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Impact of transient down-regulation of DREAM in human embryonic stem cell pluripotency☆
The role of DREAM in the maintenance of hESCs

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Little is known about the functions of downstream regulatory element antagonist modulator (DREAM) in embryonic stem cells (ESCs). However, DREAM interacts with cAMP response element-binding protein (CREB) in a Ca^{2+}-dependent manner, preventing CREB binding protein (CBP) recruitment. Furthermore, CREB and CBP are involved in maintaining ESC self-renewal and pluripotency. However, a previous knockout study revealed the protective function of DREAM depletion in brain aging degeneration and that aging is accompanied by a progressive decline in stem cells (SCs) function. Interestingly, we found that DREAM is expressed in different cell types, including human ESCs (hESCs), human adipose-derived stromal cells (hASCs), human bone marrow-derived stromal cells (hBMSCs), and human newborn foreskin fibroblasts (hFFs), and that transitory inhibition of DREAM in hESCs reduces their pluripotency, increasing differentiation. We stipulate that these changes are partly mediated by increased CREB transcriptional activity. Overall, our data indicates that DREAM acts in the regulation of hESC pluripotency and could be a target to promote or prevent differentiation in embryonic cells.
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1. Introduction

Downstream regulatory element antagonist modulator (DREAM) is a multifunctional Ca^{2+}-binding protein, in the EF-hand subfamily of neuronal calcium sensors, with specific roles in different cell compartments (Carrion et al., 1999; An et al., 2000). In the nucleus, DREAM acts as a Ca^{2+}-dependent transcriptional repressor (Carrion et al., 1999; Campos et al., 2003; Ledo et al., 2000, 2002; Rivas et al., 2004) and a transcription factor interacting directly with DNA at a specific sequence, the downregulatory element (DRE) (Carrion et al., 1999; Campos et al., 2003). Outside of the nucleus, DREAM interacts with Kv4 potassium channels, directing their trafficking to the plasma membrane and regulating channel gating properties (An et al., 2000; Takimoto et al., 2002; Fontan-Lozano et al., 2011). In addition, DREAM appears to directly or indirectly affect synaptic plasticity by modulating N-methyl-D-aspartate (NMDA) receptors (Fontan-Lozano et al., 2011; Wu et al., 2010; Zhang et al., 2010). In the cytosol, DREAM binds to presenilins (Buxbaum et al., 1998), blocks the release of Ca^{2+} from the endoplasmic reticulum, and induces apoptosis of presenilin mutants associated with Alzheimer disease (Lilliehook et al., 2002).

Expression of DREAM is also upon cyclic adenosine monophosphate (cAMP) signaling (Carrion et al., 1999). It has been reported that DREAM interacts with CREB (cAMP response element-binding protein) within its kinase-inducible domain (KID) (Ledo et al., 2002). CREB binds to cAMP-response element sequences (CRE site) (Zhang et al., 2005). To attain full transcriptional activity, CREB interacts with the histone acetyl transferase enzyme CBP dependent on Ser-133 phosphorylation (Mayr and Montminy, 2001). Unphosphorylated CREB interacts with DREAM to abolish CREB–CBP interaction in a Ca^{2+}-dependent manner (Ledo et al., 2002).

Embryonic stem cells (ESCs) display two important characteristics: self-renewal, which allows them to be indefinitely expanded while

Abbreviations: SCs, stem cells; hESCs, human embryonic stem cells; hASCs, human adipose-derived stromal cells; hBMSCs, human bone marrow-derived stromal cells; hFFs, human newborn foreskin fibroblasts; DREAM, downstream regulatory element antagonist modulator (also termed KChIP-3, potassium channel interacting protein-3, or calsenilin); CREB, cAMP response element-binding protein; pCREB, phosphorylated CREB; CBP, CREB binding protein.

☆ Author contributions in the study are as follows: A. Fontán-Lozano and A. Hmadcha designed the study, analyzed the data, and wrote the manuscript; A. Fontán-Lozano performed most of the experiments with the help of V. Capilla-Gonzalez; Y. Aguilera and N. Mellado performed the culture of hESCs; A. M. Carrión assisted in some experiments; and B. Soria and A. Hmadcha directed the study.

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maintaining the undifferentiated state, and pluripotency, which allows them to differentiate into almost all cell types. ESCs are able to differentiate into all derivatives of the three primary germ layers (ectoderm, endoderm, and mesoderm). Pluripotency distinguishes ESCs from the stem cells (SCs) in an adult organism, thus adult SCs are multipotent and differentiate into a limited number of cell types. Furthermore, ESCs in culture can differentiate spontaneously and give rise to cell types that derive from different primary germ cell layers (Hmadcha et al., 2009).

ESC differentiation requires the repression of transcription factors involved in maintaining pluripotency and the activation of developmental genes (Horrillo et al., 2013). Little is known about the relationship between CREB/CBP and SCs. A previous study showed that leukemia inhibitory factor (LIF) in mouse ESCs produces LIF-dependent phosphorylation of CREB. This process is partially under the control of the RSK2 kinase and as a consequence CREB regulates pluripotency and survival genes in mouse ESCs (Boeuf et al., 2001). CREB is also involved in differentiation and survival process of neural SCs in the evolutionary scale (Dworkin and Heath, 2007; Dworkin et al., 2009). Dworkin et al. (2009) have shown a specific role for CREB in mammalian embryonic neurogenesis. Another study associated CREB with early neurogenesis (Peltier et al., 2007) but presented only indirect evidence of PI3K/Akt stimulation activating both CREB and neurogenesis. Phosphorylation of CREB at serine 133 (Ser133) is a required step for inducing the transcription of multiple genes through the cAMP response element (CRE). Several kinases possess the capability of phosphorylating CREB at this site, including protein kinases A and C (Manier et al., 2001), MAP kinase activated protein kinase-2 (Xing et al., 1998), and Akt (Peltier et al., 2007; Caravatta et al., 2008). In particular, the kinase Akt and its activator PI3K play a significant role in multiple cellular functions, such as cell survival, proliferation, and cytoskeletal rearrangements (Cantley, 2002; Engelman et al., 2006; Xue and Hemmings, 2013). While the role of the PI3K/Akt signaling pathway in the function of adult neural stem or progenitor cells is still unclear, its role during development has been widely demonstrated. For instance, Akt is implicated in maintaining self-renewal of embryonic cortical progenitors (Sinor and Lillien, 2004) and ESC lines (Paling et al., 2004; Watanabe et al., 2006). Furthermore, the PI3K antagonist phosphatase and tensin homolog (PTEN) has been shown to negatively regulate proliferation of embryonic neural SCs (Groszer et al., 2006). The activation of the PI3K/Akt signaling pathway is also associated with neurogenesis in progenitor cells derived from the subventricular zone and in olfactory SCs (Vojtek et al., 2003). In line with these reports, Akt3-deficient mice showed reduced brain size and weight (Tschopp et al., 2005), revealing that Akt is crucial for the correct brain development. All the above demonstrate a mechanistic link between PI3K/Akt, CREB, and neurogenesis.

ESCs and cancer cells share self-renewal capacity, which allows them to be indefinitely expanded. Moreover, the recent recognition that CREB may have oncogenic properties leads us to hypothesize that over-activation of CREB could contribute to the development of some types of tumors (Ghosh et al., 2007; Abramovitch et al., 2004; Linnerth et al., 2005; Shankar et al., 2005). This suggests that CREB could have a role in regulating genes related to self-renewal. At a mechanistic level, CREB is perhaps one of the best-understood phosphorylation-dependent transcription factors. In principle, phosphorylation of CREB at Ser133 seems to be sufficient to promote target gene activation through recruitment of CBP[14]. On the other hand, unphosphorylated CREB interacts with DREAM to abolish the CREB–CBP interaction in a Ca^{2+}-dependent manner (Ledo et al., 2002; Fontan-Lozano et al., 2009).

CBP is implicated in different biological functions with an essential role in maintaining normal germ cells development. CBP is expressed in primordial germ cells (PGCs) and in ESCs but is highly expressed in PGCs compared with ESCs. Proper levels of CBP have been shown to be important for hematopoietic SCs; CBP deficient hematopoietic SCs fail to self-renew (Rebel et al., 2002). Interestingly, the deletion of CREB, which could affect CBP binding to DNA-associated transcription factors, results in apoptosis in numerous cell types (Zhang et al., 2002; Barton et al., 1996; Jaworski et al., 2003; Reusch and Klemm, 2002; Dworet and Meinkoth, 2006; Scobey et al., 2001).

In contrast, only two studies associate DREAM with SCs. First, Sanz et al. (2001) found that DREAM regulates the expression of the apoptotic protein Hrk within the hematopoietic system. Second, Cebolla et al. (2008) implicated DREAM in the differentiation of neural progenitors into astrocytes. More recently, our team described that the loss of DREAM protects the brain from degeneration during aging (Fontan-Lozano et al., 2009). DREAM could be directing a depletion of stem/precursor cell reservoirs. This may indicate a possible relationship between DREAM and SCs; our results uncover a hitherto unknown role of DREAM in regulating human ESCs (hESCs) pluripotency and differentiation.

2. Materials and methods

2.1. Ethics for the hESCs

The HS-181 hESC line was obtained from the Karolinska Institute courtesy of Dr. Outi Hovatta. The cell line was derived from a normal and healthy blastocyst donated for research in accordance with the legal requirements of the country of origin and the donors gave written informed consent. The HS-181 line is included in the European Union hESC registry (http://www.hescreg.eu/).

2.2. Cell line culture

HS-181 was cultured, as described by Hovatta et al. (2003), onto BD Matrigel™ hESC-Qualified Matrix coated flasks (BD Biosciences, San Diego, CA, USA), in human feeder-conditioned medium consisting of knockout DMEM (Gibco, Grand Island, NY, USA) supplemented with 20% serum replacement (Gibco), 2 mM L-glutamine (Gibco), 1% non-essential amino acids (Gibco), 50 U/ml penicillin (Gibco), 50 μg/ml streptomycin (Gibco), 0.1 mM β-mercaptoethanol (Gibco), and 4 ng/ml basic fibroblast growth factor (bFGF) (R&D Systems, Minneapolis, MN, USA). To promote *in vitro* spontaneous differentiation, undifferentiated colonies (hESCs at day 0: 0 d) were detached by treatment with Accutase (Gibco, Grand Island, NY, USA), bFGF was withdrawn from the medium, and cells were subjected to embryoid body (EB) formation using the hanging drop method for 48 h. Cells were then plated and incubated as floating aggregates for 13 days (hESCs at day 15: 15 d) in ultra-low attach-flasks without bFGF.

Human adipose-derived stromal cells (hASCs) (ATCC®, Manassas, VA, USA; PCS-500-011™) and human bone marrow-derived stromal cells (hBMSCs) (Lonza, Basilea, Switzerland; PT-2501) were purchased and used in experiments because they are known to contain subsets of stem cells. hASCs and hBMSCs were cultured in low glucose DMEM supplemented with 10% FBS and were characterized by their surface markers (CD13, CD29, CD31, CD34, CD45, CD73, CD90, HLA-II, and CD105) and differentiation potential after cell expansion (data not shown).

The human newborn foreskin fibroblasts (hFFs) (ATCC® CRL-2429) were cultured in DMEM supplemented with 10% FBS.

2.3. Quantitative RT-PCR

Total RNA was extracted with TRIzol® Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. cDNA was synthesized from 2 μg total RNA using MMLV reverse transcriptase (Promega, Madison, WI, USA). Quantitative real-time RT-PCR (qRT-PCR) analysis was performed on an ABI 7500 FAST Sequence Detection System (Applied Biosystems, Foster City, CA, USA) using the SensiFAST SYBR Lo-ROX Kit (Bioline, London, UK). The expression level of each gene was normalized to TATA box binding protein (TBP) mRNA levels (D101-D136, TATAA Biocenter AB, Göteborg, Sweden). The results were expressed using the ΔΔCt method as the number of the fold

change relative to the expression of control samples. Primer sequences are listed in Supplementary Table S1.

2.4. Flow cytometry

2.4.1. Cell preparations

Undifferentiated cells (0 d) were disaggregated by incubation in Accutase (5 min, 37 °C). The reaction was stopped with human feeder-conditioned medium and cells were then centrifuged and resuspended in PBS. Differentiated cells (15 d) were disaggregated by incubation in trypsin (5 min, 37 °C). The reaction was stopped with media supplemented with FBS and cells were then centrifuged and resuspended in PBS. A single-cell suspension was obtained.

2.4.2. Cell cycle analysis

Cells were fixed and permeabilized overnight at −20 °C in cold 70% ethanol. Cells were collected by centrifugation and resuspended in PBS with 1% FBS and 10 mM HEPES. Samples were labeled with propidium iodide (PI) and subjected to analysis using FL-2A to score the DNA content of the cells' PI Fluorescence (FL2) using a flow cytometer (BD FACSCalibur Cytometry System, San Jose, CA, USA). Data were processed with CellQuest Pro v 5.2.1. software (BD, San Jose, CA, USA) and analyzed in the ModFit program.

2.4.3. Apoptosis analysis

To analyze the apoptosis levels provoked by DREAM inhibition, we used the FITC Annexin V Apoptosis Detection Kit I (BD Pharmingen). Analysis was performed using flow cytometry, considering PI Fluorescence (FL2) and Annexin V-FITC fluorescence (FL1) in the flow cytometer (BD FACSCalibur Cytometry System). Data were processed with CellQuest Pro v 5.2.1. software (BD). We calculated the percentage of cells at each stage of apoptosis. Early apoptosis was defined as cells that are positive for FITC Annexin V and negative for PI (Annexin V^+/PI^-); necrosis was defined as cells that are negative for FITC Annexin V and positive for PI (Annexin V^-/PI^+); late apoptosis was defined as cells that are positive for FITC Annexin V and PI (Annexin V^+/PI^+). Total apoptosis was expressed as the sum of early and late apoptosis.

2.4.4. SSEA4 and TRA-1-60 expression analysis

Cells labeled with the fluorescent antibodies FITC Mouse anti-Human TRA-1-60 Antigen (BD Pharmingen) or FITC Mouse anti-SSEA-4 (BD Pharmingen) were analyzed using Flow Cytometry (BD FACSCalibur Cytometry System). The data were processed with CellQuest Pro v 5.2.1. software (BD).

2.5. Human pluripotent stem cell proteome array

The protein expression profiles were assayed using the specific Human Pluripotent Stem Cell Array Kit 'Proteome Profiler Array' (R&D Systems Europe, Abingdon, UK; Catalog No. ARY010) following the manufacturer's instructions. This array allowed us to simultaneously detect 15 different SCs markers related to pluripotency and differentiation. The densitometry analysis of the resulting spots was carried out using ImageJ 1.41 software (Wayne Rasband, NIH, Bethesda, MD, USA) and expressed as arbitrary units (a.u.) of spot density.

2.6. Immunofluorescence

Cells were cultured in Matrigel™-coated confocal plates. Cells were fixed for 20 min in 4% paraformaldehyde solution, washed three times with 0.05% PBS-Tween, and permeabilized for 1 h with PBS containing 0.1% Triton-X 100. After 1 h of blocking incubation with PBS supplemented with 5% goat serum and 0.05% Tween, cells were incubated with the primary antibody diluted on blocking solution overnight at 4 °C. Then, cells were washed three times with PBS and incubated with secondary antibodies for 1 h at room temperature. Next, cells were washed three times with PBS and incubated with 1 mg/ml Hoechst 33342 (DNA dye) for 5 min at RT, and finally washed three times with PBS. Digital images were obtained using a Leica SP5 confocal microscope (Leica, Mannheim, Germany) or an Olympus IX71 inverted microscope (Olympus, Tokyo, Japan). The images were quantified by ImageJ 1.41 software (Wayne Rasband, NIH, Bethesda, MD, USA). The antibodies used in this study are listed in Supplementary Table S2.

2.7. Western blot and immunoblotting

Harvested cells were lysed for protein extraction using RIPA buffer (Sigma-Aldrich), supplemented with protease and phosphatase inhibitor cocktail. Then, proteins from whole cell lysates were resolved using 10% Tris-Glycine gel electrophoresis and transferred to a polyvinylidene difluoride membrane (GE Healthcare Life Sciences, Buckinghamshire, UK). Membranes were then blocked with 5% non-fat milk and primary antibodies were probed (see Supplementary Table S2 for antibody information). Detection was performed with the appropriate horseradish-peroxidase conjugated secondary antibodies (Supplementary Table S2) using the enhanced chemiluminescence reagent (GE Healthcare Life Sciences). Densitometry analyses for the blots were performed using ImageJ software (version 1.4r; National Institute of Health, Bethesda, MD, USA).

2.8. Small interfering RNA (siRNA)

Non-specific control siRNA (Santa Cruz Biotechnology, sc-37007, Santa Cruz, CA, USA) and a recommended DREAM siRNA (Santa Cruz Biotechnology, sc-42398) were used to ensure silencing of DREAM expression. Cell transfections were carried out with siRNA and Lipofectamine 2000 Reagent (Life Technologies, Carlsbad, CA, USA) following the manufacturer's instructions. After testing different concentrations of DREAM siRNA (20, 40, and 80 pmol), the concentration of 40 pmol was selected as effective for DREAM depletion because 90% of inhibition was detected by qRT-PCR 24 h after transfection of hESCs (data not shown).

2.9. Statistical analysis

All experiments were repeated at least in triplicate ($n \geq 3$ independent experiments) and data were analyzed using the SPSS package for Windows (SPSS, Chicago, IL). Values are presented as the mean ± SEM. Statistical significance was calculated using ANOVA or *t*-test. The Mann–Whitney Rank Sum Test was used in samples that were not normally distributed or that had unequal variance.

3. Results

3.1. DREAM is expressed in human embryonic stem cells

The lack of DREAM is implicated in the delay of brain aging and in prevention of the loss of SCs with age. In this study, the results demonstrated that DREAM CREB and CBP mRNA are expressed in hESCs, and that the expression of DREAM and CBP increased when cells initiated spontaneous differentiation (Fig. 1A). In contrast, no changes in gene expression for CREB were detected and Oct4 expression was decreased when cells were cultured under conditions that promote differentiation. We also detected expression of DREAM, CREB, and CBP in the other evaluated cell types. Oct4 was only expressed in undifferentiated hESCs (0 d) and in hFFs (Fig. 1A). DREAM signaling and its related proteins were further analyzed; immunofluorescence results revealed that hESCs (0 d), hASCs, hBMSCs, and hFFs were positive for DREAM, CREB, pCREB, and CBP (Fig. 1B). The expression of DREAM coincided with a hESC specific marker, TRA-1-60 (Fig. 1C). Unfortunately, despite using different antibodies, we have been unable to detect DREAM by Western blot. Together, these data suggest that DREAM and its related genes are expressed in different cell types and indicate a possible role of DREAM in

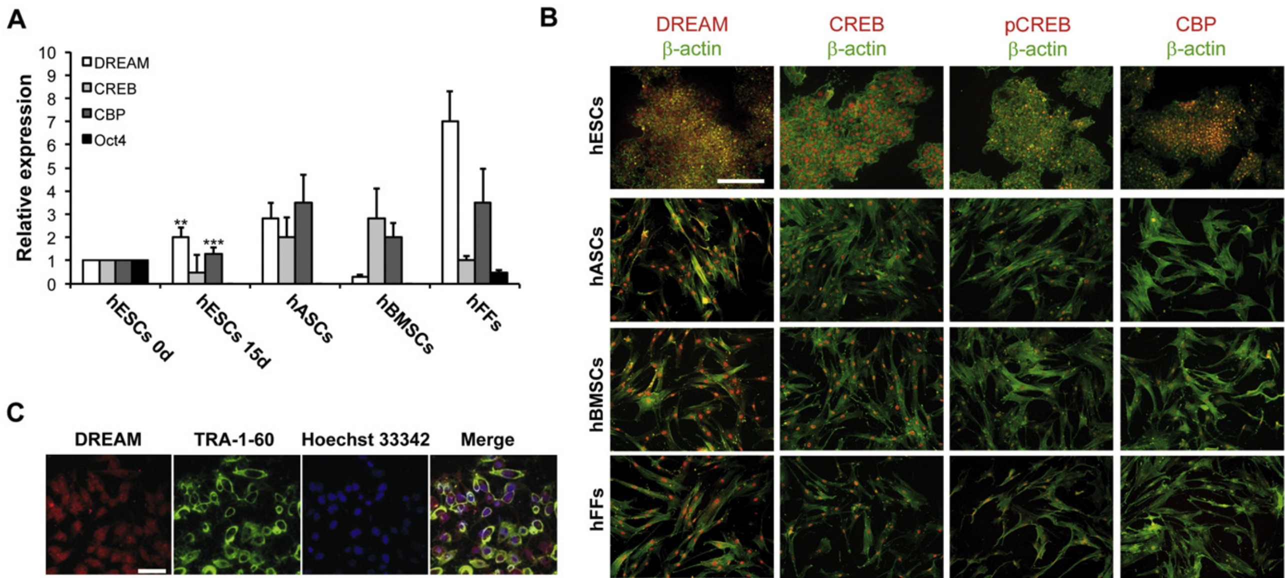


Fig. 1. DREAM protein is expressed in stem cells: A. qRT-PCR analysis of relative gene expression levels of DREAM, CREB, CBP, and Oct4 in undifferentiated hESC (0 d), spontaneously differentiated (15 d) hESCs, hASCs, hBMSCs, and hFFs. Note the statistically significant difference of undifferentiated hESCs (0 d) compared with differentiated hESC (15 d) groups for DREAM and CBP (**$p \le 0.01$ and ***$p \le 0.001$). B. Immunofluorescence detection of DREAM, CREB, pCREB, and CBP (red) expression in cultured hESCs, hASCs, hBMSCs, and hFFs. β-actin (green) was used as a fiducial marker and nuclei were stained with Hoechst 33342 (blue). Note that all four markers were expressed in all assessed cells types. The scale bar is 250 μm. C. Immunofluorescence analysis of DREAM (red) and TRA-1-60 (green) expression in hESCs. Nuclei are stained with Hoechst 33342 (blue). The scale bar is 40 μm.

hESCs when their pluripotency is diminished and cells begin to differentiate.

3.2. Spontaneous differentiation of hESCs affects DREAM expression

We evaluated the expression of DREAM and its related proteins during spontaneous differentiation. We first evaluated changes in DREAM gene expression by qRT-PCR in undifferentiated (0 d) and spontaneously differentiated (15 d) hESCs (Fig. 2A). We observed a clear increase of DREAM and CBP and no significant changes of CREB expression in differentiated cells compared with undifferentiated cells. Moreover, to confirm these changes we performed immunofluorescence in both groups to detect the DREAM protein and its related proteins, CREB, pCREB, and CBP (Fig. 2B), and quantified the fluorescence intensity (Fig. 2C). We observed that DREAM and pCREB protein levels significantly increased in spontaneous differentiation (15 d) hESCs compared with undifferentiated (0 d) cells, while CREB and CBP expression decreased. We noted that mRNA levels for CREB and CBP were not reflected in the fluorescent intensity as a surrogate for protein expression, which could be caused by post-translational events. To confirm that differentiation provokes an increase in pCREB expression, we analyzed the phosphorylation of CREB at Ser133 by Western blot and found a clear increase in pCREB (Ser133) in spontaneously differentiated cells (Fig. 2D). These results suggest a possible role of DREAM in regulation of pluripotency and differentiation, possibly through modulation of CREB–CBP.

3.3. DREAM depletion in hESCs (DREAM-siRNA)

The slight increase in DREAM observed upon hESCs entry into differentiation may suggest a role for this protein in differentiation. We proceeded to transiently inhibit DREAM expression using small interfering RNA (siRNA) against DREAM (a pool of 3 target-specific 19–25 nt siRNAs designed to knock down DREAM gene expression) and studied the knockdown effects 24 h after transfection in spontaneously differentiated cells. As shown in Fig. 3A, we obtained a reduced expression of DREAM mRNA in DREAM-siRNA transfected cells compared with Control cells and cells treated with Control-siRNA (a scrambled sequence that does not lead to specific degradation of any known cellular mRNA). To confirm the inhibition of DREAM, we detected its expression by immunofluorescence and quantified the resulting images (ImageJ software). Immunofluorescence images revealed a weak staining for DREAM and the SCs marker TRA-1-60 in DREAM-siRNA cells (Fig. 3B). Quantification revealed a significant decrease in total intensity for DREAM staining in the transfected group (DREAM-siRNA) compared with total intensity for Control and Control-siRNA staining (Fig. 3C). As DREAM is a multifunctional protein with specific roles in different cell compartments, we evaluated differential DREAM expression in the cytoplasm and nuclei by analyzing the immunofluorescence confocal images. For cells transfected with DREAM-siRNA, we obtained a significant decrease in nuclear and cytoplasmic protein fluorescence intensity (expression) compared with Control and Control-siRNA cells (Fig. 3D). These results confirm the efficacy of DREAM-siRNA used in the transient inhibition of DREAM expression in hESCs.

3.4. Cellular effects of DREAM inhibition (DREAM-siRNA)

Cell cycle-control mechanisms are functionally linked to self-renewal and differentiation in hESCs. Consequently, we used flow cytometry to evaluate if DREAM-siRNA provoked changes in a hESC cell cycle. Fig. 4A represents the percentage of cells in different cell cycle phases: G1, S, G2m (G2 and mitosis) and apoptosis in Control, Control-siRNA, and DREAM-siRNA transfected cells. No significant differences were found in the cell cycle, and we only observed a trend toward increased apoptosis in DREAM-siRNA treated cells. As DREAM protein has been considered pro-apoptotic (Lilliehook et al., 2002), we also evaluated if DREAM inhibition in hESCs could induce apoptosis using a FITC Annexin V Apoptosis Detection Kit I, which discriminates early apoptosis from late apoptosis. Fig. 4B represents the percentage of cells in early, late, or total apoptosis and necrosis for Control, Control-siRNA, and DREAM-siRNA cells. We observed an increase of early and total apoptosis in DREAM-siRNA transfected cells compared with Control and Control-siRNA cells. These data indicate that DREAM inhibition could affect hESC survival.

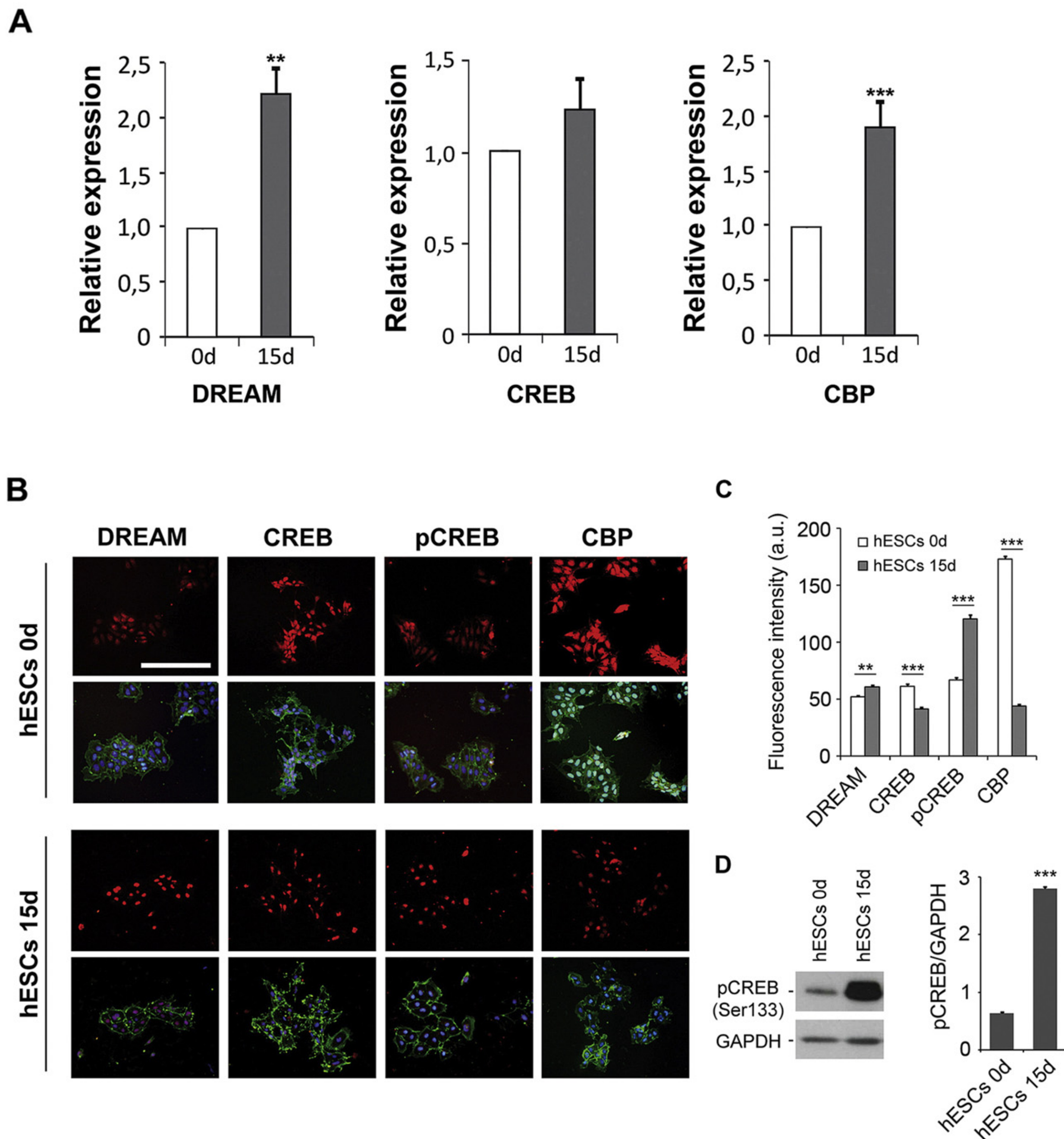


Fig. 2. Spontaneous differentiation results in changes in DREAM expression levels: A. qRT-PCR analysis of relative gene expression levels of DREAM, CREB and CBP for undifferentiated hESCs (0 d) and differentiated hESCs (15 d). Note the statistically significant increase of DREAM and CBP in differentiated hESCs (15 d) compared with undifferentiated hESCs (0 d) (**$p \leq 0.01$ and ***$p \leq 0.001$). B. Differential expression of DREAM, CREB, pCREB, and CBP (red) in cultured undifferentiated (0 d) and spontaneously differentiated (15 d) hESCs. β-actin (green) was used as a fiducial marker and nuclei were stained with Hoechst 33,342 (blue). The scale bar is 250 μm. C. Bar graph representing the fluorescence intensity in arbitrary units (a.u.) for immunostaining images in A. Note that DREAM and pCREB levels were significantly increased in spontaneously differentiated (15 d) hESCs compared with undifferentiated (0 d) cells, while CREB and CBP levels were decreased (**$p \leq 0.01$ and ***$p \leq 0.001$). D. The phosphorylation of CREB was analyzed by Western blot in undifferentiated (0 d) and spontaneously differentiated (15 d) hESCs; GAPDH was used as the internal control. Bar graph represents the resulting densitometry quantification of Western blots (***$p \leq 0.001$).

3.5. DREAM-siRNA induces modifications in hESC pluripotency

To test the effect of DREAM inhibition upon hESC entry into differentiation, we performed qRT-PCR to evaluate changes in pluripotent gene markers (Oct4, Nanog, and Sox2) and differentiation gene markers (Pax6, Nrg1, erbB2, erbB3, and erbB4) and studied the effects of siRNA transfection in undifferentiated (0 d) and spontaneously differentiated cells (15 d). As shown in Fig. 5A, pluripotent gene expression was decreased in all cell groups (Control, Control-siRNA, and DREAM-siRNA) after spontaneous differentiation. In contrast, we observed that DREAM-siRNA transfection provoked a significant increase in the expression of the differentiation gene Pax6 in spontaneously differentiated cells (15 d). Furthermore, we evaluated changes in Nrg1 and its receptors (erbB2, erbB3, and erbB4) and found that Nrg1 and erbB4 expression significantly increased with transfection (DREAM-siRNA) for differentiated cells (15 d). Overall, the expression of pluripotency markers decreased in a DREAM-independent fashion and expression of differentiation markers Pax6, Nrg1, and erbB4 was enhanced by DREAM knockdown during differentiation.

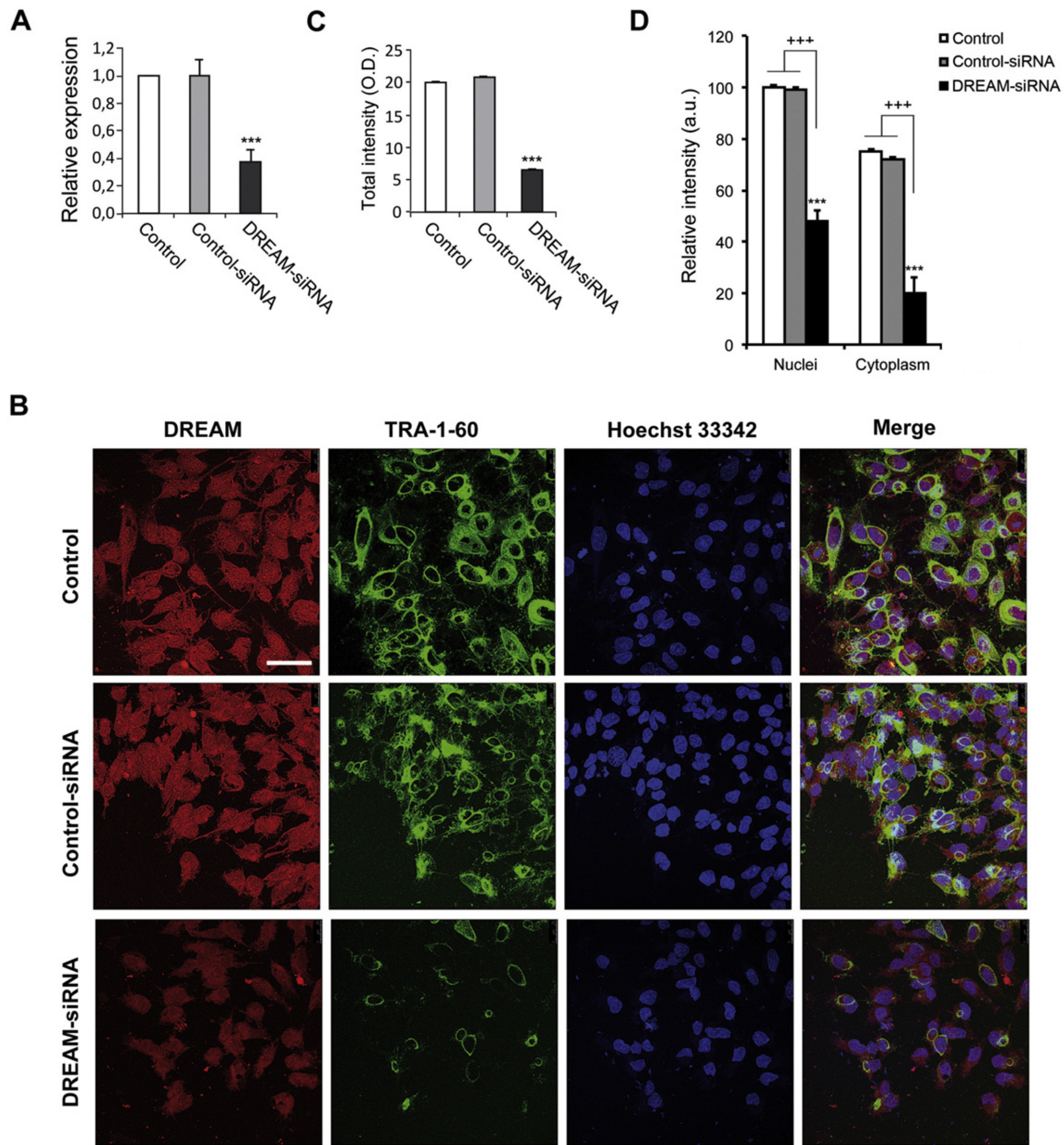


Fig. 3. siRNA-DREAM induces decreased DREAM expression in hESCs: A. Relative gene expression analysis of DREAM 24 h after transfection for Control, Control-siRNA, and DREAM-siRNA transfected hESCs. DREAM expression was significantly reduced (***$p \le 0.001$). B. Immunofluorescence analysis of DREAM (red) and TRA-1-60 (green) expression in Control, Control-siRNA, and DREAM-siRNA treated hESCs 24 h after transfection. Nuclei are stained with Hoechst 33342 (blue). Note the significant decrease in total intensity of DREAM and TRA-1-60 in the transfected group (DREAM-siRNA) compared with the Control and Control-siRNA groups. The scale bar is 50 μm. C. Bar graph representing the total fluorescence intensity of DREAM for the immunostained images in B (***$p \le 0.001$). The results are expressed as optical density (O.D.). D. Relative fluorescence intensity in arbitrary units (a.u.) of DREAM protein signal in nuclei and cytoplasm compartments for the immunostaining images in B. There were statistically significant differences between conditions and groups (***$p \le 0.001$ and $^{+++}p \le 0.001$, respectively).

The analysis of protein expression levels with a hESC-specific protein array (Fig. 5B) revealed that spontaneous differentiation (15 d) results in a decrease in expression of OCT4, NANOG, SOX2, GATA4, and OTX2 in all cell groups (Control, Control-siRNA, and DREAM-siRNA). Interestingly, there was a clear and significant decrease for protein expression of OCT4, NANOG, SOX2, SOX17, OTX2, TP63/73L, GSC, SNAIL, and VEGF/R2 in DREAM-siRNA dependent manner. However, when comparing protein levels between undifferentiated cells (0 d) and spontaneously differentiated cells (15 d), no significant differences of protein expression were detected for E-CADHERIN, AFP, GATA4, FOXA2, IFP1, and HCG in all groups (Control, Control-siRNA, and DREAM-siRNA). The protein decrease induced by DREAM-siRNA in OCT4, NANOG and SOX2 shown in Fig. 5B was not reflected in the lack of change in mRNA levels as shown in Fig. 5A.

In addition, we evaluated the percentage of cells that express SSEA4 and TRA-1-60 proteins by flow cytometry (Fig. 5C). Consistent with our results for other pluripotency markers (OCT4, NANOG, and SOX2), we observed that spontaneous differentiation (15 d) results in a clear decrease in expression for SEEA4 and TRA-1-60 in all cell groups (Control, Control-siRNA, and DREAM-siRNA). Remarkably, there was a significant decrease in expression upon reduction of DREAM (DREAM-siRNA). These data indicate that there is a variable expression of pluripotency and differentiation markers in a DREAM-independent fashion during early differentiation of hESCs.

3.6. DREAM-siRNA induces changes in CREB transcription activity

To determine whether CREB is implicated in increased expression of some differentiation markers in hESCs treated with DREAM-siRNA

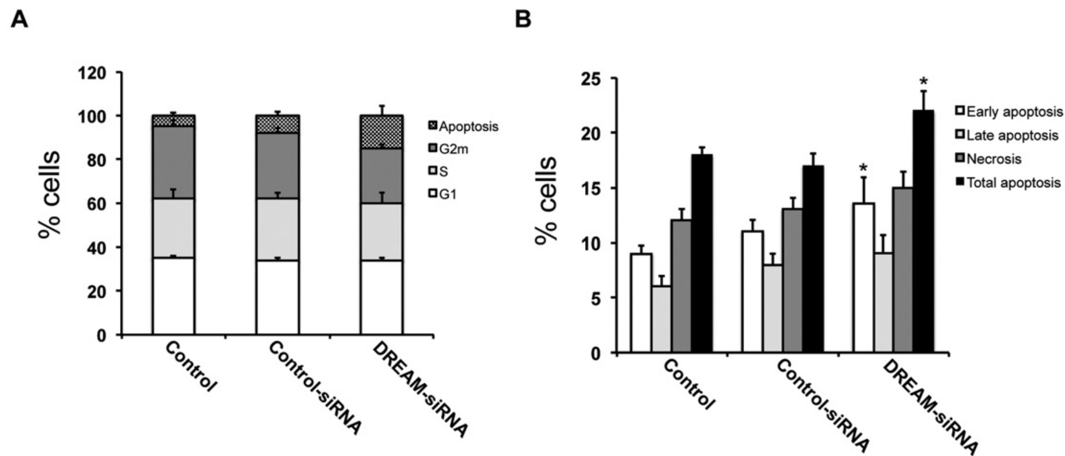


Fig. 4. Cellular effects of siRNA-DREAM: A. Bar graph obtained from cell cycle analysis of Control, Control-siRNA, and DREAM-siRNA transfected hESCs showing the percentage of cells in different phases of cell cycle (G1, S, G2m [G2 and mitosis], and apoptosis). B. Flow cytometry analysis of the percentage of cells with early, late, or total apoptosis and necrosis for Control, Control-siRNA, and DREAM-siRNA transfected hESCs. Note the statistically significant increase of early and total apoptosis in DREAM-siRNA transfected hESCs (*$p \leq 0.05$).

during differentiation, we first evaluated changes in relative gene expression for DREAM, CREB, and CBP in undifferentiated (0 d) Control, Control-siRNA, and DREAM-siRNA cells compared with differentiated (15 d) Control, Control-siRNA, and DREAM-siRNA cells (Fig. 6A). We observed that DREAM expression increased in Control and Control-siRNA cells with differentiation (15 d), but, as expected, DREAM-siRNA resulted in a decrease in DREAM expression in differentiated cells. We also observed an increase in CBP gene expression in differentiated cells (15 d) but not for cells treated with DREAM-siRNA. A non-significant increasing trend of CREB gene expression was detected between undifferentiated and differentiated cells for all conditions.

We also examined the phosphorylation of the CREB protein in undifferentiated cells (0 d) and differentiated cells (15 d). Immunofluorescence results demonstrated that the CREB signal was similar for all conditions (Fig. 6B); however, pCREB significantly increased when cells initiated spontaneous differentiation and interestingly transient inhibition of DREAM (DREAM-siRNA) significantly increased pCREB (Fig. 6C). Taken together, these results indicate that the transient inhibition of DREAM in differentiated hESCs induces an increase of CREB phosphorylation.

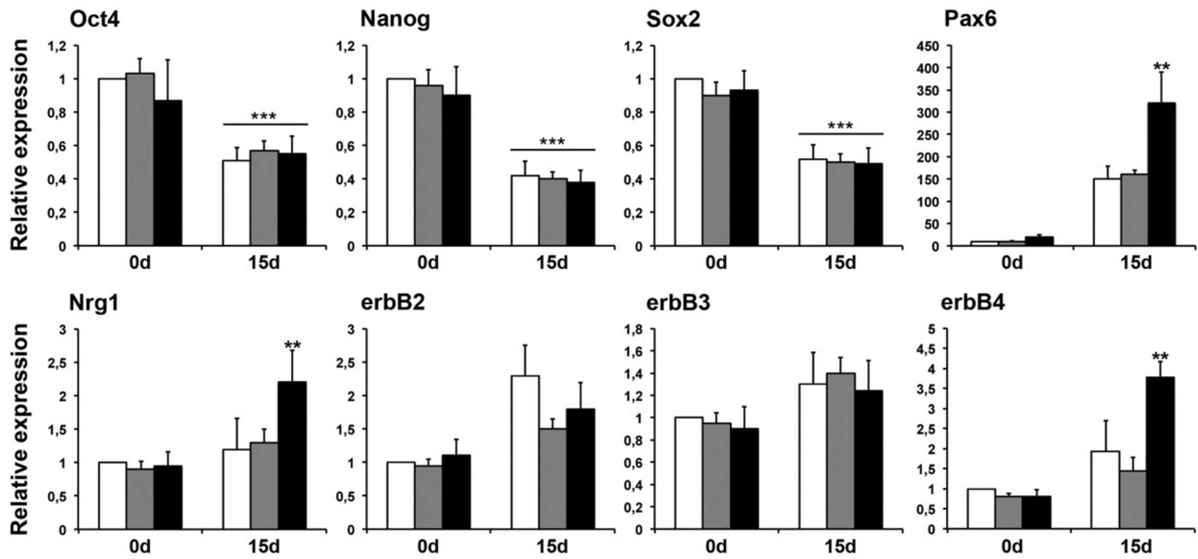
4. Discussion

Aging is a process that depends on diverse molecular and cellular mechanisms, such as genome maintenance and inflammation. Mechanisms to maintain genomic stability are thought to counteract the aging process, whereas inflammation is considered a driving force of human aging (Troen, 2003). Organisms have different anti-aging mechanisms to maintain genome integrity, such as DNA repair and cell cycle control, and to remove and recycle heavily damaged cells from the body, such as apoptosis and cellular senescence. Even if these mechanisms may be very efficient, they cannot cope with all genomic damage, leading to a gradual accumulation of DNA damage and mutations, thus contributing to organismal aging (Garinis et al., 2008). DREAM is considered a pro-inflammatory gene (Tiruppathi et al., 2014) and has a role in the modulation of inflammatory pain (Cheng et al., 2002; Jin et al., 2012), suggesting a possible implication in the aging process. Moreover, in an *in vivo* study (18-month-old *dream* $^{-/-}$ mice), we found that DREAM is a key regulator of memory and brain aging; thus, DREAM could be a potential target to not only reduce pain but also to control decline of neurons in aging (Fontan-Lozano et al., 2009), indicating a possible relationship between DREAM and SCs. However, the *ex vivo* data presented here indicates that loss of DREAM enhanced the loss of stem cell characteristics and increased the level of some differentiation markers.

Chronic inflammation has been associated with age-related decline in the function of hematopoietic SCs and tissue-specific stem/progenitor cells (Chambers et al., 2007; Lepperdinger, 2011) and has been implicated as a mediator of almost all aging-associated diseases (Gadalla et al., 2015), such as vascular diseases, diabetes, neurodegenerative diseases, and cancer (Medzhitov, 2010; Freund et al., 2010; De Martinis et al., 2005; Sarkar and Fisher, 2006). Aging is accompanied by a progressive decline in SC function, which results in less effective tissue homeostasis and repair in mammals (Jones and Rando, 2011; Capilla-Gonzalez et al., 2013). For these reasons, we checked for a possible role of DREAM in SCs. As a first approximation, we detected DREAM expression in different cell types, including hESCs, hASCs, and hBMSCs. DREAM interacted with CREB in a Ca^{2+}-dependent manner, preventing CBP recruitment. We likewise detected expression of CREB and CBP in hESCs, hASCs, and hBMSCs. All these findings point toward a possible role of DREAM in SC biology.

Aging of SCs leads to impaired self-renewal and aberrant differentiation potential (Jones and Rando, 2011). Mechanisms controlling self-renewal and the proliferative capacity of SCs are essential to maintaining functional SCs during aging. It remains unknown whether declines in SCs functionality during aging influences organismal longevity. To elucidate the role(s) of DREAM in SC pluripotency, we studied the inhibition effects of DREAM on spontaneous differentiation of hESCs. During spontaneous differentiation, the expression of DREAM increased, no changes were observed in CREB, and there was an increase in the expression of CBP. In addition, we detected a decrease in CBP protein and an increase of Ser133-CREB phosphorylation. It would appear that CREB is phosphorylated in response to DREAM inhibition. Although CBP recruitment is known to require phosphorylation of Ser133 in

Fig. 5. siRNA-DREAM alters the expression of pluripotency markers: A. Relative gene expression of pluripotency genes (Oct4, Nanog, and Sox2) and differentiation genes (Pax6, Nrg1, erbB2, erbB3, and erbB4) in undifferentiated (0 d) and differentiated (15 d) Control, Control-siRNA, and DREAM-siRNA transfected hESCs. Note the statistically significant decrease for pluripotency genes in all differentiated (15 d) cell groups compared with undifferentiated (0 d) cell groups and an increase of Pax6, Nrg1, and erbB4 in differentiated (15 d) DREAM-siRNA transfected hESCs (***$p \leq 0.001$; **$p \leq 0.01$). B. Relative protein expression levels of pluripotency markers in undifferentiated hESCs (0 d) and differentiated (15 d) Control, Control-siRNA, and DREAM-siRNA hESCs evaluated by the human pluripotent stem cell antibody array. **$p \leq 0.01$ denotes a statistically significant difference for OCT4, NANOG, SOX2, SOX17, OTX2, TP63/73L, GSC, SNAIL, and VEGF/R2 proteins. C. Quantification of the percentage of cells expressing the surface pluripotent markers SSEA4 and TRA-1-60 in undifferentiated (0 d) and differentiated (15 d) Control, Control-siRNA, and DREAM-siRNA treated cells. Significant reduction in SSEA4 and TRA-1-60 expression was detected in DREAM-siRNA cells (**$p \leq 0.01$).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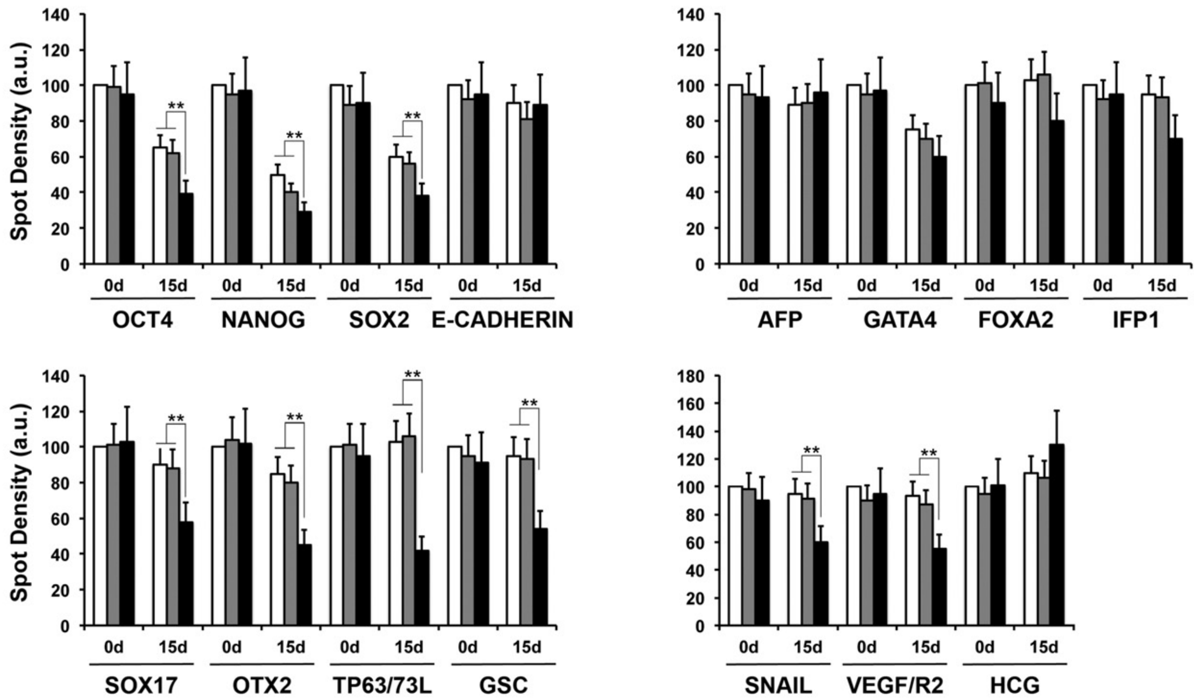
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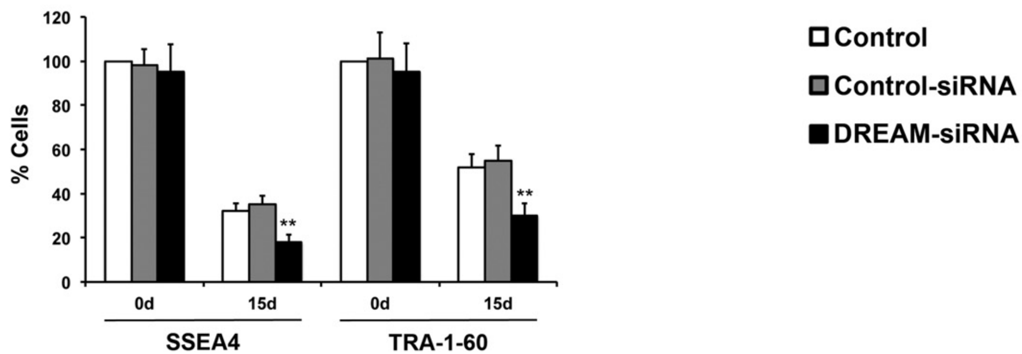
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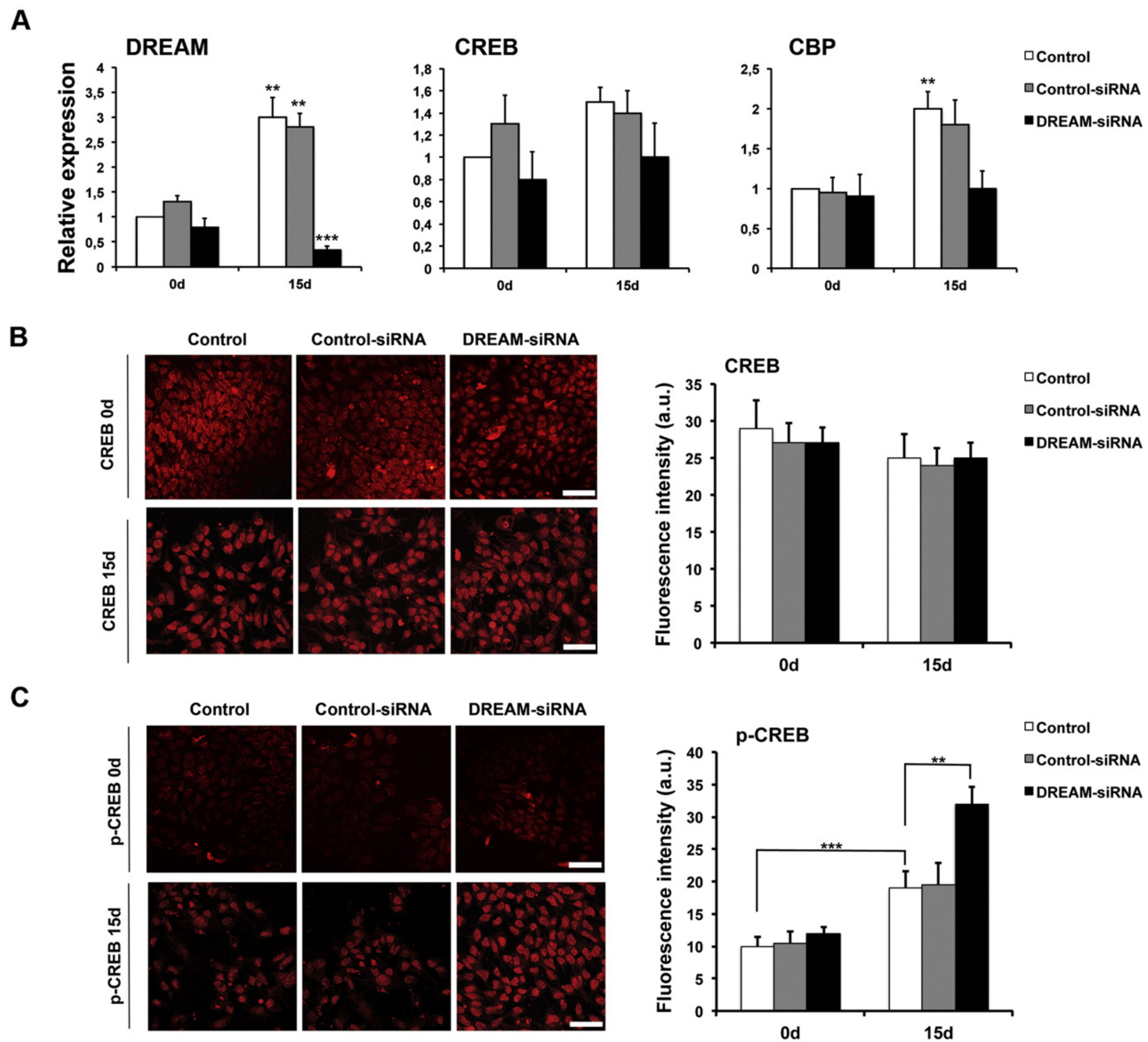

Fig. 6. siRNA-DREAM induces changes in CREB transcription activity: A. Relative gene expression of DREAM, CREB, and CBP genes in undifferentiated (0 d) and differentiated (15 d) Control, Control-siRNA, and DREAM-siRNA transfected cells. $^{**}p \le 0.01$ and $^{***}p \le 0.001$ indicate a statistically significant difference. B. Representative immunofluorescence images of CREB protein detection in undifferentiated (0 d) and differentiated (15 d) Control, Control-siRNA, and DREAM-siRNA transfected cells. The scale bar is 50 μm. The quantification of fluorescence intensity in arbitrary units (a.u.) for these images showed non-significant differences. C. Representative immunofluorescence images of pCREB protein detection in undifferentiated (0 d) and differentiated (15 d) Control, Control-siRNA, and DREAM-siRNA transfected cells. The scale bar is 50 μm. The quantification of fluorescence intensity in arbitrary units (a.u.) revealed that pCREB was significantly increased in spontaneously differentiated (15 d) hESCs compared with undifferentiated (0 d) cells and in DREAM-siRNA transfected hESCs ($^{**}p \le 0.01$ and $^{***}p \le 0.001$).

CREB, there must be additional mechanisms (perhaps further cofactor interactions) that are also required for CBP recruitment (Zhang et al., 2005; Ooi and Wood, 2008). However, we must keep in mind that CBP is essential in maintaining normal germ cell development (Elliott et al., 2007) and is necessary for neural differentiation (Rebel et al., 2002). These data could suggest the existence of mechanisms convergent on DREAM and CREB protein phosphorylation, however the role of CBP within the hESC differentiation remains to be elucidated.

Keeping SCs out of the active cell cycle phase and minimizing the risk of DNA damage may be especially important in aging and has led to a SC hypothesis of aging (Sharpless and DePinho, 2007; Beausejour and Campisi, 2006). We succeeded in transiently inhibiting DREAM expression 24 h after siRNA transfection. This inhibition of DREAM does not affect the cell cycle but provokes an increase in apoptosis. Taking this into account, programmed cell death is a fundamental process throughout mammalian development (Jacobson et al., 1997), and Caspase-3 has been shown to be crucial for mouse ESC differentiation *via* Nanog deactivation (Fujita et al., 2008). These facts continue to suggest a possible role of DREAM in hESC survival, cell proliferation, and differentiation.

We found that the lack of DREAM was associated with a decrease in protein levels of pluripotency markers and effected differentiation markers differently. CREB signaling directly regulates Pax6 and is known to control neurogenesis (Herold et al., 2011). In our case, transient inhibition of DREAM in hESCs increased the expression of Pax6, Nrg1, and erbB4 in spontaneously differentiated cells and promoted the phosphorylation of CREB. These results suggest that differentiation of hESCs could be related to DREAM inhibition, which in turn activates CREB through CREB phosphorylation.

To the best of our knowledge, this is the first study that includes DREAM in the network hierarchy controlling the pluripotency of hESCs. Our results provide new mechanistic insights regarding the function of DREAM during early hESC differentiation. However, it is crucial to dissect the additional perspectives aside from gene expression for molecular mechanisms underlying the role of DREAM in cellular pluripotency. Emerging studies show that post-translational modifications influence

the regulation of cellular pluripotency through a variety of mechanisms and provide new ways for characterizing self-renewal and differentiation of pluripotent cells (Wang et al., 2014). Therefore, DREAM could be a novel and important protein that relies on post-translational modifications; the impact of DREAM could not be directly identified at the transcriptional or translational levels in SCs. This possibility requires further investigation to determine the precise impact of DREAM and its specific targets in human pluripotent cells, with the goal of uncovering critical and yet unknown mechanisms that are responsible for tuning pluripotency in hESCs.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scr.2016.03.001.

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