Human adult germline stem cells in question

Arising from: S. Conrad *et al.* *Nature* 456, 344–349 (2008)

Conrad *et al.* have generated human adult germline stem cells (haGSCs) from human testicular tissue, which they claim have similar pluripotent properties to human embryonic stem cells (hESCs)[1]. Here we investigate the pluripotency of haGSCs by using global gene-expression analysis based on their gene array data[1] and comparing the expression of pluripotency marker genes in haGSCs and hESCs, and in haGSCs and human fibroblast samples derived from different laboratories, including our own. We find that haGSCs and fibroblasts have a similar gene-expression profile, but that haGSCs and hESCs do not. The pluripotency of Conrad and colleagues' haGSCs is therefore called into question.

Fibroblasts can be easily established from human testicular cultures[2]. Considering the similarities between haGSCs and non-testis fibroblasts, we isolated fibroblasts from human testicular biopsies and derived clusters of cells (human testicular fibroblast cells, hTFCs) from them (Fig. 1a, b). haGSCs were found to be morphologically similar, if not identical, to these hTFCs but not to hESCs (Fig. 1c).

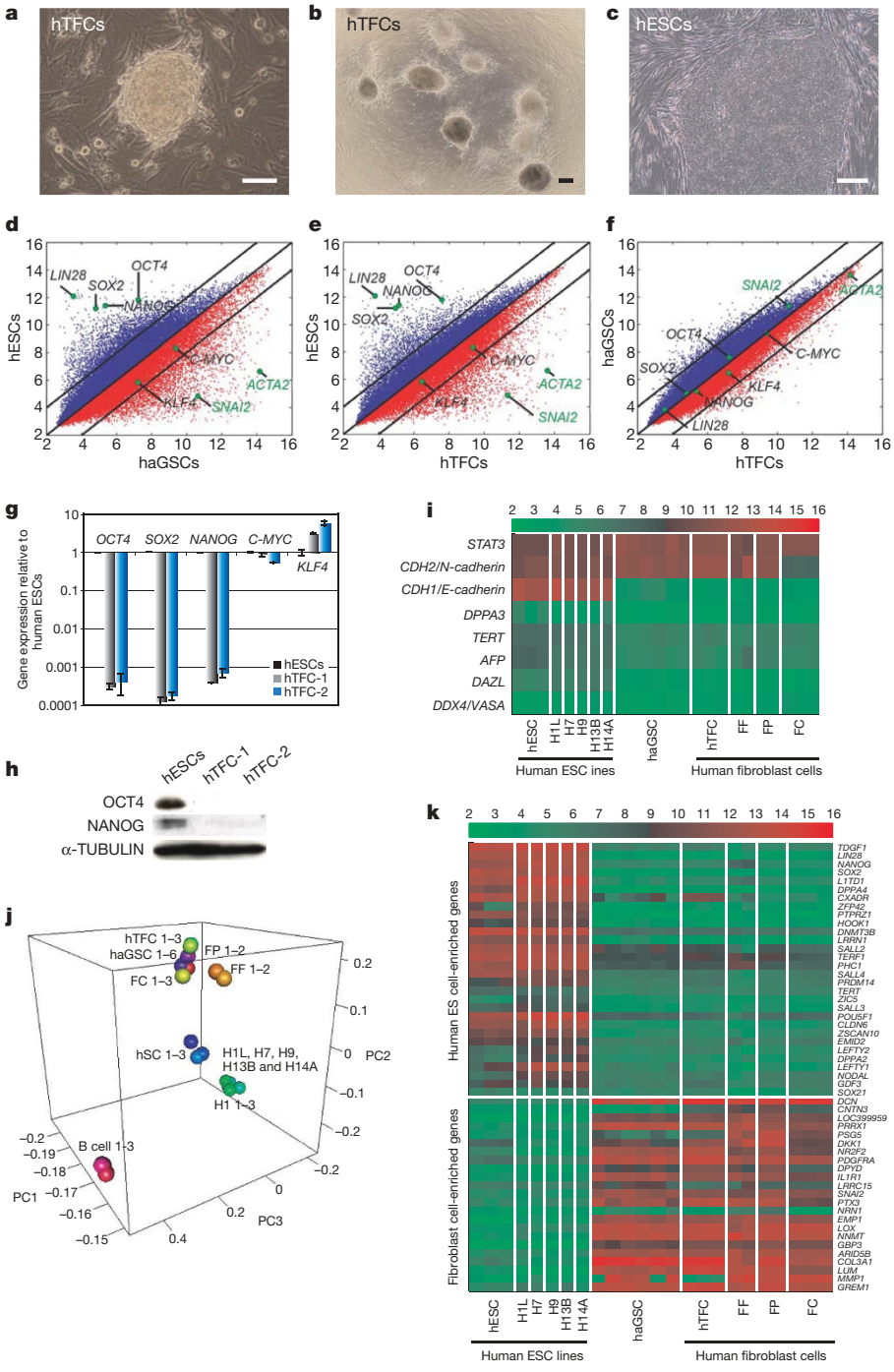

Figure 1 | Characterization of human testis-derived fibroblast cells. a–c, Phase-contrast images of human testicular fibroblast cells (hTFCs) at high (**a**) and low (**b**) magnification and of human embryonic stem cells (hESCs) (line H1) (**c**); scale bars, 200 μm. **d–f**, Scatter plots of pairwise global gene-expression comparisons: hESCs versus human adult germline stem cells (haGSCs) (**d**); hESCs versus hTFCs (**e**); and haGSCs versus hTFCs (**f**). **g**, Real-time RT–PCR analysis of hESCs and hTFCs; error bars represent standard deviation. **h**, Western blot analysis of OCT4, NANOG and α-TUBULIN. **i**, Heat map of the microarray gene-expression data of the genes discussed by Conrad *et al.*[1]. **j**, Principal component (PC) analysis of the global gene-expression profiles of hESCs, haGSCs, hTFCs and fibroblasts. **k**, Heat map of microarray gene expression of a list of human ESC- and fibroblast-enriched genes[3]. Gene-expression colour key is shown at the top in $\log_2$ scale. Microarray data were downloaded from the GEO database; accession numbers GSE11350 (haGSCs, hESCs (ES line H1), human spermatogonial cells (hSCs))[1], GSE12583 (foreskin fibroblasts (FF))[8], GSE15322 (normal colon fibroblasts (FC)), GSE15148 (ES lines H1L, H7, H9, H13B and H14A, and parental foreskin cells (FP))[3] and GSE10831 (B cells)[9].

We confirmed this similarity by comparing the global gene-expression profile of hTFCs and haGSCs using the gene array data of Conrad *et al.*[1]. Scatter plots reveal that haGSCs were remarkably similar to hTFCs and to previously described fibroblasts, but not to hESCs (Fig. 1d–f). Conrad *et al.* did not present a scatter plot analysis, which would have helped visualization of any differences between haGSCs and hESCs.

The pluripotency marker genes *POU5F1*/*OCT4*, *SOX2*, *NANOG* and *LIN28*, which are expressed in hESCs, were not expressed in haGSCs or in hTFCs, whereas the fibroblast marker genes *SNAI2* and *ACTA2* (refs 3, 4) were markedly overexpressed. Real-time analysis using polymerase chain reaction with reverse transcription (RT–PCR) confirmed the absence of *OCT4*, *SOX2* and *NANOG* expression in hTFCs (Fig. 1g).

Messenger RNA expression of *OCT4* and *NANOG* in hTFCs correlated with protein abundance, as determined by western blotting (Fig. 1h). By contrast, the western blot results of Conrad *et al.*[1] indicate that the amounts of OCT4 and NANOG protein in their haGSCs were virtually identical to those in hESCs. However, *OCT4* and *NANOG* mRNA levels, extracted from their microarray data[1], were as low in haGSCs as in hTFCs and other human fibroblasts.

This inconsistency between mRNA and protein levels in haGSCs—whether analysed by western blot or immunohistochemistry—was also evident for other genes, such as *CDH1/E-cadherin*, *DAZL* and *DDX4/VASA* (Fig. 1i). Conrad *et al.* show that *STAT3* and *CDH2/N-cadherin* are highly expressed in haGSCs, consistent with our findings (Fig. 1i). However, expression of *STAT3* and *CDH2/N-cadherin* in human ESCs was nearly identical to that in human fibroblasts (Fig. 1i). Therefore, the expression of these genes cannot be used to distinguish pluripotent cells from fibroblasts.

Principal component analysis of global gene-expression data revealed that haGSCs clustered closely with hTFCs and with previously described human fibroblasts, but not with hESCs (Fig. 1j). Heat-map analysis with pluripotent and fibroblastic genes[3] showed that haGSCs were fibroblastic, but not pluripotent, in their gene-expression profile (Fig. 1k).

Our results indicate that haGSCs are by no means similar to hESCs but that they do strongly resemble our hTFCs, which are not pluripotent. These findings are at odds with those of Conrad *et al.*[1], who report that their haGSCs are able to form teratomas, a defining feature of human pluripotent cells.

METHODS SUMMARY

hTFCs were obtained from routine biopsies taken during testicular surgery (with patients' informed written consent). Following mechanical tissue disruption and enzymatic digestion, cells were cultured in the medium described by Conrad *et al.*[1]. These experiments were approved by the local ethics council. Microarray analysis in hTFCs was performed using Human Genome U133 Plus 2.0 chip (Affymetrix) and processed as in ref. 5. The array data were deposited in the Gene Expression Omnibus (GEO) database (accession number GSE17772). All microarrays used in this study were globally normalized with the RMA algorithm implemented in R-Bioconductor[6].

Authors' note: We contacted Conrad *et al.* to obtain their haGSC lines[1]; however, the established haGSCs are not accessible at present to other laboratories because consent between Conrad *et al.* and donor patients did not include permission to distribute cells derived from the biopsies[7]. At the start of 2009, T. Skutella agreed to send us the haGSCs once he receives permission for their distribution, but we have not so far received them. We have been informed that the original consent form stipulates that all derived cell lines must be destroyed after a period of three years following biopsy.

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Conrad et al. *reply*

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Ko *et al.*[1] challenge our description of human adult germline stem cells (haGSCs)[2], indicating that we have instead cultured testis fibroblasts. However, they do not follow the experimental procedures we describe[2] and so fail to reproduce our findings.

We repeated the procedures used by Ko *et al.* that produced fibroblasts[1] by using parts of our own protocol[2] with human testis tissue, but omitting the array of selection procedures described there — such as MACS (magnetic-activated cell separation) and matrix selection of germ stem cells. We confirmed that it is indeed impossible to establish a germ-cell culture in this way, as Ko *et al.* know from their mouse studies. In the resulting cultures, the plates are overgrown with somatic cells that aggregate to clumps of cells: most of these cells are probably fibroblasts. The populations of human testicular fibroblast cells (hTFCs) described by Ko *et al.*[1] are a completely different cell population and are not comparable to haGSCs.

Fibroblasts are not germ stem cells and are unable to differentiate into cells of all germ layers, as the cells we describe did[2]. However, the microarrays on which Ko *et al.* place emphasis were only one of several molecular and functional tests we used[2] to detect similarities to human embryonic stem cells (hESCs).

According to the Microarray Facility Tübingen, one of the critical points of the challenge by Ko *et al.*[1] is that they use our data set for comparison with their own expression profiles from different cell types. The use of microarrays that are generated several days or months apart introduces systematic batch effects or non-biological differences, which make it meaningless to compare samples from different batches directly. Batch effects are inevitable when new samples or replicates are added incrementally to an existing array data set, or when a meta-analysis of multiple studies pools microarray data across different laboratories[3]. Statements about comparisons between the different data set are therefore not constructive.

We are now working on the selection and amplification of our haGSCs so that we have sufficient cell material to share with colleagues. Detailed protocols are available from us and the authors of refs 2, 4–6 for those who wish to investigate haGSCs further.

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