Can controversies be put to REST?

Arising from: S. K. Singh *et al.* *Nature* **453**, 223–227 (2008)

The contribution of REST to embryonic stem (ES) cell pluripotency has been uncertain. Two years ago, Singh *et al.*¹ claimed that *Rest*^{+/-}

and REST knock-down ES cells expressed reduced levels of pluripotency markers¹, in contrast to a prior² and subsequent reports^{3–6}. To understand the basis of this difference, we analysed the YHC334 (YHC) and RRC160 (RRC) gene-trap ES cell lines used by Singh *et al.*¹, obtained directly from BayGenomics. Both REST mutant lines generated REST-βGeo fusion proteins, but expressed pluripotency genes at levels similar to appropriately matched parental wild ES cells, consistent with expression being REST-independent.

YHC, RRC gene-trap and parental wild-type ES cells (sub-clone E14Tg2a.4, E14; www.mmrrc.org/distribution/overview_BG.html) were grown in feeder-free conditions using standard ES cell medium supplemented with leukaemia inhibitory factor (LIF). To verify the DNA sequence information provided for each cell line (BayGenomics trace 2094792394 (YHC) and 2094783300 (RRC)), RT-PCR was performed using forward primers to *Rest* exons 1–3 and reverse primers to either exon 4 or the βGeo cassette (Fig. 1a). This confirmed that the βGeo cassette is inserted within *Rest* intron 2 (YHC) and 3 (RRC) (Fig. 1b). Although Singh *et al.*¹ state that the insertion sites are within introns 1 and 2, we suspect this reflects differences in the annotation of the *Rest* locus (the Ensembl/UCSC convention is used in Fig. 1a, whereas NCBI refers to three exons lacking the non-coding exon 1). We found that YHC and RRC cells contained lower levels of *Rest* transcript (70% and 52%, Fig. 1c) and reduced abundance of full-length REST protein relative to the E14 control cells (Fig. 1d), consistent with the functional disruption of one of the *Rest* alleles in the gene-trap lines.

We analysed whether insertion of the βGeo selection cassette generated a REST-βGeo fusion product (see Fig. 1e) encoding the Sin3A-interacting repression domain⁷ and part of the REST DNA binding domain. We reasoned that chimaeric proteins of this sort could potentially explain the different phenotypes reported for these ES cell lines¹ versus heterozygous *Rest*^{+/-} ES cell lines that we and others had generated and analysed^{3,4,6}. Anti-β-galactosidase (βGal) antibody detected a protein in RRC and YHC extracts that was not present in samples from the parental E14 cell line, and had greater molecular mass (>70 kDa) but was less abundant than βGeo proteins generated by control βGeo-expressing cells (Ctrl-βGeo, Fig. 1f). These results showed that YHC and RRC cells express REST-βGeo fusion proteins, albeit at low levels, as βGal activity (by X-Gal staining) was not detected.

The phenotypic consequence of each insertion was directly evaluated by comparing the morphology and levels of pluripotency-associated markers in YHC, RRC and matched wild-type E14 ES cells. The percentage of colonies containing alkaline phosphatase (AP)-positive cells was similar in these lines (Fig. 2a), in contrast to the 50–60% reduction in AP activity for YHC and RRC claimed previously¹. Although the parental E14 and *Rest*^{+/-} lines YHC and RRC grew in flat colonies and

Figure 1 | Gene-trap ES cell lines YHC and RRC contain a disrupted *Rest* allele and express REST-βGeo fusion proteins. **a**, Diagram showing *Rest* exons, gene-trap insertion sites and primer positions. **b**, RT-PCR analysis using indicated forward primers and either R4 (top) or R-Geo reverse primer (lower panel). Ctrl, negative control. **c**, *Rest* transcript levels by real-time RT-PCR (ref. 5) in YHC and RRC relative to E14 ES cells. Error-bars show standard deviation, *n* = 3. **d**, Western blot showing levels of full-length REST (top, anti-REST from Millipore) and lamin protein (lower panel, anti-LaminB from Santa Cruz) in E14, YHC and RRC extracts (50 or 25 μg). **e**, REST protein domains relative to *Rest* gene structure with putative REST-βGeo fusion proteins shown below. **f**, Anti-βGal (Promega) western blot of E14, YHC, RRC, wild type (WT), *Rest*^{+/-}, *Rest*^{-/-} and control cells expressing unfused βGeo (Ctrl-βGeo).

Figure 2 | Rest-gene trap and parental wild-type ES cells from BayGenomics express similar levels of pluripotency-associated markers, but differ from another wild-type ES cell line. **a**, The graph shows percentages of alkaline phosphatase-positive colonies for E14, YHC and RRC ES cells. **b**, The percentage of SSEA1-positive cells ($\pm$ standard deviation) relative to background staining as determined by FACS (ref. 5). **c**, Western blot showing levels of Oct4 (top, anti-Oct4 from Santa Cruz) and Lamin protein (lower panel) in E14, YHC and RRC cells. **d**, **e**, The transcript levels of pluripotency factors (left) and differentiation-associated genes (right) were determined using quantitative RT-PCR (ref. 5). Normalized values (relative to *Hmbs*, *Ywhaz*) and standard deviation ($n = 3$) are shown relative to the matched wild-type E14 (**d**) or another wild-type ES cells line (OS25, **e**).

seemed prone to spontaneous differentiation, >90% of these cells were positive for the stem cell marker SSEA1 (Fig. 2b) and all three lines expressed very similar levels of pluripotency-associated transcription factors Oct4, Nanog and Sox2, judged by the abundance of protein and/or transcripts (Fig. 2c, d left panel). Likewise, no detectable increase in the expression of lineage markers for mesoderm, endoderm or ectoderm differentiation (*Bry*, *Gata4* and *Sox1*, respectively) was seen in *Rest*^{+/-} YHC and RRC lines, relative to the matched controls (Fig. 2d, right panel). Thus, REST- β Geo 'trapped' ES cell lines YHC and RRC are not genuinely or robustly compromised in pluripotent gene expression. Interestingly however, reproducible differences in gene expression were evident when these lines were compared to another widely used E14Tg2a-derived ES cell line, OS25. As shown in Fig. 2e, the three lines (RRC, YHC and E14) showed reduced levels of *Nanog*, *Sox2* and *Gata4* transcripts compared to OS25, equivalent expression of *Oct4* and increased abundance of *Bry* and *Sox1* transcripts. On the basis of these results it is plausible that previously reported differences in expression profile of the heterozygous *Rest* 'trapped' ES cell lines¹, could stem from intrinsic variations in sub-cloning of the original E14Tg2a line, rather than bona fide changes in *Rest* genotype (reduced REST levels) or from creation of REST- β Geo fusion proteins.

In summary, our results confirm that the *Rest* heterozygous ES cell lines YHC and RRC contain an insertion of the β Geo gene-trap cassette which disrupts one of the endogenous *Rest* alleles, resulting in reduced expression of full-length REST protein and the *de novo* expression of REST- β Geo fusion proteins. Based on a detailed phenotypic comparison of these lines alongside a matched wild-type control, we conclude however, that the insertions do not profoundly alter the expression of key pluripotency- or differentiation-associated markers. These analyses highlight the intrinsic clone-to-clone variation that occurs in ES cell cultures and underscore the importance of using matched controls for such comparisons.

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Jørgensen and Fisher¹ suggest that the discrepancy with our results² could be attributable to our use of E14Tg2a (or its derivatives, such as OS25) rather than E14Tg2a.4 as the parental control line for the *REST*^{+/−} cells (YHC and RRC). We have now reconfirmed our use of E14Tg2a.4 clonal cells as the control cells. Also, we have found that the YHC and RRC cells used in our experiments², originally purchased from Bay Genomics, differ from the YHC and RRC cells used by Jørgensen and Fisher¹ with respect to pluripotency-based on alkaline phosphatase/self-renewal assays (N. Song and S.K.S., unpublished results). We are currently using other assays to confirm these observations.

We have discovered that the original YHC and RRC cells from Bay Genomics were expanded without drug selection at MMRRRC before being made available for distribution; these were the cells used by Jørgensen and Fisher¹. Such propagation of mutant cells under self-renewal culture conditions could create an adaptive response, in which mutant cells produce changes to dampen the *REST*-mediated pathway and activate counteracting mechanisms to increase the self-renewing cell population.

To eliminate the effect of adaptive response of mutant cells to growth conditions, we grew wild-type E14Tg2a.4 mouse ES cells² and knocked-down *Rest* with small interfering RNA or short hairpin RNA. The results indicated a clear dependence of pluripotency of these

cells on *REST* protein and that this pathway involved microRNA21, supporting our original observation².

We therefore believe that growth conditions such as media, duration of growth and presence of fibroblasts can influence *REST*'s role in pluripotency. We would welcome exchange of cells and information on culture conditions to help clarify the situation.

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