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Synthesis

Cancer-Related Mutations Identified in Primed and Naive Human Pluripotent Stem Cells

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Human pluripotent stem cells (hPSCs) are known to harbor chromosomal aberrations, affecting their tumorigenic potential. We established a strategy to identify cancer-related point mutations in hPSCs, detecting recurrent mutations in over 20 genes, alongside those previously detected in p53. Importantly, naive hPSCs harbor, on average, four times more mutations than their primed counterparts, which appear primarily in pathways inhibited during naive conversion. Such cancer-related mutations should be taken into consideration in future applications, especially in clinical contexts.

Human pluripotent stem cells (hPSCs) are defined by their ability to self-renew and the capacity to differentiate into all embryonic germ layers. These traits turn them into a unique and promising resource in disease modeling and regenerative medicine. Contrary to immortalized cell lines, hPSCs are considered normal cells that maintain their genomic integrity and epigenetic landscape *in vitro*. However, studies have shown that these cells acquire various aberrations during prolonged culturing, including large chromosomal aneuploidies (Draper et al., 2004; Mayshar et al., 2010) and copy number variation (CNV) alterations (Laurent et al., 2011). Such aberrations, which are similar to those observed in germ cell tumors, were shown to gradually take over the culture, provide selective advantage to the cells, and potentially increase the risk of malignant tumor formation upon transplantation (Ben-David et al., 2014).

Point mutations found in induced pluripotent stem cells (iPSCs) were first suggested, in 2011, to arise during reprogramming or originate from their somatic origin (Gore et al., 2011). Recently, we used whole-exome sequencing (WES) to identify acquired point mutations in early passage human embryonic stem cells (hESCs) (Merkle et al., 2017). *TP53* was the only gene mutated in multiple hESC lines, with mutations previously identified in malignant tumors. Similar mutations were then found in late-passage hESCs and iPSCs using RNA sequencing. These mutations were shown to gradually take over the culture, suggesting that they provide a selective advantage to the cells (Merkle et al., 2017). However, it remains to be determined whether hPSCs acquire mutations in other cancer-related genes during prolonged culturing.

Here we established a new strategy to identify cancer-related mutations in hESCs during their propagation in culture. We show that alongside mutations in the *TP53* gene, recurrent mutations appear in at least 22 other tumor-related genes and that these mutations reoccur in iPSCs. hESCs have been suggested to have two distinct pluripotent states: a naive state, akin to inner cell mass (ICM) cells, and a primed state, similar to embryonic epiblast cells. We further show that during *in vitro* transition from primed to naive state, the cells harbor significantly more cancer-related mutations, specifically in pathways inhibited during prime-to-naive transition. These mutations hint that genetic variation acquired in culture accommodates the selective pressures imposed on the cells during this process, stabilizing the cells in their naive state. We thus broaden the spectrum of genes supporting hPSC growth and adaptation and stress the need to consider such mutations in future applications.

Identifying New Cancer-Related Mutations in hESCs

In order to identify genes that harbor cancer-related mutations during propagation of hPSCs, we initially focused on two hESC lines, WA01 and WA09, also referred to as H1 and H9, respectively. These cell lines were derived over 20 years ago and since then have been the most commonly used and studied hESC lines. Although WES of early-passage WA01 and WA09 cells did not reveal cancer-related mutations, *TP53* mutations were identified in them following culturing and selection (Merkle et al., 2017). In light of these findings, we have now obtained 178 publicly available RNA sequencing samples of these cell lines, originating from 46 studies of 41 different research groups (Table S1). In order to identify mutations that could be clinically relevant, we focused only on genes annotated as "Tier 1" in The Cancer Gene Census (CGC) of COSMIC v86 database (https://cancer.sanger.ac.uk/census) (Forbes et al., 2017). Tier 1 genes were documented as actively relevant to cancer, and mutations in them were found to promote oncogenic transformation.

RNA sequencing samples were aligned to a reference genome (GRCh38) and underwent quality control via SNP identification and removal of common polymorphic sites (see Supplemental Methods in the Supplemental Information). We focused on alterations resulting in a non-synonymous transcription or stop gains, which appeared in two samples or more (Figure 1A; Figure S1). Only positions identified in human cancers were further examined. Importantly, the number of cancer-related mutations within each sample did not correlate with the total

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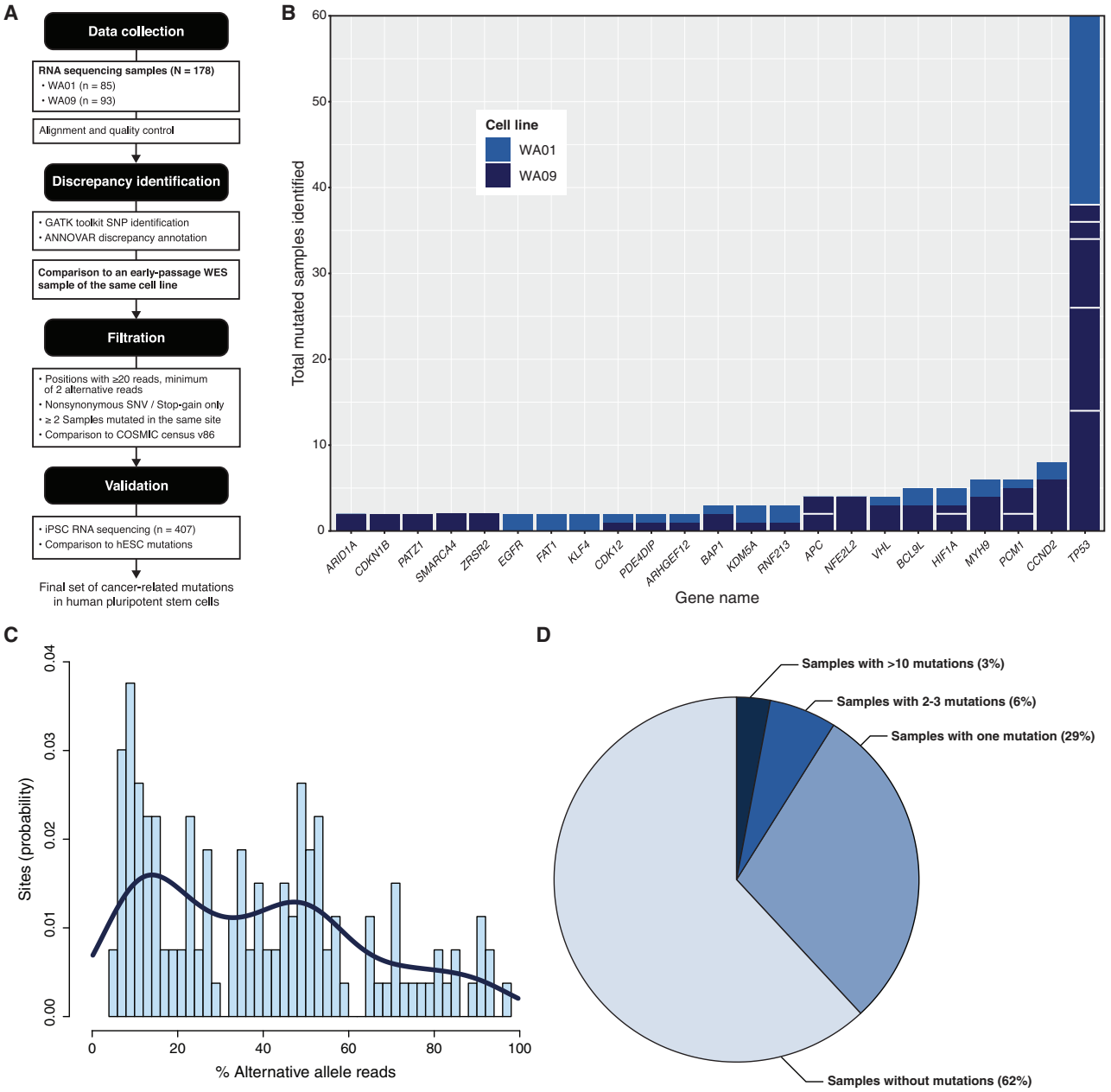

Figure 1. Identifying Cancer-Related Mutations in WA01 and WA09 hESCs

(A) A schematic workflow of the mutation identification strategy.

(B) Number of WA01 and WA09 hESC RNA sequencing samples with a mutation in a cancer-related gene. White bars separate between distinct point mutations while shades of blue represent two cell lines, WA01 and WA09.

(C) Ratio distribution of alternative (mutant) alleles in cancer-related genes in WA01 and WA09. Two visible peaks appear around 15% and around 50% of mutant reads.

(D) Number of cancer-related mutations in hESC RNA sequencing samples. At least one mutation was detected in more than one-third of the samples. See also Figure S1.

number of reads or commonly known SNP numbers (Figure S1).

We identified 31 point mutations in 22 different genes in 64 of 168 samples (Figure 1B; Table S2). *TP53* remained the most commonly mutated gene, with six individual mutations in both WA01 and WA09 (P151S, T155S, Q165K, R181H, R248Q, and R267W), four of which were previously reported (Merkle et al., 2017). We found that the genes *PCM1*, *HIF1A*, and *APC* also bear two distinct mutations. While only a single amino acid substitution occurred in the other genes, each of these mutations was identified in two to eight different hESC samples with 11 genes mutated independently in both WA01 and WA09.

We explored whether other genes, annotated as "Tier 2" in the CGC of the COSMIC v86 database, acquired mutations in WA01

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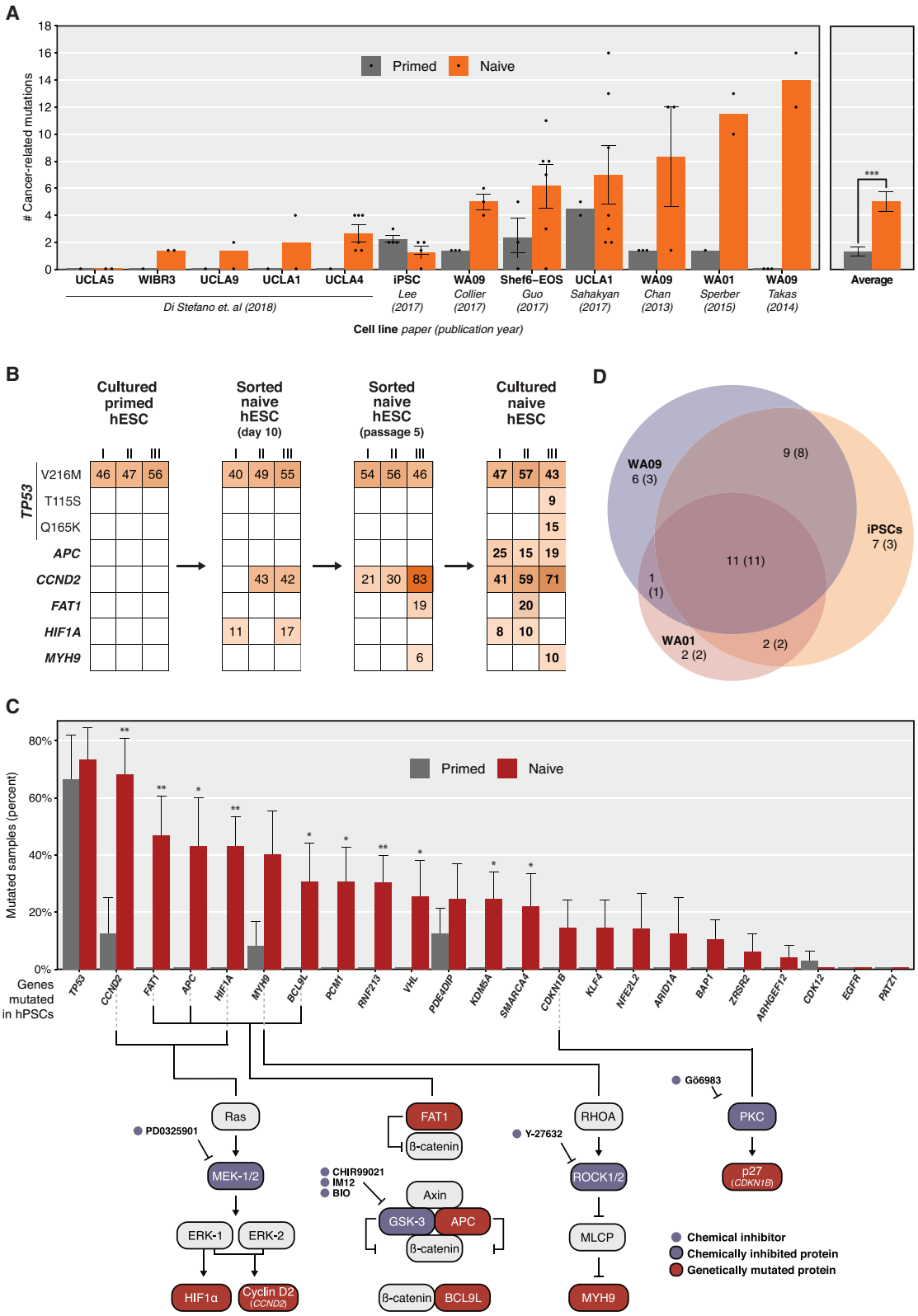

Figure 2. Cancer-Related Mutations in Naive, Primed, and Induced Pluripotent Stem Cells

(A) Naive hESCs have more cancer-related mutations on average in comparison to their primed counterparts. Black dots represent a mutation number in single samples and error bars represent the standard error of the mean. ***p < 0.001 by Student's t test.

(legend continued on next page)

and WA09. Within these 145 genes, which have less extensive evidence regarding their roles in cancer development (Forbes et al., 2017), we found a single reoccurring mutation in the *COL3A1* gene (Table S2).

RNA sequencing provides insights into the mutant allelic fraction of the population of cells it is performed from (Figure 1C). The mutated alleles found across samples were present at a various allelic fraction, ranging on average from 8.5% to 78%, with several mutations reaching close to 100% (Figure 1C; Table S2). This allelic fraction is determined by sequence mosaicism in the culture and could be affected by transcription regulation and transcript stability. Allelic fractions much lower than 50% suggest a heterogenous culture, while fractions around 50% suggest a rather homogeneous culture of heterozygous mutation. Mutant fractions higher than 50% suggest that cells started to gain homozygous mutations or underwent loss of heterozygosity, a common phenomenon in cancer identified in *TP53* mutations in culture (Merkle et al., 2017).

Overall, 62% of the analyzed samples did not contain a mutation that appears in the COSMIC Census database. However, over one-third of the hESC samples had at least one acquired mutation found on this cancer database (Figure 1D). We also noted that a small fraction of the samples (3%) harbored over ten cancer-associated mutations (Figure 1D).

Naive hPSCs Exhibit Mutations in Inhibited Cellular Pathways

In searching for shared characteristics of the five samples that acquired over ten cancer-associated mutations, we found that all of them were converted into a naive state in three distinct studies (Table S1), suggesting that this process imposed substantial selective pressure.

To expand upon this observation, we analyzed the acquired cancer-associated variants in six additional studies that included both naive and primed hPSCs from nine different cell lines (Figure 2A; Figures S2A and S2B). We show that in seven out of eight studies and in multiple cell lines, naive samples had significantly more mutations as compared with their associated primed samples (5.0 ± 0.8 compared to 1.3 ± 0.3, $p < 0.001$ in Student's t test) (Figure 2A; Figures S1C, S1D, and S2A). Furthermore, this analysis revealed that the mutations accumulated in a stepwise process, based on the analysis of cells at different stages during the transition between primed and naive hPSCs, suggesting *de novo* mutagenesis in culture (Collier et al., 2017) (Figure 2B). While only one mutation in the *TP53* gene was found in the primed cells, additional cancer-related mutations progressively appeared during naive cell selection and culturing (Figure 2B). However, the accumulation of acquired mutations during the naive transition was independent of mutations in p53 in the primed state, as similar genetic variants were acquired during the naive conversion of primed cells without any mutations.

The enhanced cancer-related mutation accumulation in naive cells is particularly notable because they have been suggested as improved precursor cells serving regenerative medicine (Weinberger et al., 2016). Naive hPSCs are derived and maintained in media supplemented with MEK/ERK and GSK3 inhibitors (2i medium) together with LIF, along with a plethora of apoptosis inhibitors and cytokines (Weinberger et al., 2016). Interestingly, we found that seven of the most frequently mutated genes in naive cells reside within four of the pathways inhibited by the media supplements inducing a naive state, suggesting that the cells harboring acquired mutations in these pathways are selected for by these conditions (Figure 2C; Figure S2B). More specifically, mutations in cyclin D2 (*CCND2*) and *HIF1A*, which appeared on average in 68% and 43% of the naive samples, respectively, mimic the effects achieved by MEK/ERK inhibitors (Minet et al., 2000; Piatelli et al., 2002). Similarly, *FAT1*, *APC*, and *BCL9L*, involved in the Wnt/β-catenin pathway, were mutated in 47%, 43%, and 31% of naive samples, respectively, and mimicked the effects achieved by various GSK3 inhibitors (Morris et al., 2013). PKC inhibitors, also commonly used in prime-to-naive conversion (Figure S2B), inhibit p27 (*CDKN1B*) (Forti and Armelin, 2011), which was mutated in multiple naive samples. Furthermore, ROCK pathway inhibitors, commonly used to prevent apoptosis in PSC cultures, inhibit *MYH9*, which was mutated in 40% of the naive samples (Figure 2C). The relations between pathways inhibited in naive cultures and enriched mutations in their proteins hint toward mutagenesis favoring the naive state. Thus, substantial selective pressure inherent to naive conversion leads to the accumulation of cells with specific acquired mutations in hPSC cultures.

A possible confounding factor for this observation is the passage number. As cells were previously shown to harbor chromosomal aberrations in late passages, it is possible that naive cells routinely harbor mutations through passaging. Notably, the vast majority of WA01 and WA09 sequencing samples (89%, 158/178 samples, Table S1) did not include information on passage number. While it is clear that all WA01 and WA09 RNA samples originated from cells in later passages than the baseline samples analyzed by WES, most samples did not have any mutations. Therefore, we cannot rule out the possibility that a high passage is a confounding factor, causing naive cells to harbor more mutations.

Cancer-Related Mutations Are Also Present in iPSCs

We next examined whether the same genes that acquired mutations in hESCs also acquired mutations in iPSCs. To this end, we used RNA sequencing data from over 400 iPSC samples originating from

(B) Accumulation of cancer-related mutations during a naive induction process. Each column represents a replicate and numbers represent the percent of mutant reads. Samples were analyzed from Collier et al. (2017).

(C) Average percent of naive hPSCs and their primed counterparts exhibiting cancer-related mutations in specific genes in individual studies. y axis represents the averaged percent of mutated samples in each individual study that included both primed and naive cells (outlined in panel A). Error bars represent the standard error of the mean. *$p < 0.05$, **$p < 0.01$ by paired samples Student's t test. Several of the mutated genes play a significant role in pathways inhibited by factors included in naive-inducing media. The chemical inhibitors are marked in purple dots, proteins directly inhibited by them are marked by a similar color, and genes mutated in hPSC samples are marked in dark red.

(D) Similar cancer-related mutations appear in hESCs and iPSCs. Numbers in brackets represent the number of mutated genes accounting for number of mutations. 178 hESC and 417 iPSC RNA sequencing samples were used in the analysis. Mutated genes were identified using WA01 and WA09 and the mutation search in them was extended in iPSCs. iPSCs did not bear mutations in *EGFR*, *CDK12*, and *PATZ1* and had unique mutations in *NFE2L2*, *SMARCA4*, and *TP53* (see also Table S2). See also Figure S2.

24 different studies and groups (Figure S2; Table S2) and found that 71% of the mutations identified in hESCs were also present in iPSCs (Figure 2D). Additional mutations in these genes were found in iPSCs, including five distinct mutations in *TP53* and additional mutations in *NFE2L2* and *SMARCA4* (Table S2; Figure S2).

As hPSCs are known to harbor typical chromosomal aberrations (International Stem Cell Initiative, 2011), we looked for additions of chromosomes 1, 12, 17, and 20 in all of the samples using RNA-sequencing-based e-Karyotyping (see Supplemental Methods in the Supplemental Information). While a small number of samples exhibited aneuploidies, over 94% of the samples with cancer-related mutations did not exhibit a large chromosomal aberration (164/173), suggesting that chromosomal aberrations were not the primary driver of acquiring such mutations (Table S1).

In contrast to hESCs, in which culture-derived mutations were facilitated by WES from early-passage cells as a baseline, mutation identification in iPSCs was constrained by the lack of appropriate comparisons. It is well established that the reprogramming process encompasses substantial selective pressure on the cells, before pluripotent-clone selection. As this process can enhance the selection of mutated clones, the list of mutations we report could be an underestimation of the actual mutational load of the cells, overshadowing selective bottlenecks inducing mutations caused by the reprogramming process.

To ensure that the cancer-related mutations identified in hPSCs do not occur in other stem cells, we analyzed 100 human mesenchymal stem cell (MSC) RNA sequencing samples from 13 different studies (Table S1). Only one cancer-related mutation (in the *ARID1A* gene), identified in hPSCs, appeared in more than a single MSC sample (Table S2). In addition, we found four distinct mutations in *TP53*, all in single samples (Table S2). These results emphasize the specificity of the reported mutations to hPSCs.

Final Remarks and Conclusions

hPSCs have immense potential for advancing regenerative medicine and for increasing our mechanistic understanding of diseases. However, culture adaptation of these cells can lead to accumulation of cells with chromosomal aneuploidy and mutations in the *TP53* gene, raising concerns regarding their reliability as research models and safety as potential therapeutic agents. In this report, we show that *TP53* is only one of several cancer-related genes gaining recurrent mutations following prolonged culturing and that these mutations further accumulate following induction of a naive state.

In our analysis, *TP53* remained the most commonly mutated cancer-related gene in hPSCs, with over ten distinct mutations in both embryonic stem cells and iPSCs (Figure 1B; Figure S2; Table S2). Indeed, an analysis of the differentially expressed genes in samples bearing mutations in *TP53* found significant enrichment for genes in the p53 signaling pathway, including *MDM2*, *FAS*, and *GADD45* (FDR-corrected $p < 8.28x10^{-3}$), and for genes involved in the regulation of cell proliferation (FDR-corrected $p < 1.27x10^{-9}$). These results echo the crucial role of p53 in cellular transformation, being the most commonly mutated gene in human cancers (Kandoth et al., 2013). Interestingly, p53 and ROCK pathways have been demonstrated to be the two major inhibitory regulators of hPSC growth (Yilmaz et al., 2018). In agreement, mutations in *TP53* and *MYH9* (the downstream effector of the ROCK pathway) were the top two mutated genes in iPSCs (Figure S2). *CCND2*, another frequently mutated gene in both hESCs and iPSCs (Figure 1B; Figure S2), has been implicated in germ cell tumorigenesis and PSC transformation (Baker et al., 2007). The mutation frequency in *CCND2* suggests that it may also provide growth advantages for hPSCs *in vitro*.

Of note is that most mutations identified are predicted to be pathogenic (i.e. result in a dysfunctional protein) by a bioinformatic analysis of their Functional Analysis through Hidden Markov Models (FATHMM) score (Table S2). In parallel, most mutant alleles were expressed in a frequency up to 50% (Figure 1C), hinting that they could have a dominant-negative effect. Indeed, 42% of the reported mutations were heterozygous in human tumors according to their COSMIC entry, and many were demonstrated to be inherited in such a manner (Table S2). Mutations in *BAP1*, for example, have disease-causing dominant effects and dramatically increase the chances of the development of renal cell carcinoma and melanoma (Bhattacharya et al., 2015). Interestingly, mutations in *BAP1* have also been shown to enhance stem-cell-like features of malignant cells such as increased self-replication and expression of typical markers, suggesting a growth advantage for cultured undifferentiated cells (Matatall et al., 2013). Although hPSCs carrying such mutations may grow better in culture, these mutations in *BAP1* could affect their differentiation, transformation, and clinical use, particularly for renal and skin-related disorders. Conversely, mutations in *SMARCA4*, a central ATPase of BAF complexes, are common in lung and blood cancers and have a dominant-negative effect. These mutations were previously shown to widely alter the cellular epigenetic landscape and enable expression changes of pro-oncogenic genes including *MYC* (Hodges et al., 2018). While loss of *SMARCA4* function could also provide growth advantages *in vitro*, it should be taken into consideration in clinical use of hPSCs, especially for lung and hematopoietic disorders. In parallel, an allelic fraction of up to 50% could also point toward a haploinsufficiency state, as previously described for *TP53* mutations in hPSCs.

The mutations described may also limit the utility of hPSCs in disease modeling. Li Fraumeni and Von Hippel-Lindau syndromes, for example, originate from mutations in *TP53* and *VHL*, respectively. Modeling other diseases with cells bearing mutations in such genes limits the ability to dissect the cause of cellular phenotypes, as those could be misleading and attributed to the erroneous gene or disease.

Our finding that about one-third of the hESC samples bear at least one cancer-related mutation (Figure 1D) is comparable to the finding that about 20% of hESC samples bear a chromosomal aberration (Mayshar et al., 2010). Both findings should draw attention to spontaneous culture adaptations and the need for routine monitoring. In contrast to aneuploidies, which are a major cause for concern regardless of the chromosomes affected, point mutations should be considered a safety concern mainly within relevant genes. We focused on mutations in cancer-related genes, as they increase the risk of cell oncogenic transformation upon transplantation. Accordingly, mutations in genes specific to certain types of cancer, exemplified by *BAP1* and renal carcinoma,

should be carefully monitored when cells are destined to be transplanted to that relevant tissue, as they could affect this tissue even when fully differentiated. Although the clinical implications of these mutations are yet to be determined, we believe that working with early-passage hPSCs is recommended, along with frequent monitoring of their chromosomal and mutational status.

Induction of human naive pluripotent state has been suggested to better mimic cells in the ICM of the blastocyst, as observed in mice (Weinberger et al., 2016). However, this induction requires a plethora of inhibitors, altering numerous cellular regulatory pathways. Multiple acquired mutations identified in hESCs originated in cells grown under naive conditions. Importantly, we show that these mutations reinforce the pathway inhibition achieved by naive-inducing small molecules (Figure 2C). We hypothesize that these mutations provide a growth advantage to the naive cells and assist them in maintaining their cellular state regardless of all external inhibitors. This adaptation is of importance as the associated increase in mutational load may affect any clinical use of naive cells.

In conclusion, we found that during prolonged culturing, hPSCs spontaneously acquire point mutations that are also found in human tumors, and in rates similar to those of chromosomal aberrations. Mutation acquisition seems to be dramatically influenced by selective pressures, culture conditions, and cell state, as seen in hPSCs grown in naive induction conditions. Although the full extent of the growth advantages provided by these mutations is to be uncovered, their alarming abundance should be considered in order to eliminate possible oncogenic cell transformation and to ensure a safe platform for regenerative medicine.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.stem.2019.09.001.

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AUTHOR CONTRIBUTIONS

Y.A. and N.B. designed the research. K.E. supplied the reference whole-exome sequencing. Y.A. established the bioinformatical pipeline and performed the analysis. Y.A. and N.B. interpreted the results and together with K.E. prepared the manuscript. N.B. supervised the study and secured funding.

DECLARATION OF INTERESTS

N.B. is CSO of NewStem Ltd.

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