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Genome-wide microarray evidence that 8-cell human blastomeres over-express cell cycle drivers and under-express checkpoints

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Abstract

Purpose To understand cell cycle controls in the 8-Cell human blastomere.

Methods Data from whole human genome (43,377 elements) microarray analyses of RNAs from normal 8-Cell human embryos were compiled with published microarrays of RNAs from human fibroblasts, before and after induced pluripotency, and embryonic stem cells. A sub database of 3,803 genes identified by high throughput RNA knock-down studies, plus genes that oscillate in human cells, was analyzed.

Results Thirty-five genes over-detected at least 7-fold specifically on the 8-Cell arrays were enriched for cell cycle drivers and for proteins that stabilize chromosome cohesion and spindle attachment and limit DNA and centrosome replication to once per cycle.

Conclusions These results indicate that 8-cell human blastomere cleavage is guided by cyclic over-expression of key proteins, rather than canonical checkpoints, leading to rapidly increasing gene copy number and a susceptibility to chromosome and cytokinesis mishaps, well-noted characteristics of preimplantation human embryos.

Keywords Aurora kinase · Cell cycle · CLOCK · Cyclins · Human embryo · RB

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Introduction

The human blastomere at the 8-cell stage of embryo development is a unique, totipotent cell. Despite the fundamental importance to human reproduction, little is known about cellular controls on gene expression and chromosome duplication in 8-Cell stage human blastomeres. Understanding cell cycle regulation in the 8-Cell is urgently needed to improve outcomes of assisted reproduction and provide insights into the cellular machinery used by the egg to reprogram nuclei from differentiated to totipotent. This information may markedly improve the efficiency of reprogramming unfertilized oocytes and somatic cells to patient-specific stem cells for research and therapy.
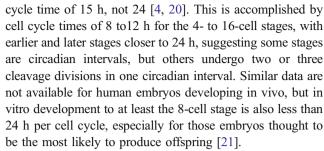
We recently reported that RB, the key G1 cell cycle checkpoint, and WEE1, a key G2 cell cycle checkpoint, were silent on microarrays of two pools of normal appearing 8-Cell stage human embryos, whereas MYC and circadian oscillators CLOCK, CRY and PER were markedly elevated [1]. Rb maintains cell cycle arrest in G1 by binding the E2F transcription factors needed for progression through G1 and the initiation of S phase. To overcome the Rb blockade, growth factors stimulate expression of CyclinD, which binds Cdk4/6 and phosphorylates Rb in a couple of locations, causing the release of E2F transcription factors that stimulate expression of

Cyclin E, which binds Cdk2 and phosphorylates Rb again, rendering it inactive. Loss of functional Rb, the first tumor suppressor gene discovered, is a characteristic of many human cancers [2], suggesting that understanding cell cycle regulation in the 8-Cell may also provide unique insights into malignant transformation. Mouse fibroblasts depleted of Rb become growth factor-independent [3], as are early cleavage stage blastomeres [4, 5], which suggests the silence of RB, also reported for early cleaving mouse embryos [6], may be characteristic of totipotent embryo cells, reflecting the lack of a need for cell growth because of their large size. Like other mammals, early human embryo cell cycles are cleavage divisions that halve the volume of daughter blastomeres. Approximately six cleavage divisions, to the 64-cell stage, are needed to reduce the size of the 120 micron diameter human egg to the 10 to 20 micron diameter of normal somatic cells and embryonic stem cells (discussed in [7]).

Consistent with the absence of Rb, CCNE was markedly elevated on the microarrays of the 8-Cells [1]. In addition to activating Cdk2, Cyclin E has Cdk2-independent functions, including regulating centrosome duplication [8]. CDK2 knock-out mice are viable, presumably because Cyclin E can also bind Cdk1, but CCNE knock-out mice die in utero [9]. Cyclin E promotes cell division, and over-expression promotes aneuploidy, as has been observed for numerous human malignancies, although the mechanisms by which Cyclin E induces tumorigenesis remain elusive [10]. The silence of Wee1, the G2 checkpoint kinase that blocks cell division by inhibiting CyclinB/Cdk1 [11], suggests that Cyclin E driven aneuploidy may go unchallenged at the G2/M transition in the 8-Cell embryo. This may help explain the heteroploidy observed in early human and non-human primate embryo blastomeres [12–14]. But given the fundamental importance to development of accurate chromosome duplication and allocation to daughter blastomeres, the absence of G1 and G2 checkpoints was a surprise. The over-expression of circadian oscillators in the 8-Cells [1] suggests they may help regulate the blastomere cell cycle, as has been demonstrated for many cell types, including mouse liver and muscle [15], rat fibroblasts [16] and human bone marrow [17]. By regulating WEE1 expression through its E-Box elements, mouse liver cell oscillators ensure mitosis is coupled to their circadian cycle [18].

The silence of WEE1 on the 8-Cell microarrays indicates this is not, however, a mechanism used by blastomeres. Moreover, despite recent evidence that circadian oscillators are over-expressed in the 8 Cells, and expression of Per is stimulated at fertilization of zebra fish eggs, heralding the onset of circadian oscillations in zebrafish embryos [19], cell division occurs at intervals much shorter than 24 h in early embryo cells, including human. In vivo developed mouse blastocysts reach 250 cells, eight cell doublings, by the time of implantation 5 days after fertilization, an average cell

cycle time of 15 h, not 24 [4, 20]. This is accomplished by cell cycle times of 8 to 12 h for the 4- to 16-cell stages, with earlier and later stages closer to 24 h, suggesting some stages are circadian intervals, but others undergo two or three cleavage divisions in one circadian interval. Similar data are not available for human embryos developing in vivo, but in vitro development to at least the 8-cell stage is also less than 24 h per cell cycle, especially for those embryos thought to be the most likely to produce offspring [21].

If the absence of G1 and G2 checkpoints accounts for rapid cell cycles in the human blastomeres, what safeguards are in place to ensure the integrity of chromosomal duplication and allocation to daughter blastomeres? Is it possible that to allow the production of sufficient embryonic proteins to signal the mother to maintain a receptive uterus, gene copy number is more important in early embryo development than accurate chromosome allocation to daughter cells? Evidence suggests that only one or two of the first 16 cells of the mouse morula give rise to the inner cell mass (ICM), which will become the embryo; the remainder of the cells become trophoblast which gives rise to placenta. Perhaps accurate chromosome allocation first becomes essential to development in the ICM, suggesting that cell cycle checkpoints first appear in ICM cells, perhaps concomitant with the need for growth factors to increase cell size, and that the cell death commonly observed in human blastocysts developed in vivo or in vitro [22, 23] reflects the elimination of defective cells, as noted for frog embryos at the midblastula transition [24].

To better understand the cell cycle in the 8-Cell human embryo, we compiled a gene data set from published high throughput strategies not feasible with human embryos that correlate cell morphology and DNA content with expression of cell cycle and cycling genes [25–27]. The compiled gene list corresponds to 3,803 gene elements in our database (8CFES) of whole human genome microarrays of four cell types: 8-Cell embryos, fibroblasts before and after induced pluripotency (iPS cells) and two lines of human embryonic stem (hES) cells (Supplemental Table 1S). We then took advantage of two recent reports of MYC transcriptome [28, 29] to assess the role of the over-expression of MYC in the 8-Cells. We report here the results of this analysis which add several gene elements to cell cycle regulation, and reveal unique gene expression characteristics of the 8-Cell human blastomere.

Methods and materials

Embryos were donated by couples undergoing infertility treatment at Alexandra Hospital in Athens Greece, as described [1, 30]. The study protocol was reviewed and approved by the ethics advisory board and human subjects research committees of Alexandra Hospital and Bedford

Research Foundation. Briefly, three embryos were selected for transfer 48 h following egg collection. Supernumary embryos were cultured an additional 24 h, and normal appearing, unfragmented, 8-Cell embryos were transferred to microfuge tubes in 2 ul of culture medium and flash frozen in liquid nitrogen for RNA extraction. A total of ten 8-Cell stage embryos from nine couples, divided into two pools of five each were used for microarray analyses. Seven of the nine couples achieved a pregnancy.

RNAs were isolated and amplified for fluorescent probe labeling and hybridization to Agilent whole genome microarrays as described [1]. Briefly, RNAs were isolated with Arcturus PicoPure according to manufacturer's instructions, examined for ratios of 18S and 28S RNAs with an Agilent Bioanalyzer to assess RNA integrity, and polyadenylated RNAs were amplified to microgram quantities according to a previously reported protocol [31] shown to be linear. Microarray hybridization was done by MoGene, St. Louis, MO, and the raw hybridization intensities normalized by Agilent Feature Extraction Software. The hybridization results were verified for standard RT-PCR housekeeping genes as described [1], and combined into a Filemaker Pro database with microarray data posted at GEO (NCBI) for fibroblast RNAs before and after induced pluripotency [32] and two human embryonic stem cell lines, H9 and HES01 [33], aligned by Agilent feature number [1]. Normalized array data for each gene element were statistically compared to develop guidelines for over- and under-detection [1]. The ratio between the two pools of 8Cell embryos of fluorescence values for each gene element was 1.0±3.0 [1]. For purposes of this report, two standard deviations (±7-fold) of the mean ratios of gene element fluorescent detection among the arrays was taken as the threshold for designation of over- or under-detection, as described [1].

Gene lists were submitted to DAVID (david.abcc.ncifcrf.gov) and Reactome (www.reactome.org) for function and ontology analyses, and hand annotated for accuracy. Because of the silence of canonical checkpoint genes, such as RB and WEE1, and the over-detection of unusual cyclins, such as Cyclin A1, standard pathway analysis tools were not relied upon.

Results and discussion

A comprehensive list of genes important to cell division was compiled from three published studies of high throughput analyses of cultured cells not possible with human embryos. U20S cells, a well studied osteosarcoma cell line, were cultured with small interfering RNA (siRNA) targeting 34,373 human genes [27]. Automated single-cell fluorescence microscopy gathered data for each siRNA: cell number; percentage of cells in G1, S and G2/M; percentage

of cells with >8N hyperploidy; and percentage of cells with cytokinetic defects. Depletion of 1,152 gene transcripts strongly affected cell-cycle progression. HeLa cells were cultured with endoribonuclease-prepared short interfering RNAs (esiRNAs) targeting 17,828 genes, followed by propidium iodide staining and flow cytometry analysis of DNA content [26]. Seven parameters were extracted from the DNA content analysis: G1, S, G2/M, 8N (polyploid), 4–8N (aneuploid), percent dead cells, plus mitotic index and cell size. This approach identified 1,351 genes involved in the HeLa cell cycle. RNAs were isolated for microarray analysis at 2 h intervals following synchronization of early passage human foreskin fibroblasts [25]; 480 cycling genes were identified.

The 3,803 gene elements in 8CFES (Supplemental Table S1) that corresponded to all the genes identified by the three studies DAVID-mapped to 2,377 genes, only 256 of which were designated GO term "cell cycle." Differential expression of the genes identified in each study is described in more detail in supplementary material (Tables S1–S3). Only 122 gene elements identified by the U2OS cell knock-down studies were also identified by one of the other studies, only 186 gene elements identified by the HeLa cell studies corresponded to elements in one of the other two studies, and only 136 of the cycling fibroblast genes corresponded to genes identified in the U2OS or HeLa cells. This striking lack of agreement among the three studies undoubtedly reflects experimental differences, but may more importantly emphasize the cell-type specificity of cell cycle control and the functional redundancy of many genes.

Genes in U20S and HeLa identified by RNA knock-down and cycled in fibroblasts

Only nineteen genes were identified by all three studies (Table S3), which meant they registered a phenotype in both U2OS and HeLa [26, 27] and their expression also cycled in the primary fibroblasts [25]. Seventeen were DAVID-annotated cell cycle (Table 1), and 12 were reported to be Myc targets [28, 29], which did not predict elevated levels of detection on the 8-Cell arrays. Interestingly, none brought about a cell cycle block in G1 and only two (CDC2 and PLK1) brought about a block in G2. This supports the cell-type specificity of gene expression needed for progression through G1 and G2.

Knock-down of eight of the cycling genes blocked HeLa and U20S in S phase, including WEE1, which was a surprise because although it is known that Wee1 phosphorylation of Cdk can arrest DNA-damaged cells in both S and G2, a role for Wee1 in normal progression through S phase has not been described. Three of the eight genes were over-detected in 8-Cells and hES cells relative to fibroblasts

Table 1 Detection levels of genes on 8CFES arrays

Cell cycle stage	Gene name	[a]CC	[b]M	Microarray fluorescence units				Entrez gene ID
				8Cell (Ave)	Fibro	iPS	hES (Ave)	
Detected in U2OS and HeLa and cycle in primary fibroblasts								
S	CDC6	y	x	8,735	281	3,165	6,673	990
S	CHAF1A	y		15,120	1,162	7,100	12,999	10036
S	RRM2	y	x	18,568	1,063	2,986	3,072	6241
S	SLBP		x	31,828	6,272	4,242	5,342	7884
S	WEE1	y	x	61	180	652	1,223	7465
G2	CDC2	y		8,742	910	3,930	5,789	983
G2	PLK1	y		42,907	1,973	8,069	14,937	5347
M	AURKB	y		10,493	136	956	882	9212
M	CDCA8	y	x	36,169	2,787	6,010	9,599	55143
M	ESPL1	y	x	5,912	476	2,562	3,227	9700
M	FBXO5	y	x	10,661	367	1,064	2,620	26271
M	SFRS3		x	35,161	23,423	42,175	68,725	6428
M	TPX2	y		21,321	1,505	3,037	5,854	22974
Detected in U2OS and HeLa								
G1	CUL2	y		3,696	385	308	573	8453
G1	KCNH5			5,949	2	3	19	27133
S,G1	RKHD2		x	7,737	34	127	120	51320
M	CYB561D1			13,547	368	254	4,617	284613
M	INCENP	y	x	8,945	241	622	1,812	3619
M	PLXNA1			242	6,425	4,546	5,157	5361
M,G1	CRKRS		x	3,645	64	96	248	51755
M,G1	WIPI1			156	17,639	1,211	2,071	55062
Detected in U2OS and cycle in primary fibroblasts								
G1	BUB1	y	x	7,848	443	3,955	5,369	699
S	GINS2	y		31,973	963	5,092	7,624	51659
S	KIFC1	y	x	13,939	119	700	1,772	3833
G2	CABYR			10,963	399	1,040	1,162	26256
G2	CDKN1B	y		37	773	595	680	1027
M	BARD1	y	x	8,486	515	1,836	2,453	580
M	CDC25B	y		28,304	4,449	538	1,061	994
M	PRKD2			9,218	597	859	2,278	25865
M	REEP4			4,258	449	281	621	80346
Detected in HeLa and cycle in primary fibroblasts								
G1	CCNE1	y	x	102,960	1,596	8,037	13,034	898
G1	HJURP			11,644	357	1,430	2,133	55355
G1	NEK2	y	x	2,792	245	595	665	4751
G1	RBBP6	y		22,492	72	226	260	5930
S	BUB1B	y	x	17,028	439	3,941	6,268	701
S	LMNB2		x	221	937	1,681	5,232	84823
S	NPAT	y		2,058	66	206	375	4863
S	PCNA	y	x	84,348	5,278	11,990	17,444	5111
G2	DTL	y	x	5,010	250	1,614	3,345	51514
G2	H1F0	y		10,711	943	344	1,098	3005
G2	TIPIN	y	x	7,815	1,060	3,315	4,746	54962
M	AURKA	y	x	46,306	1,522	3,234	5,797	6790
M	CCNA2	y		31,573	344	1,538	2,710	890

Table 1 (continued)

Cell cycle stage	Gene name	[a]CC	[b]M	Microarray fluorescence units				Entrez gene ID
				8Cell (Ave)	Fibro	iPS	hES (Ave)	
M	CDC20	y	x	29,105	688	4,203	5,406	991
M	CENPE	y		7,700	755	1,972	3,390	1062
M	CIT	y		356	3,871	1,749	4,732	11113
M	KIF23	y		22,920	1,600	4,376	5,526	9493
M	ORC1L	y	x	2,007	66	812	1,326	4998
Cycle in primary fibroblasts								
S	GMNN	y	x	62,273	2,432	9,468	19,070	51053
G2	UBE2C	y	x	144,929	2,903	12,472	28,375	11065
M	CCNB1	y	x	156,398	4,004	13,791	19,417	891
M	CKS1B	y	x	87,120	5,491	20,061	33,098	1163
M	PTTG1	y	x	579,305	15,479	31,030	41,398	9232
M	SKP2	y	x	26,057	159	1,139	1,873	6502

[a] Known cell cycle gene

[b] Myc target (27,28)

(Table 1): CDC6, an essential DNA replication licensing factor; CHAF1A, chromatin assembly factor 1, part of a nuclear complex that assembles histones onto newly replicated DNA; and RRM2, ribonucleotide reductase 2, a well known rate-limiting enzyme for DNA replication. The two genes whose knock-down blocked G2 in both HeLa and U2OS, CDC2 (CDK1, cyclin dependent kinase), and PLK1 (pololike kinase 1), were both over-detected on the 8-Cell and hES gene arrays relative to fibroblasts. Both Cdc2 and Plk1 have numerous roles in cell cycle progression [34, 35].

Six cycling genes were needed for mitosis in both cell types, one of which, AURKB, was >10-fold over-detected on the 8-Cell arrays relative to all other cell types (Table 1). Aurora kinase B is a chromosomal passenger protein whose expression for normal cytokinesis is tightly controlled: over-expression or under-expression leads to centrosome amplification, defects in spindle formation, and multinucleation [36]. Four of the six cycling mitosis genes were over-detected on both 8-Cell and hES arrays relative to fibroblasts: CDCA8 (borealin), ESPL1 (separase), FBX05 (Emi1) and TPX2. Borealin is a chromosomal passenger protein required for stability of the bipolar mitotic spindle [37] and recently reported to be essential for cleavage from the 2-cell to 4-cell stage of mouse embryos [38]; separase cleaves cohesin to allow the separation of sister chromatids [39] and cleaves the linker between mother and daughter centrioles to license centriole duplication [40]; Emi1 represses APC/C^cdh1 in G2 [36], thus blocking the degradation of Cyclin A, -B and geminin, and allowing both the formation of new DNA replication initiation sites and the prevention of re-replication of DNA before mitosis. Emi1 is essential for mouse embryo development beyond the blastocyst stage [41] and for normal development in zebrafish [42]. Failure to degrade Emi1 in prophase leads to "mitotic catastrophe", including centrosome overduplication [41]. Tpx2 is expressed exclusively at the G1/S transition and throughout G2 and activates Aurora kinase A on centrosomes to assemble mitotic spindles [43, 44].

Cell cycle genes identified by RNA knock-down in U2OS and HeLa

Fifty three gene elements were identified as inhibiting cell cycle progression in both the U2OS and HeLa cells, but were not identified as cycling by the primary fibroblast study. Only 12 DAVID-mapped to cell cycle or DNA metabolism, suggesting the discovery of many new genes important for cell cycle progression that may not have redundant functions and that should be GO designated cell cycle. Interestingly, several were not detected above background levels in 8CFES (Tables S1–S3). Twenty were reported targets of Myc [39, 40]. Eleven genes arrested G1, 10 arrested S phase, 10 arrested G2, and 22 arrested mitosis.

KCNH5 (Eag2), one of the knocked-down genes that arrested both cell types in G1, was markedly over-detected on the 8-Cell arrays (Table 1, and 2), but nearly silent on all other 8CFES arrays. Eag2 is an outwardly rectifying voltage-gated potassium channel recently shown to bind to alpha and beta-tubulin [45] suggesting it may play an important role in the cytoskeleton of the large 8-cell blastomeres. Knock-down of RKHD2 (MEX3C), the human homolog of the C. elegans Mex-3 gene, arrested U2OS in G1 and HeLa cells in S phase. One allele of

Table 2 Functional categories of cell cycle genes

Gene name	Expression			Entrez gene ID	Known CC	Myc target
	8C Over	8C,hES Over	8C Under			
Cyclin, cyclin-dependent kinase (cdk), cdk-inhibitor						
CCNE1	x			898	y	x
CDC25B	x			994	y	
CCNA1	x			8900	y	
CCNA2		x		890	y	
CCNB1	x			891	y	x
CCNB3	x			85417	y	
CCND1			x	595	y	x
CDC2		x		983	x	
CDC2L1	x			984	y	
CDKN1A, -B			x	1026	y	
CRKRS	x			51755		x
CDK6			x	1021	y	x
Kinase, kinase inhibitor, phosphatase						
AURKA,-B	x			6790, 9212	y	x
AURKC	x			6795	y	
BUB1,-1B		x		699, 701	y	x
CKS1B		x		1163	y	x
MAPK4(ERK3)	x			5596		
NEK2	x			4751	y	x
PPMID	x			8493	y	x
PLK1		x		5347	y	
PRKD2		x		25865		
SKP2	x			6502	y	x
WEE1			x	7465	y	x
Transcription, translation						
E2F3			x	1871	y	x
MYC	x			4609	y	
RB			x	5925	y	x
RBBP4		x		5928	y	x
RBBP6	x			5930		
RBM14		x		10432	y	x
RKHD2	x			51320		x
DNA replication, chromosome duplication, cohesin						
CDCA8(borealin)		x		55143	y	x
CDC6		x		990	y	x
CHAF1A		x		10036	y	x
CDC45L		x		8318	y	
CDT1		x		81620	y	x
DTL		x		51514	y	x
ESPL1(separase)		x		9700	y	x
GINS2,-3		x		51659, 64785	y	
GINS4	x			84296	y	
GMNN		x		51053	y	x
H1FO	x			3005	y	
HJURP		x		55355		
NPAT	x			4863	y	

Table 2 (continued)

Gene name	Expression			Entrez gene ID	Known CC	Myc target
	8C Over	8C,hES Over	8C Under			
ORC1L		x		4998	y	x
PCNA		x		5111	y	x
POLS	x			11044	y	x
PTTG1,-3	x			9232, 26255	y	
PTTG2	x			10744	y	
RFC3		x		5983	y	
RRM2	x			6241	y	x
TERF1			x	7013	y	
TIPIN		x		54962	y	x
STAG3,-3L3	x			10734, 442578	y	x
Centrosome, centriole, securin, spindle						
CENPE		x		1062	y	
CDC20		x		991	y	x
DLG7	x			9787	y	
INCENP	x			3619	y	x
TPX2		x		22974	y	
Ion channel, structural protein, other						
AVPI1	x			60370	y	x
BARD1		x		580	y	x
CABYR	x			26256		
FBXO5(EMI1)		x		26271	y	x
KCNH5	x			27133		
KIF23		x		9493	y	
KIFC1					y	x
LMNB2			x	84823		x
CUL2,-5	x			8453, 8065	y	
CIT			x	11113	y	
REEP4	x			80346		

RKHD2 was also markedly over-detected on the 8Cell arrays (Table 1) which is especially interesting because in combination with CUL2 (encodes the ubiquitin ligase also over-detected on the 8-Cell arrays), it has important roles in anterior-posterior cell partitioning in C. elegans embryos [46]. Only CUL2, not RKHD2 nor KCNH5, DAVID-mapped to cell cycle.

Knockdown of a cytochrome B-like gene (CYB561D1) and a CDC2-related kinase, CRKRS (CRK7), the exact functions of which are unknown, blocked mitosis in both U20S and HeLa and did not DAVID-map to cell cycle. CYB561D1 was detected at higher levels on the 8-Cell and hES cell arrays than either the fibroblast or iPS cell arrays (Table S3), whereas CRKRS was detected at higher levels on the 8-Cell arrays than all other cell types (Table 1).

Not surprising, knock-down of INCENP, inner centromere passenger protein, blocked mitosis in both U20S and HeLa, but it was surprisingly over-detected in 8-Cells relative to the other cell types, especially fibroblasts and iPS cells. In mammalian cells, 2 broad groups of centromere-interacting proteins have been described: constitutively binding centromere proteins and 'passenger,' or transiently interacting, proteins. The constitutive proteins are encoded by CENPA, -B, -C1 and -PD, none of which were identified by the RNA knock-down studies (Table S1). The term 'passenger proteins' encompasses a broad collection of proteins that localize to the centromere during specific stages of the cell cycle [47, 48]. These include CENPE and -F, cytoplasmic dyneins (e.g. DYN-C1LI2, DYNLL1, DYNLRB1, Table S1) and INCENP. The passenger proteins display a broad localization along chromosomes in the early stages of mitosis but gradually become concentrated at centromeres as the cell cycle progresses into mid-metaphase. During telophase, the

proteins are located within the midbody in the intercellular bridge, where they are discarded after cytokinesis. INCENP and CENPE knockout mice die at peri-implantation stage [49].

Cell cycle genes identified by RNA knock-down in U20S cells and cycle in fibroblasts

Eighteen genes were detected by knock-down in U2OS and also cycled in primary fibroblasts (Tables S1 and S3), of which 12 had previously been designated GO term cell cycle. Four blocked G1, two blocked S phase, four arrested G2, and eight blocked mitosis. Eleven are known cell cycle genes and seven are Myc targets.

Knock-down of BUB1brought about a G1 arrest in the U20S cells, a surprise because this gene encodes a kinase involved in spindle checkpoint activation, suggesting it plays a role in M phase. Mutations in this kinase, over-detected in 8-Cells, iPS cells and hES cells relative to fibroblasts, have been associated with aneuploidy and several forms of cancer [50].

One of the cycling genes in this group whose knock-down blocked U20S cells in S phase, GINS2, part of the DNA replication initiation complex, was over-detected on 8-Cells and hES cells, and one, KIFC1, a kinesin family member central to chromosome congression [51], was markedly over-detected on the 8-Cell arrays (Table 1). Only one of the knocked-down genes in this group that blocked U20S cells in G2 was over-expressed in the 8-Cells, CABYR, a calcium-binding protein, which did not DAVID-map to cell cycle. Originally thought to be testis specific, and a marker for testis cancer, several non-testis variants of this gene have been described [52]. CDKN1B (p27) was under-detected specifically on the 8-Cell arrays, thus removing another potential cell cycle block.

Of the 8 cycling genes in this group whose knock-down blocked mitosis, two were over-detected in 8Cells and hES cells, BARD1 and PRKD2, and two were over-detected specifically in 8Cells, CDC25B and REEP4. Bard1 is important to centrosome duplication and mitotic spindle assembly [53], and essential for mouse embryo development [54]; PRKD2 is a serine/threonine protein kinase about which little is known. CDC25B activates the cyclin dependent kinase CDC2 (CDK1) and is required for entry into mitosis; REEP4 is a membrane receptor accessory protein about which little is known.

Cell cycle genes identified by RNA knock-down in HeLa cells and cycle in fibroblasts

Fifty six genes were identified in the HeLa cells that also cycled in the synchronized primary fibroblasts (Tables S1 and S3), 32 of which DAVID-mapped to cell cycle, and 17

of which were identified as Myc targets. Fifteen blocked G1, 16 blocked S phase, five blocked G2, and 19 blocked mitosis.

Of the 15 cycling genes that blocked G1 progression in HeLa when knocked-down, two were over-detected on the 8-Cell arrays, CCNE1 and RBBP6, and two, HJURP and NEK2 were over-detected on both 8-Cells and hES cells. RBBP6 (retinoblastoma binding protein 6) is a multifunctional protein known to have Rb-independent activities [55]. The over-expression of HJURP, Holliday junction-recognizing protein, suggests the 8-Cells and hES cells may invoke a response to DNA double-strand breaks first described for cancer cells that prevents apoptosis in response to DNA damage and continues cell cycling [56]. Recent work suggests HJURP stabilizes Cenp A, the anchor protein for centrosome formation, and is therefore important for normal cytokinesis [57, 58]. NEK2 encodes a kinetochore-associated protein kinase that stabilizes chromosome attachment to spindle microtubules [59].

Of the 16 knocked-down genes that blocked HeLa in S phase, three were over-detected on the 8-Cell and hES cell arrays relative to fibroblasts, BUB1B, NPAT and PCNA, and one was markedly under-detected, LMNB2. Bub1B (BubR1) is a critical member of the spindle assembly checkpoint [60]. NPAT is an essential member of the p220 (NPAT)/HiNF-P pathway, controlled by CyclinE/Cdk2, which activates histone H4 gene expression, essential to accurate packaging of newly synthesized DNA into chromatin [61]. PCNA, proliferating cell nuclear antigen, is a member of the DNA sliding clamp family, forming a trimeric, head to tail ring around helical DNA. It is a processivity factor for DNA polymerase delta, and also has other functions in cell cycle control [62, 63].

The under-expression of LMNB2 specifically in the 8-Cells is intriguing. The lamins, A/C, B1 and B2, are intermediate filament proteins that make up the nuclear matrix next to the inner nuclear membrane. During mitosis, the lamina matrix is reversibly disassembled as the lamin proteins are phosphorylated. Structural assessment of various combinations of lamins A/C, B1 and B2 indicated polymers with B2 were "weaker" than those with B1 [64]. LmnB1 mRNA was detected on the 8CFES arrays at approximately the same level as the iPS and hES cells, all at higher levels than the fibroblasts. This suggests that the 8Cell nuclear lamina matrix may be comprised principally of LmnB1. The LMNB2 gene sequence is also a known origin of replication site, and given the rapid cell cycles of early embryos, the gene may be bound by DNA replication initiation factors nearly without interruption [65].

Of the five cycling knocked-down genes that blocked HeLa in G2, two were over-detected on the 8Cell and hES arrays: DTL, the Cdt1-targeting component of one of the ubiquitin ligases (CUL4-DDB1) responsible for degrada-

tion of Cdt1 during S phase to prevent re-replication of DNA [66], and TIPIN, encodes a replication fork binding partner of Timeless [67]. H1F0, an oocyte-specific replacement linker histone [68], was over-detected specifically on the 8-Cell array.

Of the 19 knocked-down genes that blocked HeLa in mitosis, one was over-detected on the 8-Cell arrays, AURKA, and five were over-detected on the 8-Cell and hES arrays: CCNA2, CDC20, CENPE, KIF23, and ORC1L, and one was markedly under-detected on the 8-Cell arrays, CIT, a citron kinase essential for normal cytokinesis in neuronal progenitor cells [69]. Aurora kinase A is involved in centrosome and spindle assembly and activates Plk1 at the G2/M transition [70]. Over activation of either, or both, is linked to cancer development [70]. Ccd20 is a regulatory subunit of anaphase promoting complex, APC/C, and may play a more important role in early embryo cells [71] than in somatic cells [72]; CenpE, centrosome protein E, and KIF23, are both kinesin-like motor proteins that play a role in chromosome movement [51, 73]; and Orc1l is the largest subunit of the Orc1 complex essential for the initiation of DNA replication and recently shown to also play a key role in controlling centriole and centrosome copy number in human cells [74].

Interestingly, several well described cell cycle genes shown to cycle in the primary fibroblasts were not detected by the knock-down strategies, including GMNN and CKS1B, and UBE2C over-detected on both the 8-Cell and hES arrays, and CCNB1, PTTG1 (securin) and SKP2, over-detected specifically on the 8-Cell arrays. Geminin prevents re-replication of DNA during S phase [75]; Cks1B is a kinase that blocks the inhibition of Cdks by p27 (CDKN1B) [76]. UBE2C encodes a ubiquitin-conjugating enzyme that specializes in targeting cyclins, especially G1 cyclins, for proteolysis [77]; Cyclin B1 stimulates Cdk1 during G2, and has multiple roles in the G2 to M transition [78]; securin is best known for inhibition of separase (Espl1), the enzyme that allows the separation of sister chromatids, for most of the cell cycle [79]; Skp2 is a well-studied component of a ubiquitin ligase that targets Cyclin E and p27 for proteolysis [80].

Conclusions

Using public databases of gene ontologies, combined with a gene assembly derived from high-throughput RNA knock-down and cell synchronization studies, we have identified 35 cell cycle genes over-detected on whole human genome microarrays of 8-cell stage blastomeres, 31 genes over-detected on both 8-Cell and hES microarrays, and ten genes under-detected on 8-Cell arrays (Table 2). This is by no means a comprehensive list of genes important to the cell

cycle of totipotent and pluripotent cells because of the significant number of genes with unknown function, the relatively conservative (±7-fold) criteria for classification of over- or under-detection, and the lack of verification that the microarray results reflect protein expression. It does, however, lay groundwork essential for the design of the targeted experiments possible with the small numbers of human embryos available for research.

Whether or not the genes are known targets of Myc (14 of the 35 genes over-detected on the 8-Cell arrays, 18 of the 31 over-detected on both the 8-Cell and hES arrays, and five of the ten genes under-detected on the 8-Cells) did not predict level of detection on the microarrays despite the relatively high level of detection of MYC on the 8-Cell arrays. This may relate to the observation that association with multiple transcription factors in addition to Myc correlated with high level of expression in embryonic stem cells [29].

The 8-Cell blastomere is only one or two cell cycles away from initial commitment to either outer trophoblast cells, or inner cell mass cells. That commitment carries with it a new dependence upon growth factors, such as fibroblast growth factor and insulin, for both cell lineages [81–83], in keeping with the detection of RB1 on the hES cell microarrays [1] and in mouse blastocysts [6]. Most of the daughter cells of the 8-Cell blastomere will commit to trophoblast, only a few to inner cell mass. Trophoblast stem cells will in turn undergo commitment to placenta, including initiating cycles of endoreduplication of the genome without cytokinesis, characteristic of placental cells [84].

The functions of 16 of the 35 genes over-detected on the 8-Cell arrays: AURKC, AVPI1, CABYR, CCNA1, CCNB3, CDC2L1, CRKRS, CUL2, KIFC1, RBBP6, RKHD2, PTTG2, -3, REEP4, STAG3, -3L3, are poorly understood and urgently in need of additional study. The functions in the 8-Cell blastomeres of the 19 better studied genes over-detected on the 8-Cell arrays: CCNE1, CDC25B, CCNB1, AURKA, -B, MAPK4, SKP2, MYC, NPAT, POLS, PTTG1, RRM2, DLG7, INCENP, KCNH5, PPM1D, GINS4, H1F0, and KIFC1 are also not known with certainty, although some predictions are possible.

The lack of Rb and Wee1 is similar to the absence of cell cycle checkpoints in frog embryos [24], relieves the 8-Cells from growth factor dependence for the G1 to S transition, and is consistent with the over-expression of CCNE1 and CCNA1, -2 factors essential for the G1 to S transition. RB1 was also silent, and Wee1 at threshold level of detection, on the microarrays of ovulated metaphase II human oocytes [31], indicating that unlike mouse embryos [6], these two genes are not among those that undergo degradation the first few days after fertilization of human eggs [85]. If checkpoint control in early human embryos is analogous to

frog embryo development, RB and WEE1 may be turned on at the blastocyst stage, as evidenced by their detection on the hES cell microarrays [1], and those cells with abnormal karyotype or errors in DNA replication may undergo programmed cell death [22–24].

Three other genes over-detected on the 8-Cells that were either not detected, or detected at very low levels in the human oocytes [31] are INCENP, KCNH5 and CCNA1, indicating these genes must also be upregulated following fertilization. In contrast, CCNE1, PTTG, and STAG3 were readily detected on the oocyte arrays, suggesting maternal messages could be still detected in the 8-Cells.

Frog embryo survival depends on becoming motile as quickly as possible after fertilization. Human embryo survival depends on sending immediate, sufficient and increasing molecular signals to the mother that development is progressing, or menses ensues. One obvious mechanism for accomplishing these goals is rapid duplication of gene copies early in development, which is more easily accomplished if cell cycle checkpoints are silent, and cell cycle drivers are over-expressed. But subsequent development requires faithful duplication and allocation of chromosomes, so how does the early embryo accomplish both?

Clues from the microarray data suggest G1 and S phase progression are augmented by ample Cyclin E, Cyclin A1, -2 and key DNA replication factors, such as ribonucleotide reductase and DNA polymerase S, thought to push strand synthesis through cohesin sites, Npat, important for chromatin organization, and Hjurp, thought to block cell cycle arrest by DNA strand breaks. Robust expression of geminin and its protector, Emi1, may prevent DNA re-replication errors. G2 to M phase is rushed by ample expression of Cyclin A and Cyclin B, Cdc25B, and elements important to timely proteolysis, Ube2C and Skp2, to allow the transition to metaphase. Attempts to ensure the integrity of chromosome allocation to daughter blastomeres include over-expression of chromosome glue, including Incenp and Stag proteins, as well as Pttg proteins to block separase (Espl1) activity and prevent pre-mature chromosome separation. Especially noteworthy on the 8-cell arrays are the high levels of detection of the Aurora kinase family, A, B and C, major players in centrosome duplication and formation of the mitotic spindle, as well as kinesins, KIFC1 and KIF23.

Taken together, the lack of well described cell cycle checkpoints in the presence of robust expression of cell cycle drivers and their controls in the 8-Cell blastomeres, suggest more dependence upon accurate cycling of gene expression, perhaps regulated by circadian oscillators [1], for faithful daughter cell formation than on the checkpoints imposed by growth factor dependency of later stage cells. This would have the advantage of rapid gene duplication, but the disadvantage of cleavage mishaps resulting from

even slightly out of phase or inappropriate abundance of gene expression. The phenomenon of identical twinning indicates that only half of early cleavage cells are needed for viable offspring, suggesting at least half of the blastomeres could be genetically defective without dire consequences. Studies of the relative expression in individual normal and abnormal human embryos of the genes identified by this analysis of 8-Cell blastomere gene expression will reveal which cycling elements are the most critical for development.

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