under hypoxic conditions (2 % O_2) grow faster than cells maintained in normoxia (20.9 % O_2)[1]. We chose to use a simplified version of this model to establish a protocol for setting up low-O_2 cell culture in a standard tissue culture laboratory. Since standard laboratories do not have access to controlled substances, we did not stimulate the cells with androgen before growth in hypoxic conditions.

Figure 1: The New Brunswick Galaxy 170R CO_2 incubator

We show here that low-O_2 cell culture conditions are easy to establish using the New Brunswick Galaxy 170R incubator with 1 - 19 % O_2 control. LNCaP cells grew well in both normoxia and hypoxia in this system and morphological changes were noted in low O_2 conditions. With available O_2 control from 0.1 - 19 %, the Galaxy 170R incubator provides an excellent environment with which to culture cells in a range of gas conditions.

Table 1: Materials, media and cells

Material	Supplier	Order no.
RPMI-1640	ATCC®	30-2001™
Penicillin-Streptomycin 10,000 U/mL	Life Technologies®	15140-122
Fetal bovine serum (FBS)	Life Technologies®	10437-028
1x Dulbecco's Phosphate buffered saline	Life Technologies®	14190-144
Trypsin-EDTA	HyClone®	SV30031.01
16 % Paraformaldehyde (w/v) methanol-free	Pierce™	28906
rhodamine-conjugated phalloidin	Molecular Probes®	R415
NucBlue® Fixed Cell ReadyProbes®	Molecular Probes®	R37606
T75 T-flasks*	Eppendorf	0030 711.106
6-well dishes*	Eppendorf	0030 720.105

* Currently available in China, India and Italy only

Materials and Methods

Consumable Materials

Table 1 details the consumable reagents and materials that were used in this study.

Cell Culture

Cell culture was carried out using two Galaxy 170 incubators (Eppendorf). First, the Galaxy 170R with High temperature disinfection, 4 split inner doors and 1 - 19 % O_2 control was used for culture in hypoxic conditions (Figure 1). Second, the Galaxy 170S with High temperature disinfection and 4 split inner doors was used for normoxic conditions. Setpoints on both units for temperature and CO_2 concentration were 37 °C and 5 %, respectively. For hypoxic conditions, the Galaxy 170R was set at 2 % O_2 and allowed to stabilize at setpoint for 72 h before cells were introduced into the incubator. To monitor O_2 concentration, the Galaxy 170R was connected to a computer using a RS-232/RS-422 converter (Eppendorf). Using BioCommand® SFI (Eppendorf), O_2, temperature and CO_2 process values were tracked for the course of the experiment. The O_2 concentration inside the Galaxy 170R never exceeded 2.1 %.

LNCaP clone FGC cells were acquired from the American Type Culture Collection® (ATCC®, USA #CRL-1740™) and were grown in RPMI medium supplemented with 1 % Penicillin-Streptomycin and 10 % FBS. Cells were grown in T75 flasks until 80 % confluency. At target density, the cells were disassociated from the surface by trypsinization and neutralization with FBS. After pelleting by centrifugation at 120 x g for 3 min, cells were resuspended in complete growth medium and counted using a Vi-CELL® automated cell counter (Vi-CELL XR; Beckman Coulter, Inc., USA #731050). Cells were seeded in quadruplicate 6-well dishes at a density of 300,000 cells/well and two plates were placed in the same position in both the normoxia and hypoxia incubators.

According to the protocol established by Danza and colleagues, 3 wells from both hypoxic and normoxic conditions were counted every 3 days for 9 days, generating cell concentrations per well for days 3, 6 and 9[1]. At each timepoint, the wells were also photographed using phase contrast microscopy on an Olympus® IX51 inverted microscope equipped with an Infinity2 CCD camera (Lumenera®, Canada) . The data were analyzed and statistical tests including a 2-way analysis of variance (ANOVA) were performed in Microsoft® Excel®.

Fluorescent Marker Staining

After 6 days in normoxia or hypoxia, two wells were fixed in 4 % paraformaldehyde and stained using the phallotoxin, phalloidin (which selectively binds f-actin) and the double-stranded DNA intercalator, 4',6-diamidino-2-phenylindole (DAPI; NucBlue® Fixed Cell ReadyProbes® reagent). Staining was performed exactly as the manufacturer recommended. The cells were photographed using an EVOS® LED florescence microscopy system (Life Technologies, USA #AMF4300).

Results and Discussion

LNCaP Cells Grow Normally Under Hypoxic Conditions

As the graph in Figure 2 details, LNCaP cells grown at 2 % O_2 showed growth dynamics indistinguishable from those grown in normal atmospheric O_2 (20.9 %). It is possible that we did not see the previously published growth bias in hypoxia because we did not stimulate the cells with androgens prior to exposure to O_2 deprivation. Importantly, no differences were seen in the attachment of the cells in hypoxia, as evidenced by the phase contrast micrographs in Figure 3.

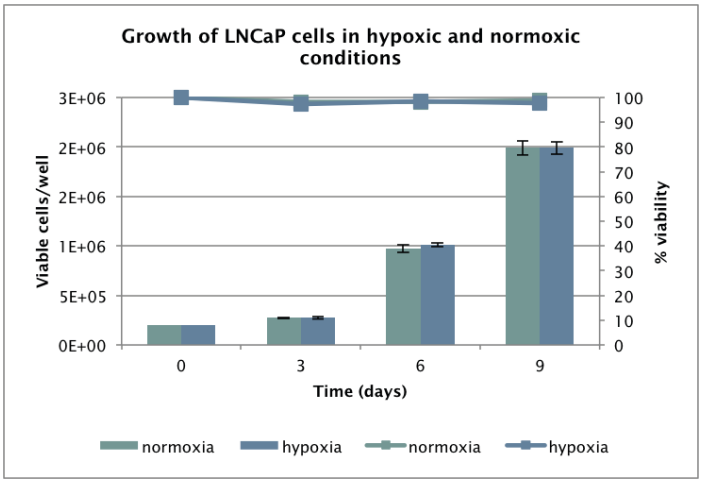

Figure 2: Growth and viability of LNCaP cells grown in hypoxia and normoxia. This graph shows the viable cell density and % viable cells in each well. Each data point represents the mean of 3 wells. Error bars indicate standard error of the mean. ANOVA analysis revealed that no significant difference was observed between the two growth conditions.

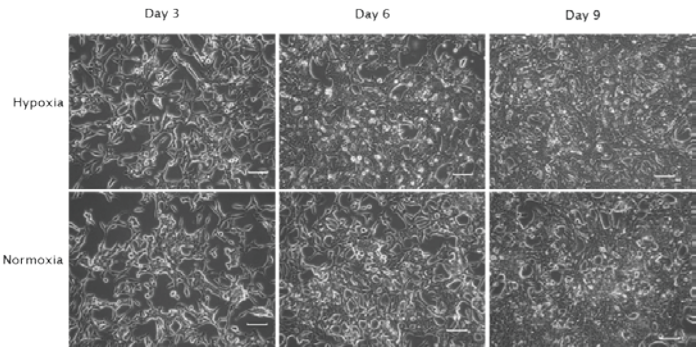

Figure 3: LNCaP cells grown in hypoxic conditions display normal attachment and homogenous growth. In the top row, LNCaP cells grown in 2 % O_2 show similar density as compared to those grown in atmospheric O_2 (bottom row). Photos were taken at 100 x magnification and the scale bar in each panel represents 100 µm.

Interestingly, we noted a significant morphology difference between the cells grown in hypoxic and normoxic conditions. Grown in atmospheric O_2 conditions, LNCaP cells are known to form large clusters or colonies of cells wherein they display no contact inhibition.

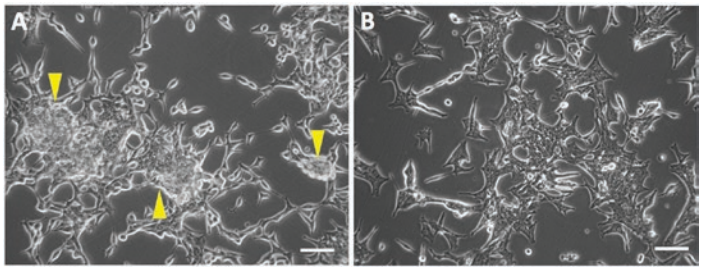

Figure 4: Growth pattern of LNCaP cells in normoxic (A) vs. hypoxic (B) conditions. Grown in atmospheric O_2 conditions, LNCaP cells make large 3-dimensional clusters (yellow arrowheads) whereas in hypoxic conditions, cells appear more spread out and do not form large colonies. Photos were collected at 100 x magnification and the scale bar in each panel represents 100 µm.

These colonies are themselves loosely attached to the substrate and can become quite large (see yellow arrowheads in Figure 4A). In contrast, LNCaP cells grown in 2 % O_2 do not form such 3-dimensional structures and tend to cover the substrate and grow in more of a packed monolayer (Figure 4B). The cells also appear more spread out and seem to display tighter adherence as evidenced by the length of time required for the cells to detach during enzymatic disassociation. To document these morphological changes, we stained cells grown in both O_2 concentrations with rhodamine-conjugated phalloidin and DAPI to visualize the actin cytoskeleton (red, Figure 5) and the cell nucleus (blue, Figure 5). As documented in Figure 5, large clusters of cells are observed in normoxic conditions while a flatter monolayer is seen in hypoxic conditions. Furthermore, the cytoplasm-to-nucleus ratio in the cells grown in 2 % O_2 appears to be larger than in those grown in 20.9 % O_2, although this observation was not quantified. We conclude that morphological changes have occurred in LNCaP cells grown with O_2 deprivation that result in diminished colony forming behavior and a flatter appearance.

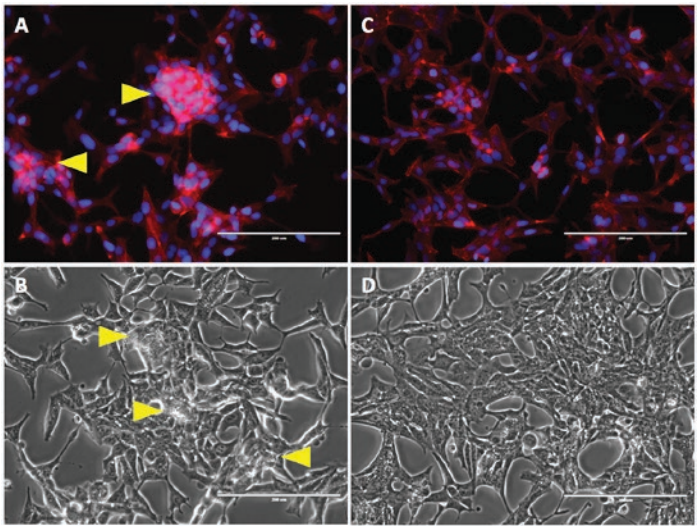

Figure 5: Growth characteristics of cells in hypoxia and normoxia. Panels A (fluorescence) and B (phase contrast) show examples of cells grown in 20.9 % O_2 for 3 days. The yellow arrowheads denote areas where 3-dimensional colonies are observed, as evidenced by the overlapping cell nuclei (blue) and high concentration of actin (red). Panels C and D show monolayers grown in 2 % O_2 where 3-dimensional growth is not seen. In addition, cells grown in hypoxia appear flatter and with larger cytoplasmic volume. The images in this figure were collected at 100 x using an EVOS LED imaging system; scale bars represent 200 µm.

Conclusion

We have shown that LNCaP cells grown in hypoxic conditions display normal growth as compared to those grown in atmospheric O_2 concentrations. Growth in 2 % O_2 also resulted in morphological changes and changes in growth characteristics such as colony formation. The ease of setup and the tight O_2 concentration control displayed by the Galaxy 170R incubator provided the ideal conditions for this experiment.

This study is a demonstration of the low O_2 feature that is available on the Galaxy 170 series incubators. Experimental conditions including O_2 concentration have not been optimized.

References

[1] Danza, G. D. (2011). Notch signaling modulates hypoxia-induced neuroendocrine differentiation of human prostate cancer cells. *Mol Cancer Res*, 230-238.

[2] Hockel, M. V. (2001). Tumor Hypoxia: Definitions and Current Clinical, Biologic, and Molecular Aspects. *J Natl Cancer Inst*, 93(4), 266-276.

[3] Kim, Y. L. (2009). Hypoxic Tumor Microenvironment and Cancer Cell Differentiation. *Curr Mol Med*, 9(4), 425-434.

[4] Wilson, W. H. (2011). Targeting hypoxia in cancer therapy. *Nature Reviews*, 11, 393-410.

Ordering Information

Description	International order no.	N. America order no.
Galaxy® 170R With high temp disinfection 1 - 19 % O_2 control Split inner doors, 4	170R230120_ B04	170R120120_ B04
Galaxy® 170S With high temp disinfection	CO170S-230-1000	CO170S-120-1000
8-port RS-232 to USB converter	P0460-7750	P0460-7750
BioCommand® SFI	M1291-1001	M1291-1001
Easypet® 3	4430 000.026	4430 000.026
Centrifuge 5810 R With 4 x 500 mL rotor, 120 V	022628179	022627082

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