Stem Cell Reports, Volume *6*

Supplemental Information

Higher-Density Culture in Human Embryonic Stem Cells Results in DNA Damage and Genome Instability

Kurt Jacobs, Filippo Zambelli, Afroditi Mertzanidou, Ilse Smolders, Mieke Geens, Ha Thi Nguyen, Lise Barbé, Karen Sermon, and Claudia Spits

Supplementary Data – Higher density culture in human embryonic stem cells results in DNA damage and genome instability

Supplementary Table 1: Overview of biological and technical replicates.

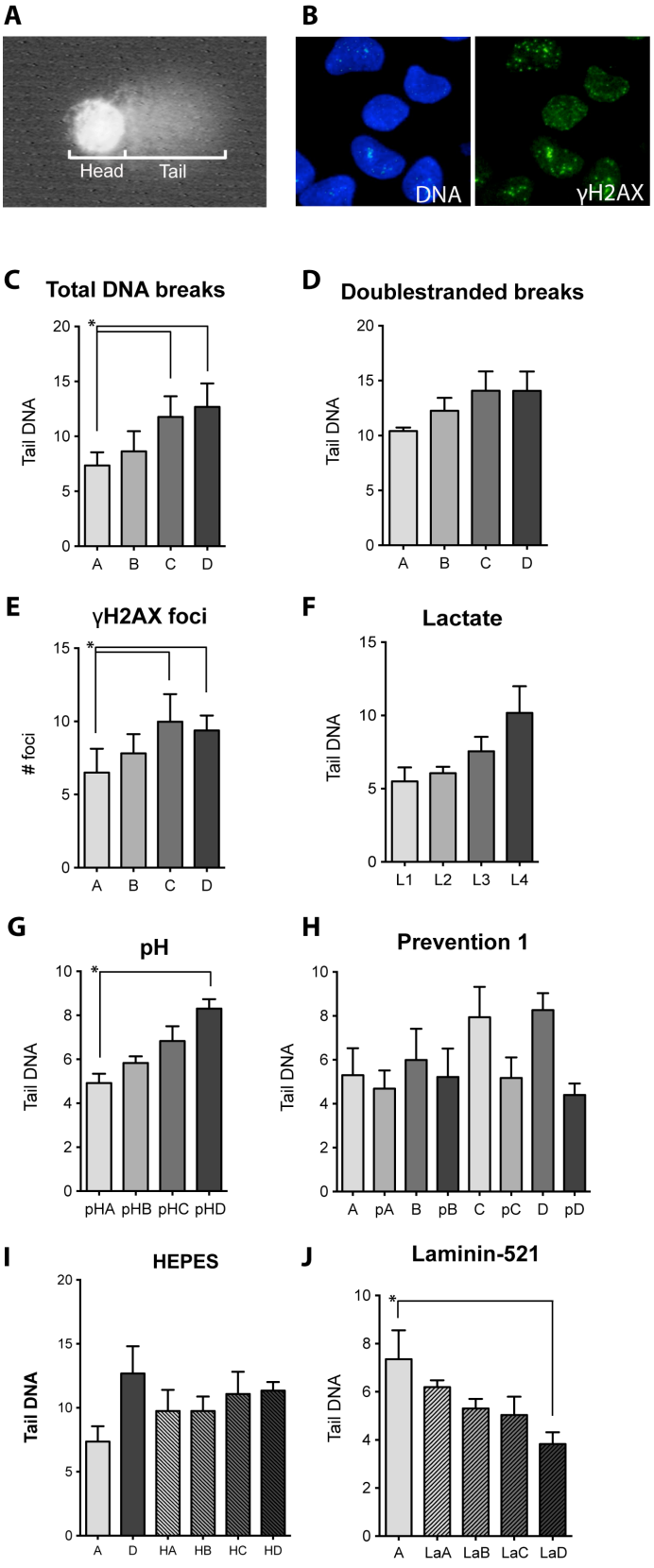
The hESC lines used in this study are listed per type of experiment and assay, with the number of biological replicates per cell line between brackets. A biological replicate is defined as a full and independent experiment. In the case of biological replicates involving the same hESC line, the cell cultures in different conditions were initiated on different days for each biological replicate. For each culture condition within one experiment, at least three wells were pooled to obtain sufficient material and reduce possible technical variation, as explained in the materials and methods section. The minimum number of analyzed cells or events per condition per replicate is listed under technical replicates.

ROS stands for 'Reactive Oxygen Species', PI for 'propidium iodide', qRT-PCR for 'quantitative real-time PCR', aCGH for 'array-based comparative genomic hybridization', HPLC for 'high performance liquid chromatography', FISH for 'fluorescent in situ hybridization'.

	Assay	**Biological replicates**	**Technical replicates**	**Figure**
DNA damage	Alkaline Comet Assay	VUB07 (1), VUB14 (2), VUB31 (3)	≥200 cells per condition	1B,E ; S1C
	Neutral Comet Assay	VUB14 (2), VUB31 (1)	≥200 cells per condition	1C,F ; S1D
	γH2AX foci quantification	VUB07 (2), VUB14 (2)	≥1000 cells per condition	1D,G ; S1E
Metabolism	Annexin V/PI Assay	VUB14 (2), VUB31 (2)	≥2500 cells per condition	3A
	ROS analysis	VUB14 (1), VUB31 (3)	≥10000 cells per condition	3C
	Cell Cycle Profiling	VUB14 (2), VUB31 (2)	≥10000 events per condition	3B
	qRT-PCR	VUB14 (1), VUB31 (2)	triplicates	S3
Chromosomal content	single-cell aCGH	VUB31	29 cells in condition A 30 cells in condition D	2
Medium analysis	HPLC (day 5)	VUB07 (3), VUB14 (3), VUB31 (3)	duplicates	3D,E
	Glucose and Lactate (day 5)	VUB07 (3), VUB14 (3), VUB31 (3)	duplicates	3F,H
	Folic acid (day 5)	VUB07 (1), VUB14 (2), VUB31 (2)	duplicates	Not shown
	pH (day 5)	VUB14 (3), VUB31 (2)	duplicates	3G
	Lactate and pH (daily)	VUB14 (2), VUB31 (1)	single measurement	3I,J
Lactate series	Alkaline Comet Assay	VUB31 (3)	≥200 cells per condition	4A,B
	pH and Lactate Assay	VUB31 (3)		Table S3B
pH series	Alkaline Comet Assay	VUB31 (3)	≥200 cells per condition	4C,D
	pH and Lactate Assay	VUB31 (3)		Table S3C
Prevention 1 series	Alkaline Comet Assay	VUB31 (3)	≥200 cells per condition	5A
	pH and Lactate Assay	VUB31 (3)		Table S3D
Prevention 2 series	FISH	VUB14 (2), VUB31 (2)	≥400 cells per condition	5B
HEPES series	Alkaline Comet Assay	VUB07 (1), VUB14 (1), VUB31 (1)	≥100 cells per condition	5E
	Cell Cycle Profiling	VUB07 (1), VUB14 (1), VUB31 (1)	≥10000 events per condition	5F
Laminin-521 series	Alkaline Comet Assay	VUB07 (1), VUB14 (1), VUB31 (1)	≥100 cells per condition	5H
	Cell Cycle Profiling	VUB07 (1), VUB14 (1), VUB31 (1)	≥10000 events per condition	5J
	FISH	VUB07 (1), VUB14 (1), VUB31 (1)	≥400 cells per condition	5I

Supplementary Figure 1: Absolute values for DNA damage assays

A,B) representative pictures for an analysed cells for the COMET assay (A) and the γH2AX staining (B). C-J) Plots of the absolute values (means ± SEM) used to calculate the relative values presented in the main figures. C) corresponds to Figure 1B, D) corresponds to Figure 1C, E) corresponds to figure 1D, F) corresponds to Figure 4A, G) corresponds to Figure 4C, H) corresponds to figure 5A, I) corresponds to Figure 5D, J) corresponds to Figure 5F.



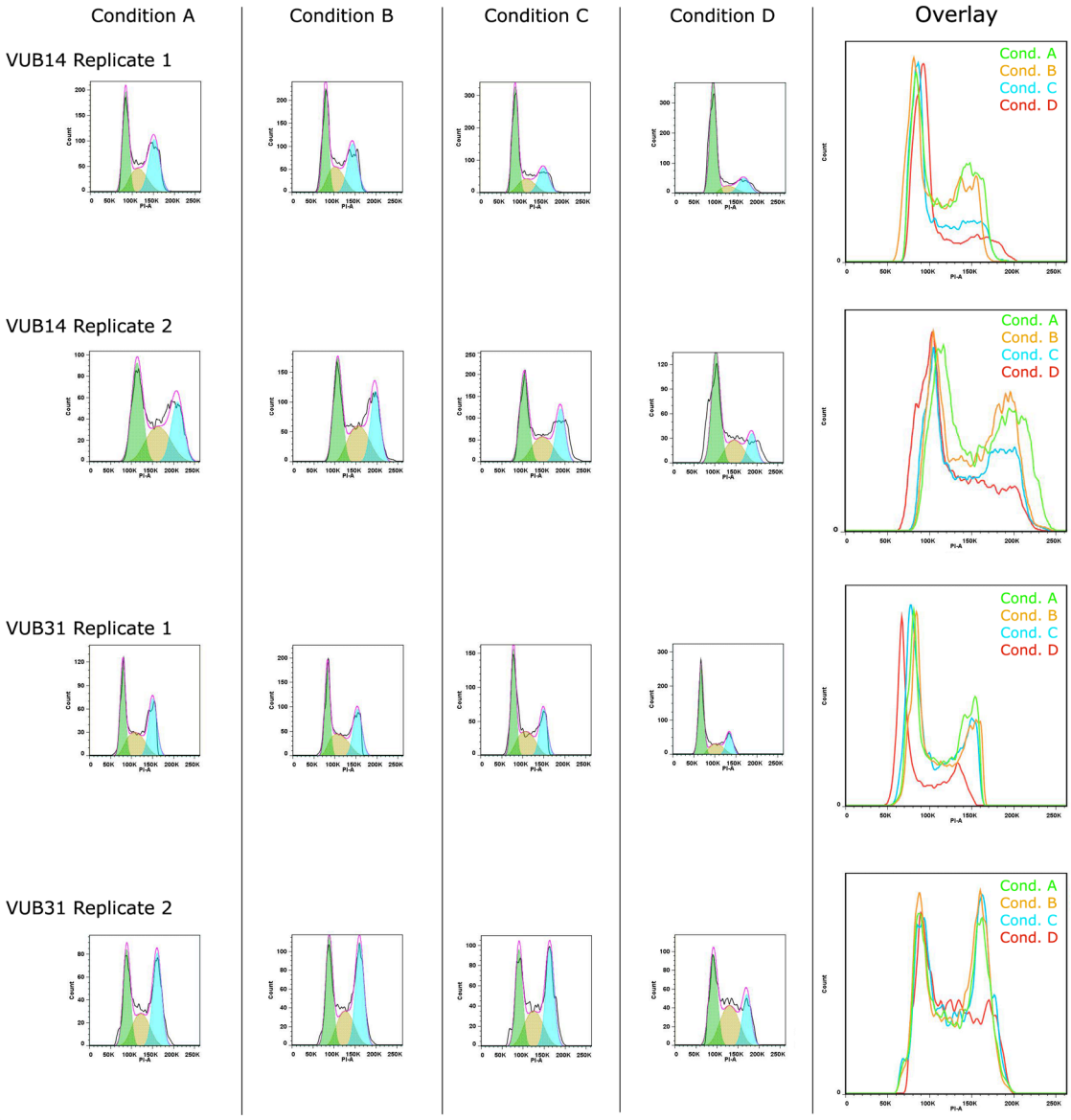
Supplementary Table 2: Overview of chromosomal content and breakpoints of the detected CNV's.

All hESC with a normal (balanced female) or abnormal (unbalanced) chromosomal content are listed, and details on the detected CNVs are given. Chr. stands for chromosome, Mb stands for Megabase.

		Breakpoints			
Sample	**Chromosomal content**	**Chr.**	**StartPosition**	**EndPosition**	**Size (Mb)**
Condition A					
Balanced cells (N = 29)	Balanced female				
Condition D					
Balanced cells (N = 20)	Balanced female				
Unbalanced cell (N = 10)					
VUB31_D_01	Unbalanced female, dup(1()q21.1q44)	1	144041843	248776442	104.7
VUB31_D_03	Unbalanced female, del(3)(p26.1)	3	233708	7685894	7.4
VUB31_D_05	Unbalanced female, dup(6)(p12.3p21.31), dup(10)(p11.21q23.32)	6	36144335	51262134	15.1
		10	37583025	93835221	56.3
VUB31_D_09	Unbalanced female, dup(1)(q21.1q44)	1	144041843	248776442	104.7
VUB31_D_10	Unbalanced female, dup(3)(q21.3q23),dup(15)(q21.2q23), dup(18)(q21.32q23)	3	125916715	140768168	14.9
		15	51921437	62998451	11.1
		18	58803741	77856022	19.1
VUB31_D_12	Unbalanced female, dup(1)(q21.1q44)	1	144041843	248351025	104.3
VUB31_D_24	Unbalanced female, del(3)(p26.3)	3	233708	658659	0.4
VUB31_D_25	Unbalanced female, dup(9)(p22.3), dup(10)(p11.1p11.21)	9	104476	15320901	15.2
		10	37455731	38148165	0.7
VUB31_D_27	Unbalanced female, dup(1)(p13.2q44)	1	114680507	248776442	134.1
VUB31_D_28	Unbalanced female, dup(1)(q21.1q44)	1	144041843	248776442	104.7

Supplementary Figure 2: Overview of the flow cytometry profiles for the cell cycle analysis

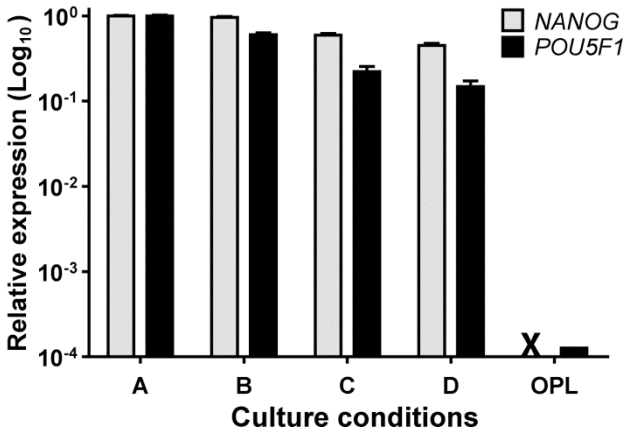
Cell cycle profiles of hESC (N = 2 for VUB14 and N = 2 for VUB31) growing in the four culture densities A - D. The fractions of cells in G1 (green), S (yellow) and G2/M (blue) were determined by the Dean-Jett-Fox model using Flowjo software. An overlay of all cell cycle fractions is provided for each replicate.



Supplementary Figure 3: mRNA quantification of the pluripotency markers *NANOG* and *POU5F1*.

Messenger RNA of *NANOG* and *POU5F1* was quantified by quantitative real-time PCR for VUB14 (N = 1) and VUB31 (N = 2) grown in the four culture densities. The gene-expression is plotted relative to that of cells growing in culture condition A (lowest density), and in a $\log_{10}$ scale. OPL stands for osteoprogenitor-like cells, which are differentiated hESC. X stands for undetectable levels of expression. Results are presented as means ± SEM.

All hESC groups show at least a 4000-fold higher expression as compared to OPLs ($p \leq 0.01$; two-way ANOVA). Nevertheless, when comparing the highest density condition (D) to the lowest (A) there was a trend to a lower expression of these markers in condition D (a two-fold change for *NANOG* ($p \leq 0.01$; two-way ANOVA) and a six-fold change for *POU5F1* ($p \leq 0.01$; two-way ANOVA). Taken together, this indicates that whilst the analyzed cells were still undifferentiated, there appears to be a small loss in pluripotency in the highest density condition. However, the change in cell cycle profile (see Supplementary Fig. 1b) cannot be accounted for by the relative loss of pluripotency only, as testified by the insignificant differences in *NANOG* and *POU5F1* expression between C and D, while, in contrast to condition D, the cell cycle profile for condition C is still indistinguishable from condition A.



Supplementary Table 3: Overview of lactate concentration and pH of all analyzed samples.

Lactate concentrations and pH values of the medium at the time of analysis of (a) the culture density series, (b) the lactic acid addition experiments, (c) the modified pH experiments, (d) the prevention experiments, (e) the four cell densities grown in medium supplemented with 25mM HEPES and (f) the four cell densities grown on Laminin-521. All values are presented as mean ± SEM.

a)

	MEF only	A	B	C	D
Lactate (mmol/L)	2.22 ± 0.33	2.66 ± 0.39	4.91 ± 0.78	10.16 ± 1.34	14.39 ± 0.71
pH	7.44 ± 0.03	7.29 ± 0.03	7.25 ± 0.03	7.10 ± 0.03	6.90 ± 0.03

b)

	L1	L2	L3	L4
Lactate (mmol/L)	0.87 ± 0.15	5.07 ± 0.15	9.73 ± 0.20	13.77 ± 0.03
pH	7.37 ± 0.01	7.29 ± 0.01	7.19 ± 0.01	7.06 ± 0.01

c)

	pHA	pHB	pHC	pHD
pH	7.37 ± 0.01	7.25 ± 0.01	7.07 ± 0.02	6.79 ± 0.05

d)

	A	pA	B	pB	C	pC	D	pD
Lactate (mmol/L)	2.25 ± 0.67	0.15 ± 0.15	4.78 ± 0.84	0.90 ± 0.11	9.43 ± 0.34	3.45 ± 0.89	13.73 ± 0.10	3.98 ± 0.94
pH	7.24 ± 0.01	7.32 ± 0.02	7.21 ± 0.02	7.30 ± 0.03	7.08 ± 0.04	7.24 ± 0.02	6.90 ± 0.06	7.18 ± 0.03

e)

	HA	HB	HC	HD
pH	7.34 ± 0.01	7.33 ± 0.01	7.19 ± 0.02	6.94 ± 0.03

f)

	LaA	LaB	LaC	LaD
pH	7.41 ± 0.01	7.40 ± 0.01	7.10 ± 0.01	6.93 ± 0.01

Supplementary Table 4: Overview of all abnormalities detected with FISH

a) All abnormalities found using interphase-FISH for chromosome 18 in density condition A and D and their respective rescue conditions. The experiment was performed in triplicate (N = 2 for VUB31 and N = 1 for VUB14) and the sum of all abnormalities found over the three experiments is listed here.

b) All abnormalities found using interphase-FISH for chromosome 18 in laminin-cultured hESC of conditions LaA and LaD. The experiment was performed in triplicate (VUB07, VUB14 and VUB31) and the sum of all abnormalities found over the three experiments is listed here.

p+ and p- stand for gain and loss of the p-telomere respectively. q+ and q- stand for gain and loss of the q-telomere respectively. 18+ and 18- stand for trisomy and monosomy 18 respectively.

a)

	Total number of cells	p+	p-	q+	q-	18+	18-
A	1203	4	3	4	3	0	0
pA	938	3	0	8	0	0	0
D	1510	5	5	12	3	2	2
pD	1207	3	5	8	0	0	0

b)

	Total number of cells	p+	p-	q+	q-	18+	18-
LaA	1200	4	5	8	6	0	2
LaD	635	1	2	2	4	0	1