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Controlling the Survival of Human Pluripotent Stem Cells by Small Molecule-Based Targeting of Topoisomerase II Alpha

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ABSTRACT

Pluripotent-specific inhibitors (PluriSIns) make a powerful tool to study the mechanisms controlling the survival of human pluripotent stem cells (hPSCs). Here we characterize the mechanism of action of PluriSIn#2, a compound that selectively eliminates undifferentiated hPSCs, while sparing various other cell types derived from them. Toxicogenomic analysis predicts this compound to be a topoisomerase inhibitor. Gene expression analyses reveal that one of the human topoisomerase enzymes, topoisomerase II alpha (*TOP2A*), is uniquely expressed in hPSCs: *TOP2A* is highly expressed in undifferentiated cells, is downregulated during their differentiation, and its expression depends on the expression of core pluripotency transcription factors. Furthermore, siRNA-based knock-down of *TOP2A* in undifferentiated hPSCs results in their cell death, revealing that *TOP2A* expression is required for the survival of these cells. We find that PluriSIn#2 does not directly inhibit TOP2A enzymatic activity, but rather selectively represses its transcription, thereby significantly reducing TOP2A protein levels. As undifferentiated hPSCs require TOP2A activity for their survival, TOP2A inhibition by PluriSIn#2 thus causes their cell death. Therefore, TOP2A dependency can be harnessed for the selective elimination of tumorigenic hPSCs from culture. STEM CELLS *2014; 00:000–000*

INTRODUCTION

Human pluripotent stem cells (hPSCs) are a promising source of cells for therapy due to their ability to self-renew and to differentiate into any cell type. However, residual undifferentiated hPSCs in culture may give rise to tumors upon transplantation, and thus pose a major safety concern[1]. Selective chemical ablation of undifferentiated hPSCs could effectively alleviate the risk of tumor formation following the transplantation of hPSC-derivatives into patients[2]. We have recently conducted a high-throughput screen of over 52,000 small molecules, and identified 15 pluripotent-specific inhibitors (abbreviated as "*PluriSIns*"). These small molecules selectively eliminate undifferentiated hPSCs from culture, while sparing various cell types differentiated from them[3].

We characterized the mechanism of action of one of these compounds, PluriSIn#1, identifying it as a stearoyl-coA desaturase (SCD1) inhibitor, thus revealing a unique dependency of hPSCs on the synthesis of the fatty acid oleate[3]. We figured that other PluriSIns might work through different mechanisms of action, and that studying these small molecules could shed light on additional control mechanisms of hPSC survival. We decided to focus on PluriSIn#2, also known as 1-Phenylcarbamoyl-5-fluorouracil (**Fig. 1A**), for the following reasons: a) its biological activity is unknown; b) it doesn't contain the structural moiety phenylhydrazine, shared by several other PluriSIns, which is suspected to be related to inhibition of oleate synthesis[3]; and c) structure-based query of the CAS databases revealed that 1-carbamoyl-5-fluorouracil derivatives may exhibit antitumor activity *in vitro* and *in vivo*[4,5].

MATERIALS AND METHODS

Detailed Materials and Methods are presented in the Supporting Information.

RESULTS AND DISCUSSION

PluriSIn#2 selectively eliminates undifferentiated hPSCs

Based on our high-throughput screen data[3], we found that PluriSIn#2 effectively eliminates undifferentiated hPSCs; human embryonic stem cells (hESCs) are highly sensitive to this compound, and lose their sensitivity with differentiation, whereas human somatic cells gain this sensitivity following reprogramming into induced pluripotent stem cells (hiPSCs) (**Fig. 1B**). A 24h exposure of PluriSIn#2 (20μM) was sufficient to induce massive cell death in hPSCs, but didn't affect the viability of various other cell types derived from them (**Fig. 1B-C**). Interestingly, hESC-derived neural stem cells were sensitive to PluriSIn#2, albeit not as much as their undifferentiated counterparts (**Fig. 1C**), suggesting that PluriSIn#2 may be less suitable for elimination of hPSCs from cultures of differentiating neural cells.

PluriSIn#2 is predicted to be a TOP2 inhibitor

In order to gain biological insights into PluriSIn#2 mechanism of action, we first examined in an oleate rescue assay[3] whether PluriSIn#2 exerts its cytotoxic effect on hPSCs through inhibition of SCD1. Oleate could not decrease cell death in culture following exposure to PluriSIn#2, as it does following exposure to PluriSIn#1, indicating that PluriSIn#2 works through a different mechanism of action (**Fig. 1D**).

We therefore applied an unbiased toxicogenomic approach, in order to detect the gene expression changes induced by the compound. Following a 12h exposure of undifferentiated hPSCs to PluriSIn#2 (20μm) or to control conditions, RNA was extracted from the cells, and gene expression levels were measured by expression microarrays. The list of differentially expressed genes (*i.e.*, genes that were >2-fold up-regulated and down-regulated in PluriSIn#2-treated hPSCs) was then subjected to a connectivity map (cmap) analysis, which aims to discover pathways perturbed by small molecules of unknown activity by comparing the induced gene expression signature to that of known compounds[6]. Remarkably, this analysis revealed that the cellular effect of PluriSIn#2 is highly similar to that of DNA topoisomerase inhibitors, with extremely high connectivity scores (33-fold enrichment, $p<1*10^{-4}$). Importantly, the most similar compound to PluriSIn#2, daunorubicin, as well as two other compounds in this list, are well-known TOP2 inhibitors (**Fig. 1E**). Therefore, the connectivity map analysis predicts PluriSIn#2 to be a novel TOP2 inhibitor.

TOP2A is uniquely expressed in undifferentiated hPSCs

TOP2 enzymes modulate DNA topology by cleavage of both DNA strands, and are involved in many cellular processes, such as DNA repair, transcription and replication[8]. Human TOP2 family members include topoisomerase 2A (TOP2A), which is the main topoisomerase involved in replication, and topoisomerase 2B, (TOP2B) which is involved in DNA repair and transcription. Therefore, activity of TOP2A characterizes dividing cells, whereas TOP2B is also active in post-mitotic tissues[8]. We compared the gene expression levels of *TOP2A* and *TOP2B* between undifferentiated hPSCs and 11 differentiated tissues, using gene expression microarray data[9]. This analysis revealed that *TOP2A* is highly expressed in undifferentiated hESCs and hiPSCs, but is transcribed at below-threshold levels in all adult tissues (except testis), whereas *TOP2B* is expressed in all of the analyzed tissues (**Fig. 2A**). We confirmed the highly differential expression of *TOP2A* by qRT-PCR, using a cDNA panel of adult tissues (**Fig. 2B**).

Next, we examined the down-regulation of *TOP2A* throughout differentiation of hPSCs into the three germ layers. This analysis showed that *TOP2A* was significantly down-regulated during the first few weeks of endodermal and mesodermal differentiation, but remained high in early hESC-derived neural stem cells (**Fig. 2C**). *TOP2A* is thus uniquely expressed in undifferentiated hPSCs and in their early proliferating neural derivatives. The sensitivity of various cell types to PluriSIn#2 (**Fig. 1C**) is therefore congruent with their TOP2A gene expression levels (**Fig. 2A-C**).

As TOP2A has recently been shown to contribute to gene expression regulation in mouse ESCs[10], we next asked whether it could play a similar role in the pluripotency circuitry of hPSCs. A query of the embryonic stem cell database (ESCDb)[11] suggested that *TOP2A* may be regulated by several pluripotency factors, including *NANOG* and *OCT-4*. In line with this prediction, knockdown of either *NANOG* or *OCT-4* in undifferentiated hPSCs resulted in rapid down-regulation of *TOP2A* (**Fig. 2D**), confirming the regulation of *TOP2A* by these pluripotency factors, either directly through promoter binding or indirectly through their regulation of the pluripotent state.

TOP2A is required for the survival of hPSCs

As our data highlighted the unique expression of *TOP2A* in undifferentiated hPSCs, and its rapid downregulation at the onset of differentiation, we next aimed to examine the effect of *TOP2A* down-regulation on their survival. Indeed, siRNA-based knockdown of *TOP2A* in undifferentiated hESCs significantly reduced the viability of the cells (**Fig. 3A-B**). In line with these findings, we found that undifferentiated hPSCs are much more sensitive than 14d-differentiating hPSCs to the TOP2 inhibitor salicylate, which was recently found to preferentially inhibit the alpha isoform[12] (**Fig. 3C**). We conclude that undifferentiated hPSCs depend on TOP2A activity for their survival.

PluriSIn#2 is a transcriptional inhibitor of TOP2A

Given the dependency of hPSCs on TOP2A activity, and the finding that the cellular effect induced by PluriSIn#2 is highly similar to that of known TOP2 inhibitors, we next turned to examine whether PluriSIn#2 could be a novel selective TOP2A inhibitor. There are two types of TOP2 inhibitors: TOP2 poisons, which target the topoisomerase-DNA complex and lead to DNA cleavage, and TOP2 catalytic inhibitors, which disrupt the catalytic turnover of the protein. To examine both possibilities, we applied the "trapped in agarose DNA immunostaining" (TARDIS) assay, which quantifies topoisomerase-DNA covalent complexes in single cells[13]. Whereas increasing concentrations of the etoposide led to increased TOP2A-DNA adducts, no increase was identified following PluriSIn#2 exposure (**Fig. 4A**), indicating that PluriSIn#2 is not a TOP2A poison. Whereas high concentration (100μM) of ICRF193 decreased the etoposide effect, pluriSIn#2 couldn't lead to a similar rescue (**Fig. 4B**), indicating that PluriSIn#2 is not a TOP2A catalytic inhibitor either.

A DNA decatenation assay confirmed that PluriSIn#2 is not a direct inhibitor of TOP2A enzymatic activity. Increasing concentrations of recombinant human TOP2A led to increasing decatenation of kinetoplast DNA, whereas the known TOP2 inhibitor etoposide prevented this decatenation (**Fig. 4C**). In contrast, high concentration (50μM) of PluriSIn#2 did not jeopardize TOP2A enzymatic activity in this assay (**Fig. 4C**), in line with the TARDIS results.

Therefore, we examined whether PluriSIn#2 could be an indirect inhibitor of TOP2A, exerting its cellular effect through transcriptional inhibition rather than by direct interaction with the enzyme. Cells were exposed to PluriSIn#2 (20μM) for 12h, and the RNA levels of *TOP2A* and *TOP2B* were then quantified by qRT-PCR. Interestingly, PluriSIn#2 led to a significant reduction in the transcript level of *TOP2A*, but not *TOP2B* (**Fig. 4D**). Western blot with a specific antibody against TOP2A showed that PluriSIn#2, but not PluriSIn#1, reduced TOP2A expression at the protein level as well (**Fig. 4E**), confirming that PluriSIn#2 is an indirect inhibitor of the enzyme. Further research is warranted to unravel the molecular mechanism underlying the selective transcriptional inhibition of *TOP2A* by PluriSIn#2.

CONCLUSIONS

This study reveals the dependency of undifferentiated hPSCs on TOP2A, and characterizes PluriSIn#2 as a novel transcriptional inhibitor of TOP2A in these cells. Notably, *TOP2A* is over-expressed in many types of tumors[14], and its expression levels determine the response to TOP2-based chemotherapies *in vitro* and *in vivo*[15]. Targeting TOP2A as an anticancer strategy, however, is compromised by its high sequence similarity to that of TOP2B, creating a pressing need for more selective inhibitors of TOP2A [8]. Although several preferential inhibitors of TOP2A have been reported[12,16,17], none of them was shown to induce selective cell death of cancer cells. PluriSIn#2, which kills undifferentiated hPSCs by selectively attenuating the transcription and consequently the protein expression of TOP2A, may therefore be of potential interest to cancer research as well.

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DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

AUTHOR CONTRIBUTIONS

U.B.-D. and N.B. conceived and designed the research, analyzed the data and wrote the manuscript. U.B.-D. performed the experiments. I.G.C. and C.A.A. performed and analyzed the TARDIS experiments.

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Figure 1. PluriSIn#2 selectively eliminates undifferentiated hPSCs, and is predicted to be a TOP2 inhibitor.
(A) The chemical structure of PluriSIn#2, also known as 1-Phenylcarbamoyl-5-fluorouracil. (B). Dose response curves of PluriSIn#2 in hESCs (red) hiPSCs (orange), 8d-differentiated endodermal progenitor cells (dark blue) and the fibroblasts from which the hiPSCs were generated (light blue). (C) Quantification of cell death, by an ATP-based luminescence cell viability assay, in various cell types exposed to PluriSIn#2 (20μM) for 24h. Data represent an average of triplicates from one cell line (except for hESCs, where data represent triplicates from two cell lines), error bars show s.e.m. ESCs, human embryonic stem cells; NSCs, hESC-derived neural stem cells; MSCs, hESC-derived mesenchymal stem cells; EPCs, hESC-derived endodermal progenitor cells; Cardio, hiPSC-derived cardiomyocytes; Hepato, hESC-derived hepatocytes; Fibro, fibroblasts used for the generation of the hiPSCs. (D) Quantification of the relative viability of undifferentiated hESCs exposed to PluriSIn#1 (20μM) or to PluriSIn#2 (20μM) for 24h, in the absence (red) or the presence (black) of oleic acid (100μM). Data represent an average of triplicates from two cell lines, error bars show s.e.m. *, p=0.002. (E) The 10 "hits" ranked by the connectivity map as most similar to PluriSIn#2, in terms of the global gene expression changes induced by them. The mean connectivity score represents the degree of similarity (on a scale of 0 to 1). TOP2 inhibitors are denoted with red asterisks, and TOP1 inhibitors are denoted with orange asterisks. 6 out of the 10 "hits" – representing 5 out of 8 distinct compounds most similar to PluriSIn#2 – represent known DNA topoisomerase inhibitors (33-fold enrichment, $p<1*10^{-4}$). Three of these compounds are known DNA topoisomerase type II (TOP2) inhibitors; another compound in this list, hycanthone, was previously suggested to inhibit TOP2 [7], but is not a canonical topoisomerase inhibitor, and is thus not marked with an asterisk.

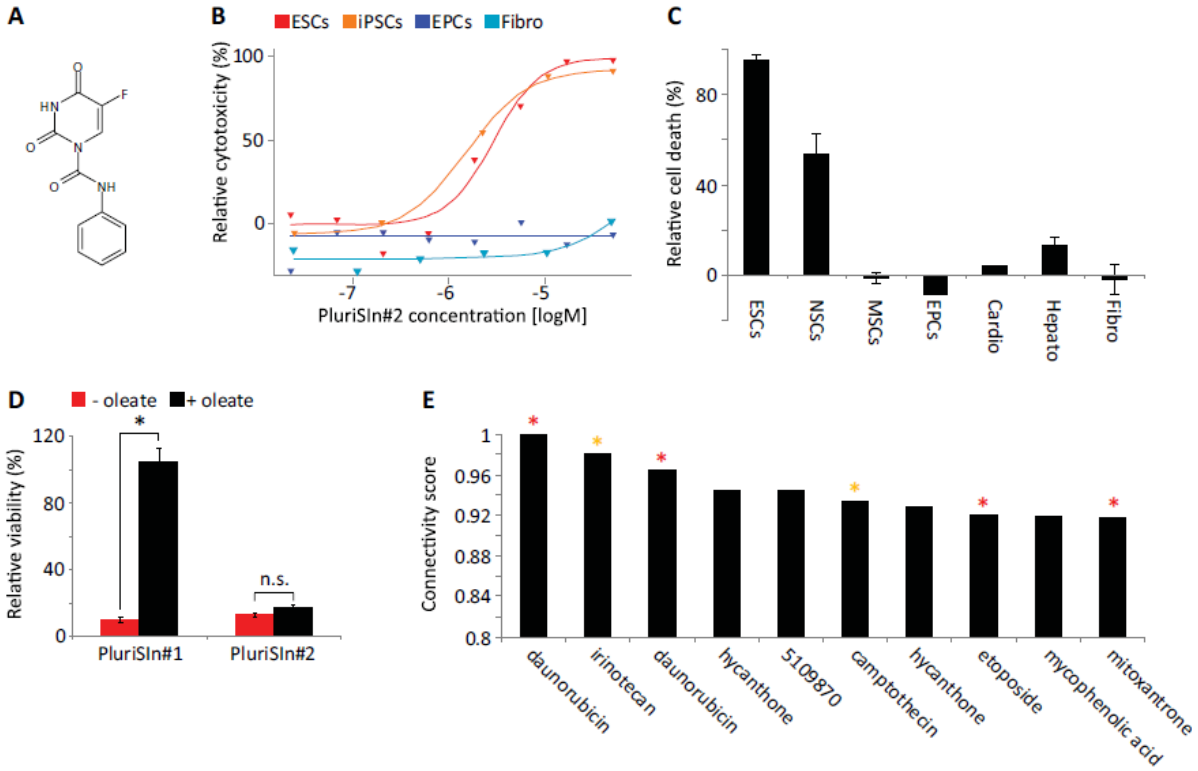

Figure 2. *TOP2A* is highly expressed in hPSCs, is downregulated during their differentiation, and is associated with expression of core pluripotency factors

(A) Quantification by gene expression microarrays of the expression levels of *TOP2A* and *TOP2B* in undifferentiated hPSCs and in various adult cell types. The dashed line denotes the threshold expression level below which genes are considered not to be expressed. Data represent an average of triplicates from one cell line, error bars show s.e.m. (B) Quantitative RT-PCR measurement of the relative gene expression levels of *TOP2A* in undifferentiated hPSCs and in various adult cell types. Data represent an average of triplicates from one cell line, error bars show s.e.m. (C) Quantification by gene expression microarrays of the expression levels of *TOP2A* throughout differentiation into the three germ layers. Data are derived from the NCBI Gene Expression Omnibus (GEO) database: endodermal differentiation data are obtained from accession number GSE14897 (H9_undifferentiated vs. H9_d20_hepatic differentiated); mesodermal differentiation data are obtained from accession number GSE7214 (hES_SA01 vs. MSC_SA01); neural differentiation data are obtained from accession number GSE9940 (H9 hES cells vs. H9 hES cell derived d17 definitive neural epithelial). Data represent an average of triplicates from one cell line (except for the neural cells, where data represent an average of sextuplicates), error bars show s.e.m. *, p=0.001 and $p=5*10^{-4}$ for endodermal and mesodermal differentiation, respectively. (D) Quantitative RT-PCR measurement of the relative gene expression levels of *NANOG*, *OCT-4* and *TOP2A*, 72h following their transfection with mock-siRNA (black), with siRNA against *OCT-4* (dark gray), or with siRNA against *NANOG* (light gray). Data represent an average of triplicates from one cell line, error bars show s.e.m. *, p=0.03, p=0.03, p=0.01, p=0.017, p=0.03 and p=0.05 for *NANOG*, *OCT-4* and *TOP2A* levels in OCT-4 and NANOG knockdown, respectively. The statistically significant, yet partial, down-regulation of *TOP2A* at the very onset of differentiation, suggests that this is a gradual process that begins immediately after the initial perturbation of the pluripotency circuitry and ends days later.

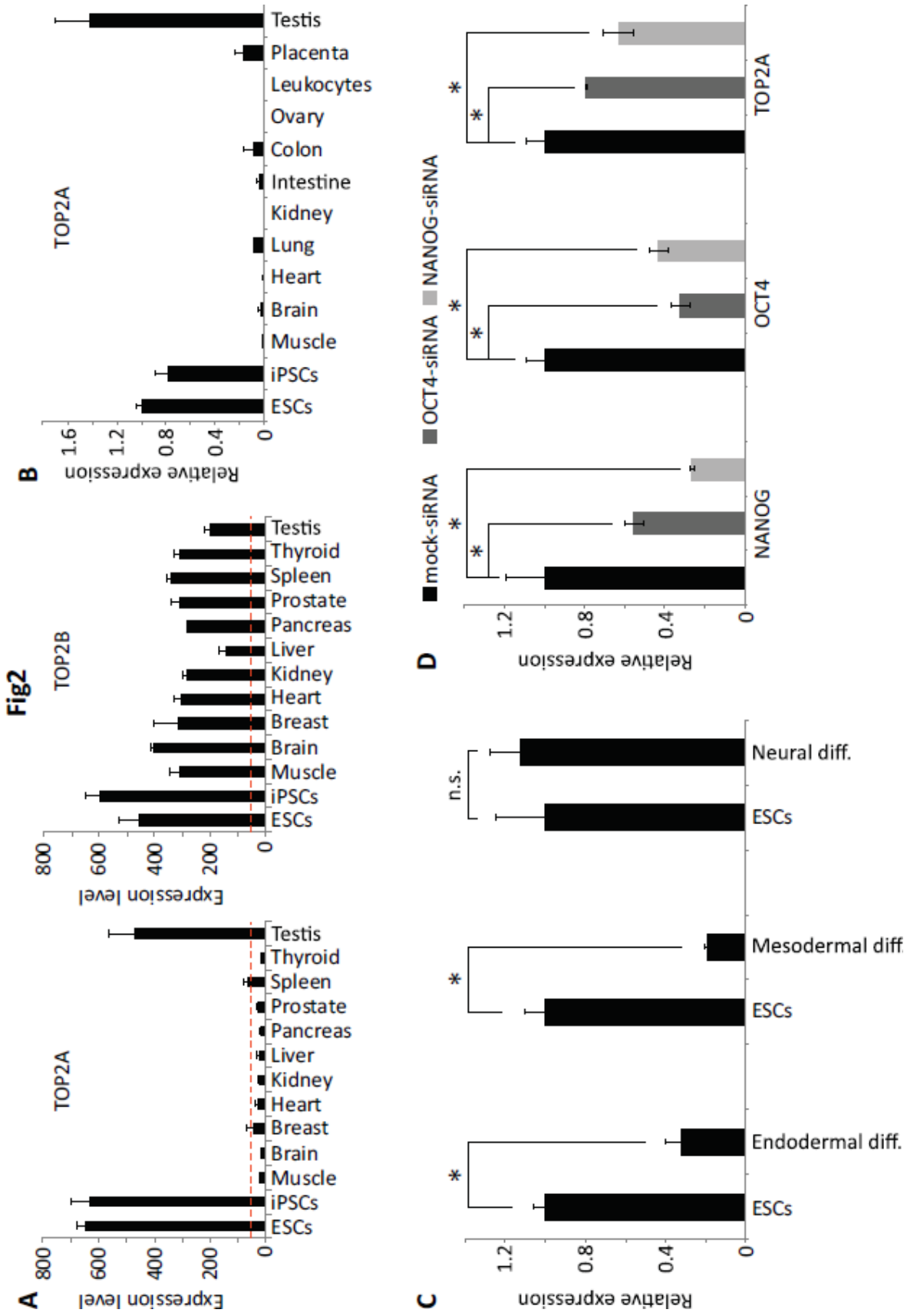

Figure 3. TOP2A is required for the survival of hPSCs.
(A) Quantitative RT-PCR measurement of the relative expression levels of *TOP2A* in hESCs, 72h following their transfection with mock-siRNA or with siRNA against *TOP2A*. Data represent an average of a triplicate from two cell lines, error bars show s.e.m. *, p=0.008. (B) Quantification of the relative viability of undifferentiated hESCs, 72h following their transfection with mock-siRNA or with siRNA against *TOP2A*. Data represent an average of a quadruplicate from two cell lines, error bars show s.e.m. *, $p=5*10^{-4}$. (C) Quantification of the relative viability of undifferentiated hESCs (red) and 14d-differentiating cells (black), 24h following their exposure to increasing concentrations of the preferential TOP2A inhibitor salicylate. Data represent an average of a triplicate from two cell lines, error bars show s.e.m. *, p=0.004 and $1*10^{-4}$ for 13.3mM and 40mM, respectively.

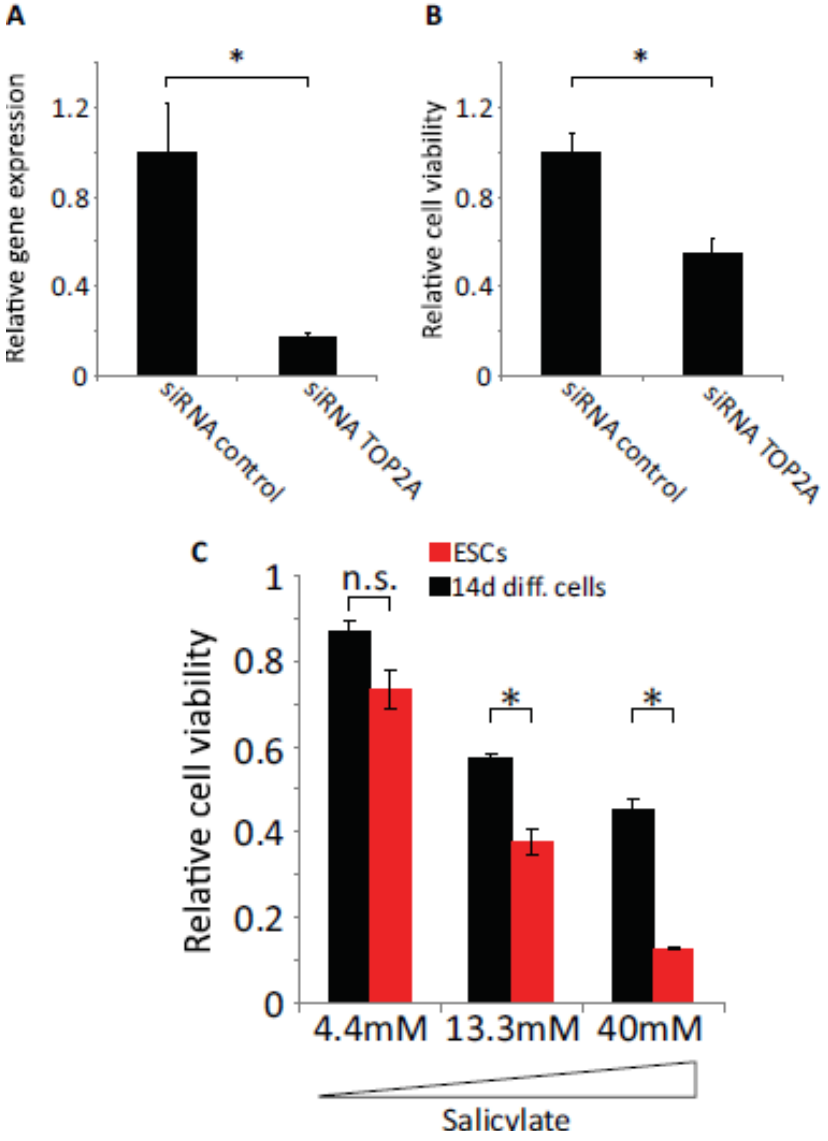

Figure 4. PluriSIn#2 is a transcriptional inhibitor of TOP2A.
(A) TARDIS fluorescence intensity measurement in K562 cells exposed at the shown concentrations to DMSO (control condition), to the known TOP2 poison etoposide, or to PluriSIn#2. Following 1h exposure, cells were embedded in agarose on slides and soluble cellular constituents were extracted leaving genomic DNA in place. TOP2A-DNA adducts remaining on the genomic DNA were quantified by immunofluorescence. *,$p=2*10^{-5}$ and $p=1*10^{-7}$ for 10μM and 100μM of etoposide, respectively. (B) TARDIS fluorescence intensity measurement in K562 cells exposed for 1h to vehicle (DMSO), ICRF193 (100μM) or PluriSIn#2 (100μM), before the addition of the etoposide for 2h at the concentrations shown. *,$p=5*10^{-4}$ and p=0.006 for 10μM and 100μM of etoposide added after ICRF193 exposure, respectively. (C) Analysis of TOP2A enzymatic activity by a decatenation assay, using kinetoplast DNA as a substrate. The ability of purified human TOP2A to decatenate kinetoplast DNA was evaluated in the presence of control conditions (DMSO), of PluriSIn#2 (50μM), or of etoposide (50μM). Lane 1: decatenated kinetoplast DNA marker; lane 2: linear kinetoplast DNA marker; lane 3: Catenated DNA without TOP2A; lanes 4-6: catenated DNA with 0.4 Unit (lane 4), 1.3 Unit (lane 5) or 4 Unit (lane 6) of TOP2A in solvent (DMSO) control conditions; lane 7: catenated DNA with 4 Unit of TOP2A in the presence of PluriSIn#2 (50μM); lane 8: catenated DNA with 4 Unit of TOP2A in the presence of etoposide (50μM). (D) Quantitative RT-PCR measurement of the relative expression levels of TOP2A and TOP2B in hESCs exposed to PluriSIn#2 (20μM) for 12h. Data represent an average of triplicates from two cell lines, error bars show s.e.m. *, $p=8*10^{-4}$ (E) Top: western blots with a specific antibody against human TOP2A, showing the protein expression levels in two cell lines of undifferentiated hESCs exposed to control conditions (DMSO), to PluriSIn#1 (20μM), or to PluriSIn#2 (20μM) for 12h. Bottom: quantification of TOP2A protein levels by band intensity comparison. Ponceau staining was used to normalize quantification. Data represent an average of two cell lines, error bars show s.e.m. Note that a ~40% reduction in TO2A protein levels was measured already 12h after the exposure of hPSCs to PluriSIn#2, at which time point cell viability was not yet jeopardized.