SUPPLEMENTARY INFORMATION

Embryonic Stem Cell Proliferation Stimulated By Altered Anabolic Metabolism From Glucose Transporter 2-Transported Glucosamine

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List of Supplementary Materials

Supplementary Table 1 (Related to Figure 1)

Y and Autosomal Genotype Results

Cell line	***Zfy***	***Glut2***
LG-ESC	+	+
LG-ESC-2	-	+
LG-ESC-3	-	+
D3 ESC	+	+

SUPPLEMENTARY DATA

Supplementary Table 2 (Related to Figure 5)

Heat map of statistically significant biochemicals profiled in this study.

Statistical trend-value difference approaching significance. (p ≤ 0.05, fold change > 1.0) (p ≤ 0.05, fold change < 1.0) (0.05 < p ≤ 0.10, fold change > 1.0) (0.05 < p ≤ 0.10, fold change < 1.0)

Blue shaded cells indicate significant (p≤0.05) ANOVA effect.

Data normalization: Bradford protein concentration

Pathway Sort Order	Super Pathway	Sub Pathway	Biochemical Name	Platform	Comp ID	KEGG	HMDB	ANOVA Contrasts: Fold of Change (+)GLU(+)GLCN / (+)GLU(-)GLCN	(-)GLU(+)GLCN / (-)GLU(-)GLCN	(-)GLU(-)GLCN / (+)GLU(-)GLCN	(-)GLU(+)GLCN / (+)GLU(+)GLCN	Two-Way ANOVA: Glucose Main Effect	Glucosamine Main Effect	Glucose / Glucosamine Interaction	Statistical Values: ANOVA Contrasts (+)GLU(+)GLCN / (+)GLU(-)GLCN p-value	q-value	(-)GLU(+)GLCN / (-)GLU(-)GLCN p-value	q-value	(-)GLU(-)GLCN / (+)GLU(-)GLCN p-value	q-value	(-)GLU(+)GLCN / (+)GLU(+)GLCN p-value	q-value	Two-Way ANOVA: Glucose Main Effect p-value	q-value	Glucosamine Main Effect p-value	q-value	Glucose:Glucosamine Interaction p-value	q-value
36	Amino Acid	Glutamate Metabolism	glutamate	LC/MS pos	57	C00025	HMDB00148	0.16	1.12	0.39	0.65				0.0102	0.0185	0.5379	0.1213	0.0000	0.0000	0.0079	0.1481	0.0000	0.0000	0.1338	0.0499	0.0237	0.0136
37			glutamine	LC/MS pos	53	C00064	HMDB00641	0.62	0.81	0.42	0.56				0.0396	0.0473	0.3661	0.0900	0.0008	0.0002	0.0115	0.1829	0.0001	0.0001	0.0389	0.0196	0.3773	0.1368
296		Urea cycle; Arginine and Proline Metab	arginine	LC/MS pos	1638	C00062	HMDB00517	0.69	0.82	0.97	1.15				0.0229	0.0337	0.3067	0.0791	0.5537	0.0929	0.4260	0.9963	0.8833	0.2773	0.0220	0.0118	0.3290	0.1223
298			ornithine	GC/MS	1493	C00077	HMDB03374	0.60	1.88	0.21	0.64				0.0068	0.0151	0.0025	0.0011	0.0000	0.0000	0.0325	0.3352	0.0000	0.0000	0.7632	0.1996	0.0002	0.0002
299			proline	LC/MS pos	1898	C00148	HMDB00162	0.70	1.44	0.37	0.75				0.0359	0.0462	0.0400	0.0135	0.0000	0.0000	0.0958	0.6742	0.0000	0.0000	0.9706	0.2365	0.0051	0.0035
731	Carbohydrate	Glycolysis, Gluconeogenesis, and Pyr	glucose	GC/MS	38507	C00031	HMDB00122	1.08	5.62	0.19	0.97				0.4002	0.2321	0.0000	0.0000	0.0000	0.0000	0.7465	0.9963	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
732			glucose-6-phosph	GC/MS	31260	C00668	HMDB01401	1.11	1.79	0.50	0.80				0.4533	0.2485	0.0003	0.0002	0.0000	0.0000	0.0583	0.4807	0.0000	0.0000	0.0017	0.0014	0.0197	0.0118
735			fructose-6-phosph	GC/MS	12021	C05345	HMDB03124	1.00	6.22	0.16	0.98				0.9906	0.4024	0.0000	0.0000	0.0000	0.0000	0.7827	0.9963	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
742			1,3-dihydroxyacet	GC/MS	35963	C00184	HMDB01882	1.08	1.76	0.43	0.89				0.6859	0.3212	0.0035	0.0014	0.0001	0.0000	0.0655	0.5188	0.0001	0.0001	0.0160	0.0090	0.0536	0.0271
748			lactate	GC/MS	527	C00186	HMDB01100	1.03	1.47	0.49	0.70				0.8559	0.3711	0.0127	0.0047	0.0001	0.0000	0.0237	0.3047	0.0000	0.0000	0.0522	0.0246	0.0863	0.0415
751			glycerate	GC/MS	1572	C00258	HMDB01139	1.21	1.19	0.72	0.71				0.0937	0.0891	0.0829	0.0247	0.0043	0.0012	0.0050	0.1463	0.0002	0.0002	0.0197	0.0107	0.9633	0.2760
756		Pentose Phosphate Pathway	ribose 5-phosphat	GC/MS	561	C00117	HMDB00618	1.33	2.40	0.40	0.72				0.1648	0.1321	0.0937	0.0270	0.1486	0.0290	0.2500	0.9902	0.0719	0.0338	0.0348	0.0178	0.8245	0.2458
759			sedoheptulose-7-p	GC/MS	35649	C05382	HMDB01068	1.41	2.93	0.39	0.82				0.1124	0.1033	0.0012	0.0006	0.0046	0.0012	0.2854	0.9963	0.0066	0.0040	0.0010	0.0010	0.1543	0.0670
765		Pentose Metabolism	ribose	GC/MS	12080	C00121	HMDB00283	1.21	4.09	0.31	1.02				0.2394	0.1735	0.0000	0.0000	0.0000	0.0000	0.9650	0.9963	0.0000	0.0000	0.0000	0.0000	0.0000	0.0001
873		Aminosugar Metabolism	glucosamine	GC/MS	18534	C00329	HMDB01514	1.13	2.26	1.00	1.49				0.0353	0.0462	0.0001	0.0001	1.0000	0.1514	0.0232	0.3047	0.0975	0.0448	0.0001	0.0001	0.0975	0.0451
883			N-acetylglucosam	LC/MS neg	15107	C00357	HMDB02917	2.36	13.80	0.18	1.09				0.0000	0.0002	0.0000	0.0000	0.0000	0.0000	0.6864	0.9963	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
908	Energy	TCA Cycle	citrate	GC/MS	1564	C00158	HMDB00094	0.96	4.70	0.12	0.58				0.7077	0.3290	0.0000	0.0000	0.0000	0.0000	0.0067	0.1481	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
917			fumarate	GC/MS	1643	C00122	HMDB01134	0.83	1.63	0.49	0.95				0.5002	0.2649	0.0584	0.0186	0.0033	0.0009	0.5284	0.9963	0.0108	0.0062	0.3614	0.1163	0.0713	0.0355
918			malate	GC/MS	1303	C00149	HMDB00156	0.89	4.07	0.10	0.45				0.4009	0.2321	0.0000	0.0000	0.0000	0.0000	0.0007	0.0354	0.0000	0.0000	0.0005	0.0005	0.0000	0.0001
928		Oxidative Phosphorylation	pyrophosphate (P	GC/MS	2078	C00013	HMDB00292	0.94	1.11	0.68	0.80				0.7133	0.3298	0.8739	0.1722	0.2559	0.0475	0.5318	0.9963	0.2162	0.0861	0.8824	0.2192	0.7100	0.2286
929			phosphate	GC/MS	11438	C00009	HMDB01429	0.87	0.80	1.07	0.99				0.3089	0.1973	0.0864	0.0256	0.7187	0.1142	0.6978	0.9963	0.9841	0.3005	0.0577	0.0264	0.5973	0.2004
2064	Nucleotide	Purine Metabolism	adenosine	LC/MS pos	555	C00212	HMDB00050	0.87	1.26	0.69	1.00				0.2973	0.1959	0.1353	0.0368	0.0084	0.0022	0.7662	0.9963	0.0339	0.0182	0.7347	0.1952	0.0781	0.0382
2065			adenine	GC/MS	554	C00147	HMDB00034	0.63	1.75	0.36	1.01				0.0049	0.0125	0.0013	0.0006	0.0000	0.0000	0.7920	0.9963	0.0000	0.0000	0.6832	0.1860	0.0001	0.0001
2095		Purine Metabolism, Guanine containi	guanosine	LC/MS pos	1573	C00387	HMDB00133	0.81	5.53	0.14	0.97				0.0418	0.0492	0.0000	0.0000	0.0000	0.0000	0.5778	0.9963	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
2096			guanine	LC/MS pos	32352	C00242	HMDB01192	0.79	0.97	0.82	1.01				0.0310	0.0411	0.8189	0.1634	0.0467	0.0100	0.9760	0.9963	0.1441	0.0625	0.0861	0.0362	0.1552	0.0670
2127		Pyrimidine Metabolism, Uracil-Contai	uridine	LC/MS neg	606	C00299	HMDB00296	1.73	8.09	0.20	0.93				0.0013	0.0057	0.0000	0.0000	0.0000	0.0000	0.4491	0.9963	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
2128			uracil	GC/MS	605	C00106	HMDB00300	1.71	5.72	0.33	1.09				0.0001	0.0006	0.0000	0.0000	0.0000	0.0000	0.4910	0.9963	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
2149		Pyrimidine Metabolism, Cytidine cont	Cytidine 5'-monop	LC/MS pos	2372	C00055	HMDB00095	1.08	3.99	0.27	0.93				0.6095	0.2915	0.0000	0.0000	0.0000	0.0000	0.5856	0.9963	0.0000	0.0000	0.0000	0.0000	0.0000	0.0001
2152			cytidine	LC/MS pos	514	C00475	HMDB00089	0.71	0.89	0.79	0.90				0.0028	0.0084	0.0446	0.0146	0.0229	0.0055	0.2461	0.9902	0.0176	0.0099	0.0008	0.0008	0.3799	0.1369
2168		Pyrimidine Metabolism, Thymine conta	thymidine	LC/MS neg	39539	C00214	HMDB00273	0.87	4.14	0.16	0.77				0.2614	0.1808	0.0000	0.0000	0.0000	0.0000	0.0285	0.3258	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
2169			thymine	GC/MS	604	C00178	HMDB00262	0.82	1.57	0.43	0.82				0.3150	0.1973	0.0434	0.0144	0.0001	0.0000	0.1229	0.7035	0.0002	0.0002	0.4353	0.1350	0.0356	0.0192

Supplementary Table 3 (Related to Methods)

Immunoblot Antibodies

Antibody	Dilution	Species	Source
Anti-GLUT1	1:500	Mouse	Abcam (ab40084)
Anti-GLUT2	1:1000	Rabbit	Santa Cruz Biotechnology (sc-9117)
Anti-GLUT3	1:1000	Mouse	Santa Cruz Biotechnology (sc-74399)
Anti-PCNA	1:2000	Mouse	Santa Cruz Biotechnology (sc-56)
Anti-β-ACTIN	1:5000	Mouse	Sigma (A5441)
Anti-HK1	1:500	Rabbit	Cell Signaling Technology (2024)
Anti-HK2	1:500	Rabbit	Cell Signaling Technology (2867)
Anti-PKM1/2	1:500	Rabbit	Cell Signaling Technology (3190)
Anti-PKM2	1:500	Rabbit	Cell Signaling Technology (4053)
Anti-PFKP	1:500	Rabbit	Cell Signaling Technology (8164)
Anti-PDHA	1:500	Rabbit	Cell Signaling Technology (3205)
Anti-LDHA	1:500	Rabbit	Cell Signaling Technology (3582)
Anti-GlcNAc	1:1000	Mouse	Santa Cruz Biotechnology (sc-59624)
Anti-OXPHOS cocktail	1:500	Mouse	Abcam (ab110413)
Anti-NANOG	1:1000	Rabbit	Cell Signaling Technology (8822)
Anti-SOX2	1:1000	Rabbit	Cell Signaling Technology (2748)
Anti-OCT4	1:1000	Rabbit	Santa Cruz Biotechnology (sc-9081)
Anti-Mouse IgG (HRP-coupled)	1:3000	Goat	Santa Cruz Biotechnology (sc-2055)
Anti-Rabbit IgG (HRP-coupled)	1:3000	Donkey	GE Healthcare (NA934V)

Supplementary Table 4 (Related to Experimental Procedures)

Immunoprecipitation antibodies

Antibody	Amount	Species	Source
Anti-OCT4	1 μg	Rabbit	Santa Cruz Biotechnology (sc-9081)
Anti-NANOG	1:100 (vol:vol)	Rabbit	Cell Signaling Technology (8822)
Anti-SOX2	1:100 (vol:vol)	Rabbit	Cell Signaling Technology (2748)
Anti-GlcNAc	1 μg	Mouse	Santa Cruz Biotechnology (sc-59624)
Nonimmune Mouse IgG	1 μg	Mouse	Santa Cruz Biotechnology (sc-2025)
Nonimmune Rabbit IgG	1 μg	Rabbit	Calbiochem (12-370)

SUPPLEMENTARY METHODS

LG-ESC Culture and Transfection

Murine LG-ESC were grown in low glucose DMEM (Life Technologies) with 15% fetal calf serum (Atlanta Biologicals) as described[1] ± GlcN (Sigma), added at 0.8 mM unless otherwise indicated. To maintain glucose concentrations at 5.5 mM in cultures lasting more than 2 days, media glucose concentrations were measured with a blood glucose meter (Roche Diagnostics) and then media were brought to 5.5 mM by adding appropriate volumes of 25% glucose as described[2]. Cultures are tested for mycoplasma contamination using the MycoAlert Mycoplasma Detection Kit (Lonza).

G2KD-LG-ESC were generated by stably transfecting LG-ESC with pSUPER (OligoEngine) containing a short hairpin RNA (shRNA) against *Glut2/Slc2a2*[3] as described[4]. A control cell line, C-LG-ESC, was generated by transfecting with empty pSUPER vector. OGT-KD-LG-ESC cells were derived by stably transfecting LG-ESC with doxycycline (Dox)-inducible pSingle (Clontech) containing one of three shRNA sequences against *Ogt* mRNA that were previously reported[5] or a scrambled shRNA sequence (Sc-LG-ESC)[6]. shRNA expression was induced with 1 µg/ml Dox). 1-10 mM alloxan (Sigma) was added to LG-ESC cultures to inhibit OGT enzyme activity.

LG-ESC Sex Determination

The sex of each cell line was determined by performing PCR of genomic DNA for *Zfy*, a Y chromosome marker as described[7,8] except *Glut2* as an autosomal PCR control[9].

^{3}H-GlcN Transport Assay

3 x 10^5 cells were cultured in 35 mm dishes for 48 hr in 5.5 mM glucose media. Cells were incubated with 10 µCi D-[6-^{3}H(N)]GlcN (PerkinElmer) in 0.8 mM GlcN with 5 mM or 16 mM 2-deoxy-D-glucose for 20 min. Reactions were stopped and cells were solubilized as described[10]. 950 µl of cell lysate were counted using a liquid scintillation counter. 50 µl of cell lysate were used to measure protein concentration with Protein Assay Dye Reagent (Bio-Rad).

2-deoxy-D-Glucose Transport Assay

3 x 10^5 cells were cultured in 35 mm dishes for 48 hr in 5.5 mM glucose media, then were incubated with 0.125 mM or 0.4 mM of the fluorescent 2-deoxy-D-glucose analog, 2-NBD-glucose (2-deoxy-2-[(7-nitro-2,1,3- benzoxadiazol-4-yl)amino]-D-glucose, Cayman Chemical) in 5 mM or 16 mM 2-deoxy-D-glucose, respectively, $\pm$ 0.8 mM GlcN, for 20 min. Reactions were stopped by solubilizing cells according to the manufacturer's recommendations. 485/535 nm (excitation/emission) was measured using 50 µl of cell lysate in a 96 well plate. Protein was assayed as above.

Real Time RT-PCR

Total RNA was extracted after 4 days of culture using Ultraspec reagent (Biotecx Laboratories, Friendswood, TX) and 200 ng were reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit (Life Technologies). Real time PCR was performed using TaqMan PCR Master Mix (Life Technologies) as described[11]. Primers and FAM-labeled probes for *Nanog* (Mm 02384862), *Sox2* (Mm 00488369), *Oct4* (Mm

00658129), and VIC-labeled probe for *rRNA* (4310893E) were obtained from Life Technologies.

Extracellular Flux (XF) Analysis

3 x 10^3 cells were cultured for 12 hr then were washed with DMEM (0 glucose) with 143 mM NaCL and incubated for 1 hr in DMEM (5.5 mM glucose with 2 mM glutamine) $\pm$ 0.8 mM GlcN in a 37^o oven. Extracellular acidification rates (ECAR) and oxygen consumption rates (OCR) were measured using a Seahorse Extracellular Flux Analyzer (XF24) 6 times over 1.5 hr. DNA was extracted with 50 mM NaOH heated to 95 °C for 30 min followed by neutralization with 1M Tris-Cl pH 6.8. DNA concentrations were measured by NanoDrop™ (Thermo Scientific).

Metabolic Profiling

Six replicate 10 cm culture dishes of 5 x 10^6 cells were grown under 4 culture conditions ((+)GLU(-)GLCN; (+)GLU(+)GLCN; (-)GLU(-)GLCN; (+)GLU(+)GLCN), in which cultures were grown in 5.5 mM glucose $\pm$ 0.8 mM GlcN for 47 hr, and then media were replaced with the same media, or 0 glucose media $\pm$ GlcN, for the final h of culture. Cells were harvested by scraping into PBS, pelleted, flash frozen in liquid nitrogen, and stored at -80 °C until analysis by Metabolon, Inc. Three independent platforms were employed for untargeted metabolic profiling: ultrahigh performance liquid chromatography/tandem mass spectrometry (UHLC/MS/MS) optimized for basic species, UHLC/MS/MS optimized for acidic species, and gas chromatography/mass spectrometry (GC/MS). Samples were processed essentially as described previously[12,13]. For UHLC/MS/MS

analysis, aliquots were separated using a Waters Acquity UPLC and analyzed using an LTQ mass spectrometer (Thermo Fisher Scientific, Inc.), which consisted of an electrospray ionization (ESI) source and linear ion-trap (LIT) mass analyzer. The MS instrument scanned 99-1000 *m/z* and alternated between MS and MS^2 scans using dynamic exclusion with approximately 6 scans per second. Derivatized samples for GC/MS were separated on a 5% phenyldimethyl silicone column with helium as the carrier gas and a temperature ramp from 60°C to 340°C and then analyzed on a Thermo-Finnigan Trace DSQ MS (Thermo Fisher Scientific, Inc.) operated at unit mass resolving power with electron impact ionization and a 50-750 atomic mass unit scan range. Metabolites were identified by automated comparison of the ion features in the experimental samples to a reference library of chemical standard entries that included retention time, molecular weight (*m/z*), preferred adducts, and in-source fragments as well as associated MS spectra and curated by visual inspection for quality control using software developed at Metabolon[14]. Experimental samples and controls were randomized across a one-day platform run. Any missing values were assumed to be below the limits of detection and for statistical analyses and data display purposes, these values were imputed with the compound minimum (minimum value imputation). Statistical analysis of log-transformed data was performed using "R" (http://cran.r-project.org/). Two-way ANOVA was performed to determine main effects of glucose and GlcN and glucose:GlcN interaction. Student t-test was performed to compare data between experimental groups. Multiple comparisons were accounted for by estimating the false discovery rate (FDR) using q-values[15].

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