Suggested co-optimization of ultralow oxygen and frequent media change to enable eSET: lessons from embryonic stem cells where frequent Medium Changing decreases G1 cell-cycle delays as cells enter Warburg anabolism.

ESC, TSC and embryo anabolism requires rapid media change.

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ABSTRACT

Introduction

Human preimplantation culture for single blastocyst transfer is suboptimal: IVF blastocyst formation rate is ~50% with even lower implantation and birth rates. Culture optimization should improve blastocyst and pregnancy rates. Media is changed infrequently, and labs improving blastocyst rates with ultralow (2%) O2 don't change media. Here we modeled development of embryonic stem cells (ESC) from blastocysts to show that lactate-producing Warburg anabolism increases with time, and media change is needed to prevent G1 cell cycle delays.

Methods

Fluorescence ubiquitinated Cell-cycle indicator (FUCCI) mouse ESC were cultured 72hr in a live imager where fluorescent green ESC are in S-G2-M-phase, and non-Green are in G1 phase of cell cycle. ESC cultured at 20% O2 were recorded for confluence and number of green cells every 2hr where media was changed with two frequencies: every 24hr or 12hr. After 72hr culture, ESC cultured with 24hr frequency were assayed by RNAseq using Illumina NovaSeq6000 for bulk transcriptome or single cells (sc)RNAs after 10X Genomics cell sorting. In RNAseq studies media components were changed to emulate normal stemness and development.

Results

Discussion

Transcriptomic studies confirm reported low mitochondrial charge and high lactate starting at blastocyst and increasing after. Previous reports showed optimization of trophoblast stem cells to near-in vivo growth at 2% O2, but morbidity instead without 12hr feeding-frequency. Together, data suggest blastocyst-derived cell culture will optimize at 2% with frequent media change.

RESULTS

FUCCI ESC confluence/growth is a sigmoidal, exponential curve with 24hr media changes but surprisingly green FUCCI ESC decrease and then increase 5-7fold before and after media change: suggesting G1 delay. This amplitude of G1 delay decreases significantly with 12hr medium change although growth rate didn't increase, perhaps due to confounding variables. The transcriptome of 24hr group showed ESC moving from Naïve to Formative pluripotency during culture (E3.5/4.5 ICM to E5.5 progression), and the expression of 18 Warburg genes increase 2fold during this development.

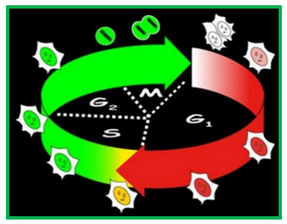


Figure 1 (above). Fluorescent-ubiquitinated cell cycle indicator (FUCCI) reporter transgenes report preparatory G1 phase of cell cycle in red, and DNA synthesis (S phase) and progression of the cell cycle through M-phase (mitotic cells completing cell cycle) in green. The other figures here show only the number of green ESCs (green objects) progressing through the cell cycle measured in time-lapse every 2hrs in a live imager. Confluence was also measured as an assay of cell growth.

Figure 2A (right). ESC grew exponentially (confluence) but surprisingly the expected parallel exponential increase in green cells progressing through the cell cycle did occur. Instead, there was a nadir of cells in green cell cycle progression in the unfed state and there was a 3fold change (FC) increase of green cells after feeding. Thus, ESC were tested for confluence every 2hr to assay growth, and by green fluorescence to test for cells progressing through cell cycle to division. **2B.** The large amplitude of Fed/unfed state green with 24hr decreases significantly with 12hr feeding and hypothetically a smooth green line like the expected one in figure 2A would occur if optimized microfluidics changed media and stirred media around embryos. .

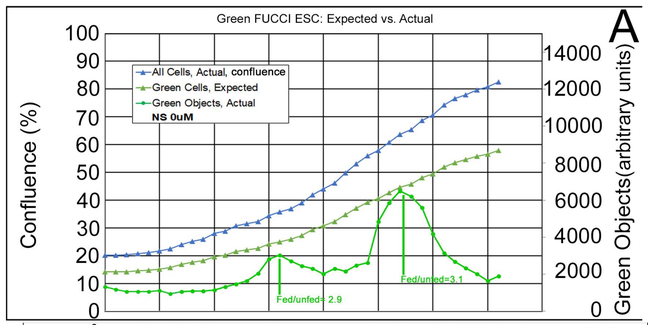
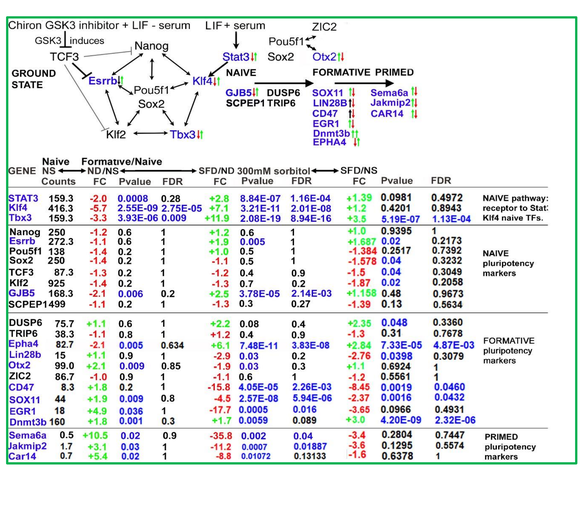


Figure 3 (left). **1)** Three core transcription factors maintaining naïve pluripotency/Normal stemness (NS) in E4.5 embryo and 8 downstream markers of this pluripotency are highly expressed and thus ESC culture on normal stemness conditions emulate the ICM of the preimplantation embryo **2)** When growth factors that maintain naïve pluripotency are removed for three says expression of all 3 core transcription factors and 8 markers **d**ecreases (**red FC** is decreased) and in 7/10 markers of normal differentiation/ND/Formative pluripotency increase (green FC is increase). **3)** the data suggest that ESC culture under (NS) conditions emulate the ICM/ESC lineage of the preimplantation E4.5 embryo and under ND conditions ESC lineage emulates the E5.5 embryo.: restriction of ESC from naïve to formative pluripotency state has occurred. .



GENE	Naive NS Counts	Formative/Naive ND/NS FC	Pvalue	FDR	SFD/ND 300mM sorbitol FC	Pvalue	FDR	SFD/NS FC	Pvalue	FDR	
STAT3	159.3	-2.0	0.0008	0.28	+2.8	8.84E-07	1.16E-04	+1.39	0.0981	0.4972	NAIVE pathway: receptor to Stat
Klf4	416.3	-5.7	2.55E-09	2.75E-05	+7.1	3.21E-11	2.01E-08	+1.2	0.4201	0.8943	
Tbx3	159.3	-3.3	3.93E-06	0.009	+11.9	2.08E-19	8.94E-16	+3.5	5.19E-07	1.13E-04	Klf4 naive TFs.
Nanog	250	-1.2	0.6	1	+1.2	0.6	1	+1.0	0.9395	1	
Esrrb	272.3	-1.1	0.6	1	+1.9	0.005	1	+1.687	0.02	0.2173	
Pou5f1	138	-1.4	0.2	1	+1.0	0.5	1	-1.384	0.2517	0.7392	
Sox2	250	-1.4	0.2	1	-1.1	0.5	1	-1.578	0.04	0.3232	NAIVE pluripotency markers
TCF3	87.3	-1.3	0.2	1	-1.2	0.4	0.9	-1.5	0.04	0.3049	
Klf2	925	-1.4	0.2	1	-1.3	0.7	0.2	-1.87	0.02	0.2058	
GJB5	168.3	-2.1	0.006	0.2	+2.5	3.78E-05	2.14E-03	+1.158	0.48	0.9673	
SCPEP1	499	-1.1	0.2	1	-1.3	0.3	0.27	-1.39	0.13	0.5634	
DUSP6	75.7	+1.1	0.6	1	+2.2	0.08	0.4	+2.35	0.048	0.3360	
TRIP6	38.3	-1.1	0.8	1	+1.2	0.4	0.9	-1.3	0.31	0.7678	
Epha4	82.7	-2.1	0.005	0.634	+6.1	7.48E-11	3.83E-08	+2.84	7.33E-05	4.87E-03	FORMATIVE pluripotency markers
Lin28b	15	+1.1	0.9	1	-2.9	0.03	0.2	-2.76	0.0398	0.3079	
Otx2	99.0	+2.1	0.009	0.85	-1.9	0.03	0.3	+1.1	0.6924	1	
ZIC2	86.7	-1.0	0.9	1	-1.1	0.6	1	-1.2	0.5561	1	
CD47	8.3	+1.8	0.2	1	-15.8	4.05E-05	2.26E-03	-8.45	0.0019	0.0460	
SOX11	44	+1.9	0.009	0.8	-4.5	2.57E-08	5.94E-06	-2.37	0.0016	0.0432	
EGR1	18	+4.9	0.036	1	-17.7	0.0005	0.016	-3.65	0.0966	0.4931	
Dnmt3b	160	+1.8	0.001	0.3	+1.7	0.0059	0.089	+3.0	4.20E-09	2.32E-06	
Sema6a	0.5	+10.5	0.02	0.9	-35.8	0.002	0.04	-3.4	0.2804	0.7447	PRIMED pluripotency markers
Jakmip2	1.7	+3.1	0.03	1	-11.2	0.0007	0.01887	-3.6	0.1295	0.5574	
Car14	0.7	+5.4	0.02	1	-8.8	0.01072	0.13133	-1.6	0.6378	1	

RESULTS

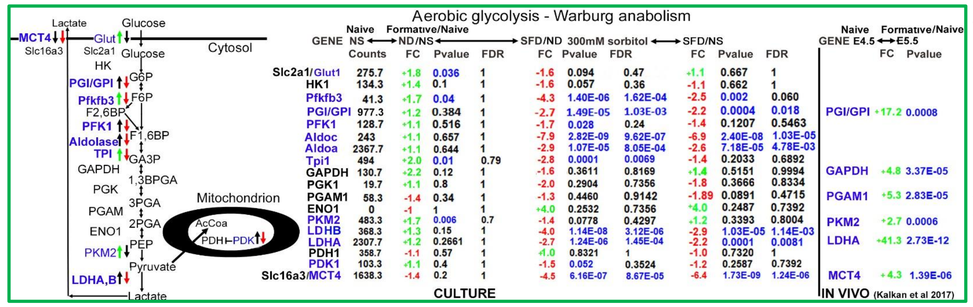


Fig.4. 1) Aerobic glycolysis is strongly expressed in NS/Naïve pluripotent ESCs resembling ICM of blastocyst and with counts in ND counts column all >10 except 1 gene. **2)** Compared with NS, expression increases (**green FC** is increased) in ND/Formative pluripotent ESC resembling E5.5 embryos, with 14/18 genes increasing and an average or 2fold increase for all 18 genes. **3)** Note that Warburg anabolism creates Lactate which must be removed. **4)** On the far right are data for NS and ND/NS fold increase reported only for significantly upregulated genes (Kalkan et al 2017). from E4.5 and E5.5 embryo cells ex vivo assayed by single cell RNA/seq. These data agree with our data for increases in Warburg from E4.5 to E5.5. .

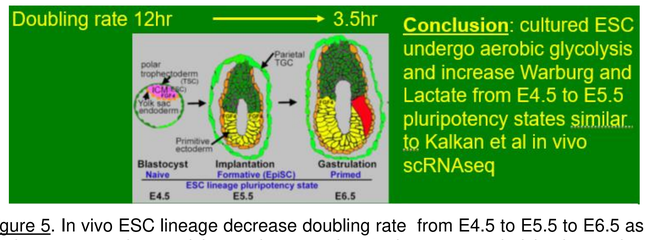


Figure 5. In vivo ESC lineage decrease doubling rate from E4.5 to E5.5 to E6.5 as pluripotency restricts and increasing growth rate is accompanied by increasing Warburg anabolism and increasing lactate (Van Blerkom 2006, Houghten and Leese 1996). Lactate decreases media pH and even at ambient O2 frequent media change should maintain ESC lineage progressing through the cell cycle.

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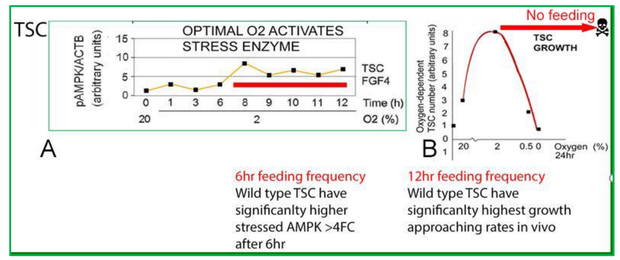

Figure 6. Complementing ESC studies here where media change optimizes culture at 20% oxygen, previous studies have optimized trophoblast stem cells (TSC) culture at ultralow oxygen and shown that rapid TSC growth approximated those in vivo occur at 2%, but only if media is changed every 12hr. In other studies, with TSC cultured at 2% oxygen, dangerously high stress enzyme (AMPK) levels occurred 6hr after last feeding. (Yang et al, 2017, Zhou et al, 2011, Bolnick et al 2017).

CONCLUSION

1) Even ESC cultures at 20% show cell cycle delay if not fed frequently.
2) Past reports show that TSC culture optimizes at ultralow oxygen, near in vivo growth rates.But, only if media is change every 12hr, And other studies of TSC suggest that stress enzyme activation can be prevented only if media is changed every 6 hr.
3) ESC culture model emulates embryos and their changes in stemness (next to last figure) and Warburg anabolism (last figure) as embryos develop from preimplantation to post implantation pluripotency states. Increasing Warburg from E3.5 to E4.5 to E5.5 suggest increasing lactate will need removal in cultured embryos.
4) Past reports show that fastest growth of culured embryos, most comparable to age-matched embryos ex vivo, is supported by microfluidics with user controlled, Piezo-pin mediated pulsatile flow rate microfluidics.
5) Five recent reports suggest that ulra-low oxygen may improve human embryo Culture, but none of these change media.
6) Taken together data from ESC and TSC, isolated from blastocysts, suggest that blastocysts should be cultured at 2% O2, but need media media change.

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