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INDUCTION OF A TROPHOBLAST-LIKE PHENOTYPE BY HYDRALAZINE IN THE P19 EMBRYONIC CARCINOMA CELL LINE

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Abstract

Chemicals that affect cellular differentiation through epigenetic mechanisms have potential utility in treating a wide range of diseases. Hydralazine decreases DNA methylation in some cell types but its effect on differentiation has not been well explored. After five days of exposure to hydralazine, P19 embryocarcinoma cells displayed a giant cell morphology and were binucleate, indicative of a trophoblast-like morphology. Other trophoblast-like properties included the intermediary filament Troma-1/cytokeratin 8 and the transcription factor Tead4. A decrease in CpG methylation at three sites in the TEAD4 promoter and the B1 repeated sequence was observed. Knocking down expression of Tead4 with siRNA blocked the increase in Troma-1/cytokeratin 8 and over expression of Tead4 induced the expression of Troma-1/cytokeratin 8. Cells treated for 5 days with hydralazine were no longer capable of undergoing retinoic acid-mediated neuronal differentiation. An irreversible loss of the pluripotent transcription factor Oct-4 was observed following hydralazine exposure. In summary, hydralazine induces P19 cells to assume a trophoblast-like phenotype by upregulating Tead4 expression through a mechanism involving DNA demethylation.

Keywords

Hydralazine; trophoblast; Tead4; P19 cells; DNA methylation

INTRODUCTION

Interest in the epigenetic basis of diseases such as neurodegenerative diseases, schizophrenia, autoimmunity and cancer has prompted interest in identifying drugs which target enzymes involved in regulation of the epigenome. The aim is to identify drugs that could alter the differentiation pattern of cells through effects on their methylome. In cancer

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treatment, drugs that alter differentiation will likely produce fewer secondary effects than chemicals that are cytotoxic. Interestingly, many chemicals that have recently been shown to modify histone and DNA methylation have been previously used both clinically and in the laboratory for studies on cellular differentiation. The effectiveness of sodium valproate, for example, in epilepsy treatment is thought to be due to increasing levels of the inhibitory neurotransmitter GABA by direct inhibition of GABA transaminase [1] and succinic semialdehyde dehydrogenase [2] but a secondary mechanism could be through inhibition of histone deacetylase [3]. Other chemicals such as the short chain fatty acid butyric acid [4, 5] and fungistatic antibiotics trichostatins were first shown to induce differentiation of leukemic cells to hematopoietic lineages [6] before the demonstration that they inhibit histone deacetylation [7-9]. In changing DNA methylation, 5′azacytidine has been shown to induce differentiation in a several cell culture models including the P19 cell line to cardiomyocytes [10], and Ch310T1/2 cells to striated muscle, adipocytes, and chondrocytes [11]. The chemical is now recognized as a powerful inhibitor of DNA methyltransferase 1, though it was synthesized as an antimitotic for use in chemotherapy [12].

Several non-nucleoside chemicals, such as hydralazine (Hyd), procaine, and (−)-epigallocatechin-3-gallate [13], have also been shown to decrease DNA methylation. Hyd was suggested to increase expression of CD70 by decreasing promoter methylation in T-derived lymphocytes [14] and to induce expression of adenomatous polyposis coli by decreasing the methylation of its promoter sequence in HeLa and CaSki cells [15]. More recent studies have shown decreases in methylation of sarcoplasmic reticulum Ca^{2+} ATPase in cardiomyocytes [16] but, unlike 5′azacytidine, the mechanism is unclear. In T cells, Hyd was suggested to decrease DNA methylation by inhibiting extracellular related kinase activity [17] whereas in CHO cells investigators found a decrease in expression of DNA methyltransferase 1 [18]. A molecular modelling approach indicates that Hyd and 5′azacytidine interact within the DNA methyltransferase catalytic site [19]. Another substantial benefit is that Hyd has been used to treat hard-to-control blood pressure for over thirty years and few side effects have been reported [20]. Whether Hyd alters cell differentiation has been largely unexplored.

We examined the effect of Hyd on early embryonic differentiation using the P19 embryonic carcinoma cell line. P19 cells are an appropriate model for our study due to their mulipotency. P19 cells are capable of differentiating to neural [21, 22] and mesenchyme lineages [23, 24] in vitro. We reasoned that P19 cells and other multipotent cell types have the greatest potential to differentiate in response to drugs that target the epigenome. In this study, we demonstrate that low concentrations of Hyd decrease DNA methylation at several loci and induced P19 cells to express trophoblast-like properties.

MATERIALS AND METHODS

P19 cell culture

P19 cells were obtained from ATCC (Manassas, VA) grown in α-MEM, 7.5 % bovine calf serum and 7.5% foetal bovine calf serum (growth media) at 37°C 5% CO2. They were maintained in culture for up to 16 passages by passing cells by scraping. Cells were plated at

a density of approximately 4000 cells/cm^2 for the different assays. Hyd was obtained from Sigma and prepared fresh in phosphate buffered saline prior to treatment.

Retinoic Acid Treatment and neuronal differentiation

Cells were plated at 10^6 cells/ bacteriological 10 cm petri plate in growth media with 500 nM retinoic acid to allow the formation of embryoid body-like structures. After 3 days, spherical embryoid bodies were harvested, dissociated and re-plated at 200,000 viable cells per poly-L-lysine (0.1%) coated glass coverslips in 24-well dishes. The cells were cultured in Neurobasal Media supplemented with B27 to promote neuronal differentiation.

Haemotoxylin/eosin staining

Cells were allowed to attach overnight prior to treatment and exposed to either 10 μM Hyd or 10 μM 5′Aza up to 5 d. After treatment the cells were washed with PBS and fixed in 4% paraformaldehyde. The fixed cells were permeabilized in 100% methanol and stained with Eosin and Haemotoxylin. Coverslips were viewed on a Zeiss Axioplan microscope. The percentage of cells which altered morphological appearance was obtained by counting a minimum of 100 total cells from 3 separate experiments, cells were characterised as altered when they demonstrated a larger cell body, bi or multinucleation and expression of Troma-1/ cytokeratin-8 staining.

Immunocytochemistry

Cells were washed and fixed with 4% paraformaldehyde for 15 min and permeabilized/ blocked in 5% normal goat serum, 0.1% triton-x 100 in PBS. Monoclonal antibodies against Troma-1/cytokeratin-8 (Developmental Studies Hybridoma Bank, The University of Iowa, Ames, IO) at 1:25 dilution, rabbit antibody against Oct4 (ab19857, Abcam, Cambridge, MA) at 1:100 dilution, a mouse monoclonal antibody against β-III tubulin clone SDL.3D10 (Sigma), and a monoclonal Tead4 antibody at 1:100 (H00007004-M01, Abnova) were obtained. Coverslips were incubated with primary antibody overnight at 4°C, washed in PBS, and incubated with secondary antibody (rabbit anti-mouse IgG labelled with FITC or goat anti-rabbit IgG labelled with rhodamine) at a 1:200 dilution for 2 h at room temperature. The coverslips were washed three times and mounted with Prolong gold with DAPI (Invitrogen, Carlsbad, CA). The slides were viewed on the Zeiss axioplan microscope.

Western blotting

Cells were scraped and lysates were prepared in 60 μl of RIPA (PBS, 1% Igepal, 0.5% w/v Deoxycholic acid, 0.1% SDS) buffer with protease inhibitor cocktail set I and phosphatase inhibitor cocktail set II (Calbiochem, La Jolla, CA). Pellets were sonicated to complete lysis. Protein concentration was determined by Bradford assay using Bovine Serum Albumin (BSA) as the standard. Cell lysates were boiled for 4 min in sample buffer containing 0.31 M Tris-HCl pH 6.8, 1% SDS, 5% glycerol, 3.6 M β-mercaptoethanol, 5 mM PMSF, and 0.05% bromophenol blue. 25 μg of protein were separated on 4-20% gradient gels (Cambrex, East, Rutherford, NJ) at 120 V. The proteins were transferred to nitrocellulose membranes at 100 V for 90 min. Nitrocellulose membranes were blocked with Odyssey blocking buffer (Licor) and incubated with appropriate primary antibody overnight.

Secondary antibody (Licor) was diluted 1:10000 in 1:1 Odyssey buffer: PBS and 0.1% Tween and incubated with membrane for 1 h. The level of antibody binding was measured on the Licor Odyssey system and normalized to β–actin.

Transfections

A Tead4 mammalian expression plasmid was obtained from Open Biosystems (MHS1010-58160). This plasmid expresses the full length TEAD cDNA in a pCMV-Sport6 plasmid. The plasmid was transfected at a concentration of 200 ng/cm^2 with Lipofectamine 2000. An empty pCMV-sport6 plasmid was used as a negative control and was transfected at the same concentration. The TET expression vector expressing the catalytic domain of TET was a gift from the laboratory of Dr. Hongjun Song [25]. siRNA transfections were performed also using Lipofectamine 2000 (Invitrogen). For Tead4 knockdown, siRNA Silencer Select Pre-silencer siRNA s74939 was transfected at a concentration of 5 nM (Ambion). Control siRNA #2 was also used at the same concentration to control for nonspecific effects (Ambien).

RT-PCR

RNA was isolated using Tirol reagent (Invitrogen) according to the manufacturer's instructions. 5 μg of RNA was converted to cDNA using the superscript II kit (Invitrogen). Rt-PCR was performed with Platinum Taw (Invitrogen), 1.5 mM MgCl2, 200 nM dots, 200 nM each primer, 1x PCR buffer.

The primer sequences used were:

*TEAD*4	F-5′-CAACCTGGAACATCCCACGAT-3′
	R-5′-GAAAGCCGAGAACTCCAACAT-3′
Troma1	F-5′-ACCGCCTGCAGGAGATTGAAGCC-3′
	R-5′-GCCATCTCCCCACGCTGCTCA-3′
CDX2	F-5′-AGACAAATACCGGGTGGTGTA-3′
	R-5′-CCAGCTCACTTTTCCTCCTGA-3′
HAND1	F-5′-CATCATCACCACTCACACCC-3′
	R-5′-GACCTGGGCTCTGACTCGG-3′
EOMES	F-5′- GGCAAAGCGGACAATAACAT-3′
	R-5′- GAGACAGGCGCTTTCTCTTG-3′

Methylation Specific PCR

PCR for methylated and methylated B1 repetitive elements was carried out as previously described [26]. Briefly DNA was extracted from treated P19 cells using the Gene lute Mammalian DNA extraction kit (Sigma) and quantified. 2 μg of DNA was bisulphite modified by incubation overnight at 50 ᶜo. with 0.5 mM hydroquinone and 2.5 M Sodium bisulfide. The bisulphite modified DNA was isolated using the Wizard DNA clean-up system (PR omega, Madison, WI) and stored at −20 ᶜo. until use. PCR amplification was performed in a 25 μl reaction mixture containing Platinum Taq with 0.1 μM of each primer.

Primer sequences;

B1 unmethylated F 5′-TAACCTCAAACTCAAAAATCCACC-3′

B1 unmethylated R 5′-GTTGGGTGTAGTGGTATATATTTTTAATTTTA-3′

B1 methylated F 5′-CTCGAACTCAAAAATCCGCC-3′

B1 methylated R 5′-GTCGGGCGTAGTGGTATATATTTTT-3′.

PCR products were resolved on a 1.5% agarose gel and visualized under UV light.

Pyrosequencing

One microgram of DNA was bisulfite converted using the EpiTect Bisulfite kit (Qiagen). Quantitative DNA methylation analysis was conducted by pyrosequencing a 31 base pair sequence using 15 ng of bisulphite treated DNA. Methylation of three CpG's was measured using the following sequencing primer: GGGGAGGGCTGGCGAGGGAAAGCGGTGGCGG. PCR was carried out for 45 cycles of 45 seconds at 94°C, 45 seconds at 55°C, and 45 seconds at 72°C, with a final extension step at 72°C for 1 minute using the mouse Tead4 Pyromark assay (Qiagen).

DNA Dot Blot

P19 cells were exposed to Hyd for up to 8 hrs and DNA was immediately isolated using the GenElute mammalian DNA genomic DNA miniprep kit (Sigma). The DNA was denatured with NaOH and heat and a vacuum manifold was used to immobilize the DNA on nitrocellulose paper. DNA was fixed to the membrane by vacuum baking at 80°C. The membrane was then blocked and probed with antibodies against 5 hydroxymethylcytosine (Active Motif) and single stranded DNA (Abcam) for normalization. Binding was visualized using the Licor Odyssey.

In vivo treatment of mice with Hyd

Female Swiss Webster mice (Charles River) at 6 weeks of age were treated with 200 μg/L Hyd in the drinking water for one week and mated overnight. At day 4.5 p.c., pregnant mice were anesthetized and given an intercardiac injection of 0.1% solution of Chicago blue to stain implantation sites. After 30 seconds, uteri were removed, cleaned and weighed and homogenised for Western blotting.

RESULTS

Hyd decreases DNA methylation and cell morphology

Hyd has been reported to decrease DNA methylation in some cell lines but not others [27]. The Methylation specific-PCR assay was used to qualitatively assess the effects of Hyd on P19 cells by comparing methylated and unmethylated forms of the B1 repetitive element. In controls and cultures treated with Hyd for 15 minutes, very little unmethylated B1 sequence was detected compared to the level of methylated sequence. After 60 minutes of treatment,

the levels of unmethylated and methylated B1 sequence were similar. Even after 4 hours, the level of unmethylated B1 sequence was elevated (Figure 1A).

We also made a qualitative assessment of differentiation by examining morphological changes in cells treated with Hyd. P19 cells have the potential of differentiating to neurons and muscle cells, both cell types display distinct and well characterized morphologies. Morphological changes were observed in a subpopulation of P19 cells treated with Hyd but the changes were not reminiscent of neurons or muscle. These cells were larger than untreated cells and multi nucleated (Figure 1B). The morphology of the cells was similar to reports describing induction in differentiation of mouse embryonic stem cells to trophoblasts by knocking out Oct4 expression [28, 29]. We found that 11±3% of cells underwent the morphological transition characterised by a larger cell body, flattened appearance and multinucleation. In the absence of Hyd, cells displaying the morphological transition were not observed. Similarly, the morphological changes were not observed with the DNA methyltransferase 1inhibitior 5′azacytidine. It is possible that the differences between 5′ azacytidine and Hyd were due to the mechanisms by which these two chemicals decrease methylation or that effects on morphology were unrelated to decreases in DNA methylation.

Hyd treatment increases Tead4 expression and decreases methylation at the TEAD4 promoter

We further investigated the effects of Hyd in inducing trophoblast-like properties. Tead4 is one of the earliest transcription factors to appear during embryogenesis and was proposed to initiate the differentiation of trophoblasts [30]. An increase in Tead4 mRNA levels was observed at one hour and 24 hours after treatment with Hyd (figure 2A). The levels returned to basal levels within 72 hr of treatment. An increase in levels of Tead4 protein was observed at 72 hr after treatment figure 2B). Because Hyd decreases methylation at the B1 repeated sequence, we investigated whether it decreased methylation specifically at the TEAD4 promoter. Three CpG sites were identified in the TEAD4 promoter as possible sites of demethylation and were assessed by pyrosequencing (figure 3). A significant decrease was observed in the level of methylation at site 2 at one hour and decreases were observed at the other sites at 2 hours after treatment with Hyd. Levels of methylation remained lower than control at even 8 hours. Similarly, a 24 hr treatment with 5′azacytidine resulted in decreases in methylation of all three CpGs in the TEAD4 promoter.

Hyd modifies expression of trophoblast intermediate Troma-1/cytokeratin-8 levels and transcription factors—The intermediary filament Troma-1/cytokeratin-8 is commonly used as a marker to study trophoblasts [31]. Indeed, the expression of Troma-1/cytokeratin-8 was detected by immunocytochemistry in P19 cells treated with Hyd (figure 4A). Interestingly, only cells displaying morphological alterations including increased size and multinucleation expressed Troma-1/cytokeratin-8. In contrast, cells that did not undergo the morphological transformation expressed Oct4 but not Troma1/cytokeratin-8. Increased levels of Troma-1/cytokeratin-8 were confirmed on Western blots (figure 4B). Interestingly, lower levels of Oct4 and higher levels of Troma-1/cytokeratin-8 were observed in homogenates prepared from early embryo implantation sites isolated from dams given Hyd before coitus and 3 days post coitus (supplementary figure 1).

Other trophoblast transcription factors were expressed but their responsiveness to Hyd varied. For example, increased expression of Cdx2 was observed but the expression of Eomes was initially very high and at three days after Hyd treatment. Very little expression of Hand1 was detected in naïve cells or at 3 days after Hyd treatment. In contrast, increases in levels of Hand1, Eomes, and Cdx2 occur during differentiation of mouse embryonic stem cell lines to trophoblasts induced by knocking down Oct4 [32] or by inducing with gelatin [33]. These differences could reflect differences in the types of cells in the trophectoderm lineage being formed [34] or differences due to the stimulus and/or the cell line.

Tead4 modifies expression of Troma-1/cytokeratin-8

The influence of Tead4 on Troma-1/cytokeratin-8 expression was examined by knocking down *Tead4* and expressing high levels of Tead4. We were able to reduce the level of *Tead4* mRNA at 24 hr after transfecting cells with a Tead4 siRNA (figure 5 C). Interesting, Hyd failed to induce expression of Troma-1/cytokeratin-8 in P19 cells expressing lower levels of Tead4 (Figure 5A) although increases were observed in cells transfected with control siRNA. Cell transfected with the Tead4 expression vector displayed an increase in Troma-1/cytokeratin-8 expression (figure 5B).

Hyd treated cells lose their multipotent potential and no longer differentiate to neural cells

The decreases in expression of Oct4 suggested that Hyd treatment blocks P19 cells' multipotency. To examine the effect of Hyd on neuronal differentiation, P19 cells were pre-treated with 10 μM Hyd for 3 d and then treated with retinoic acid for 3 d (without Hyd). Cells pre-treated with Hyd failed to form the spherical embryoid body structures that normally form after treatment with retinoic acid (not shown). When the Hyd pre-treated cells were re-plated on poly-lysine coated plates, neurites were not observed (figure 6A). Cells Hyd pre-treatment responded to retinoic acid and displayed outgrowths characterized by beta-III tubulin staining.

The failure to respond to retinoic acid even when Hyd was withdrawn suggests that the effects of Hyd on multipotency were irreversible. Oct4 levels began to decline at 72 hours after Hyd was added to P19 cell monolayers and remained below basal levels at 120 hours after treatment (Figure 6B). In order to investigate the reversibility of the loss of Oct4, cells were treated with Hyd for 4 days, passaged and then allowed to grow for a further 4 days without Hyd. We also found that levels of Oct4 in Hyd treated cells did not increase even after Hyd was withdrawn. Cells treated with Hyd for 4 d and replated in fresh media without Hyd for 5 d displayed levels of Oct4 that were similar to cells treated with Hyd for 5 d (Figure 6C).

DISCUSSION

Here we demonstrate that Hyd induces the P19 embryonic carcinoma cell line to display a trophoblast-like phenotype. In embryogenesis, the development of trophoblasts is the first identifiable differentiation event occurring at the blastocyst and will give rise to the few differentiated cell types in the foetal side of the placenta. The trophoblast-like properties expressed by P19 cells include increases in the trophoblast transcription factor Tead4 and the

expression of the intermediary filament Troma-1/cytokeratin-8. The large size and multinucleation displayed by cells positive for Troma-1/cytokeratin-8 are also features resembling trophoblasts [35, 36]. Cells expressing Troma-1/cytokeratin-8 no longer expressed Oct4 and total levels of Oct4 were much lower. Indeed, the expression of the trophoblast-like phenotype was associated with the loss in potential to differentiate to neurons and possibly other cell types. Similarly, a previous study reported the induction of trophoblast giant cells by P19 cells ectopically expressing an Oct4 dominant negative though the expression of biochemical features specific to trophoblast-like cells were not studied [37].

The expression of Troma-1/cytokeratin-8 was modified by Tead4. Knocking down Tead4 by siRNA reduced expression and over expressing *Tead4* increased expression. *Tead4* belongs to the family of TEAD/TEF-1 (transcriptional enhancer factor-1) transcription factors, a four member family, that bind to the same DNA consensus sequence [38]. To our knowledge Tead4 is the earliest transcription factor activated after fertilization [35]. Other investigators also observed a relation between Tead4 and the induction to a trophoblast lineage. Mouse embryonic stem cells from Tead4 −/− mice failed to differentiate to trophoblast stem cells or express the early Cdx2 transcription factor [30]. Indeed, Tead4 is required for the expression of two parallel trophoblast signalling pathways transcription, one is regulated by Cdx2 [35] [30] and the other is regulated by Gata3 [39]. A model has been developed suggesting that Tead4 triggers a critical event early in the establishment of trophoectoderm, sometime during the 8-cell to morula transition, but does not regulate expression of all genes involved in formation of trophectoderm [30].

One possible mechanism for the increases in Tead4 expression is through demethylation. Three CpG sites within the TEAD4 promoter displayed less methylation in cells treated with Hyd for 2 hours. A decrease in methylation could be due to a passive or active mechanism. Passive mechanisms would be caused by a decrease in the activity of DNA methyltransferases, which methylate during DNA synthesis and DNA repair (Leonhardt, Page et al. 1992) (Mortusewicz, Schermelleh et al. 2005). A passive mechanism, however, would be less likely because very few cells are going through S phase in 1-2 hours of Hyd treatment. An active mechanism might involve hydroxymethylation by the TET followed by deamination and base excision repair pathway. An increase in levels of 5-hydroxymethylcytosine (supplementary data figure 3) were not observed though it might be possible that Hyd had direct interactions with methylcytosine [40] or provoking a signalling pathway and downstream events not yet identified. Hyd was suggested to decrease DNA methylation by inhibiting the ERK pathway in T cells [14]. Hyd is also known to activate hypoxia inducible factor [41] and disrupt calcium signalling [16].

In summary, Hyd induces DNA demethylation and expression of trophoblast-like properties in P19 cells. Similarly, Hyd was shown to induce demethylation in a number of other cell types though it is not active in all cell types [27]. By understanding the mechanism by which Hyd induces DNA demethylation, we could better predict the types of tumors that would be responsive to Hyd and its potential role in epigenetic modulation of disease states.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

Hyd	hydralazine

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Highlights

- Hydralazine decreases DNA methylation in P19 cells
- Hydralazine induces P19 cells to express trophoblast-like properties
- Tead 4 is required to express trophoblast-like properties

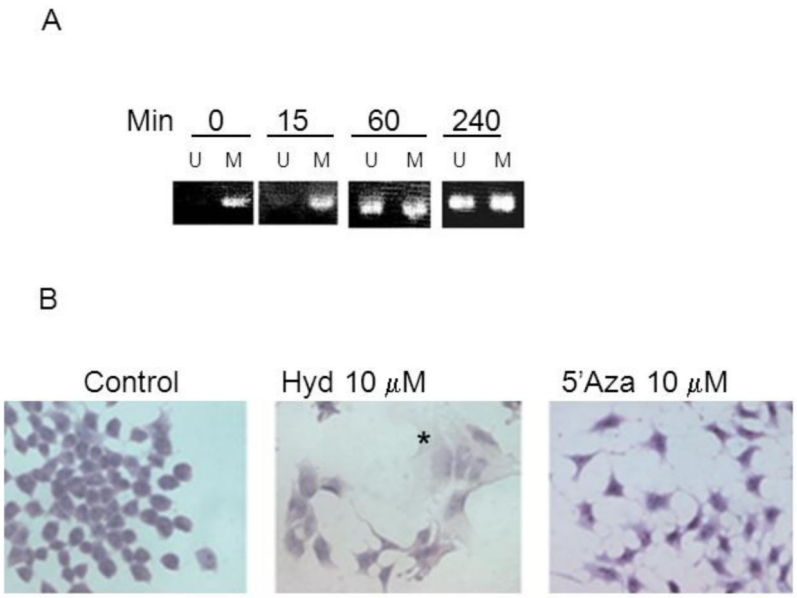

Figure 1. Hyd treatment modifies morphology and increases unmethylated B1 sequence in P19 cells

Methylation specific PCR was performed on DNA isolated from untreated P19 cells (0) or treated with Hyd for 15, 60, 240 minutes. PCRs were performed for both methylated (M) and unmethylated (U) forms of the B1 repetitive element. PCR product was resolved on a 1.5% agarose gel and visualized under UV light. (A). Cultures treated 5 days with 10 μM Hyd or 10 μM 5′azacytidine were stained with Eosin and Haemotoxlin. * indicate larger multinucleated cells (B).

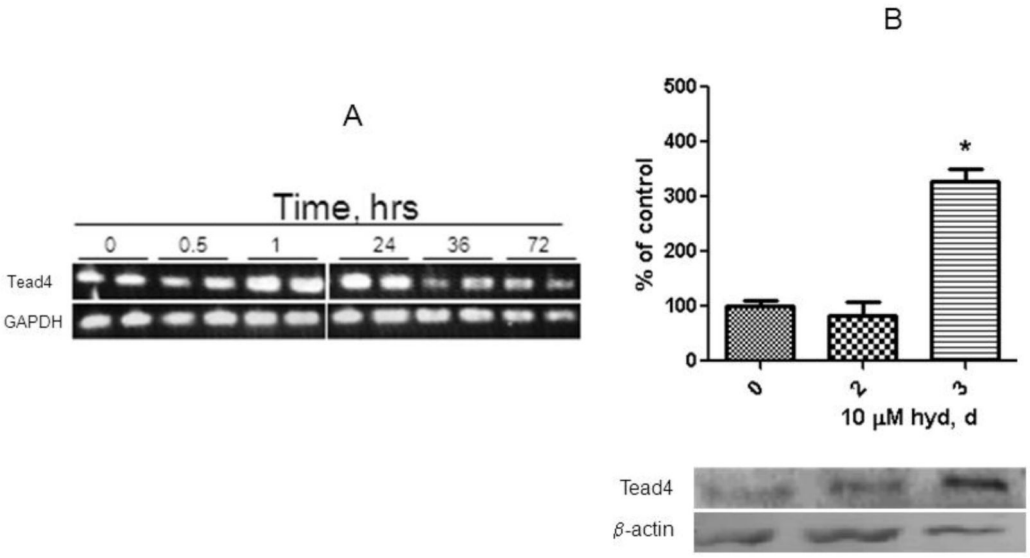

Figure 2. Hyd increases expression of Tead4

Tead4 mRNA was analysed by rt-PCR in P19 cells treated with Hyd for up to 3 d (A) and protein levels were determined by Western blots (B). Results for the western blot are normalized to β-actin and are expressed as percentage of the control and presented as a mean of triplicate cultures ± S.E.M. Representative blots of single cultures are shown below the graphs. * $p<0.05$ using ANOVA with Dunnetts multiple comparisons post hoc test. A representative blot is shown below the graph.

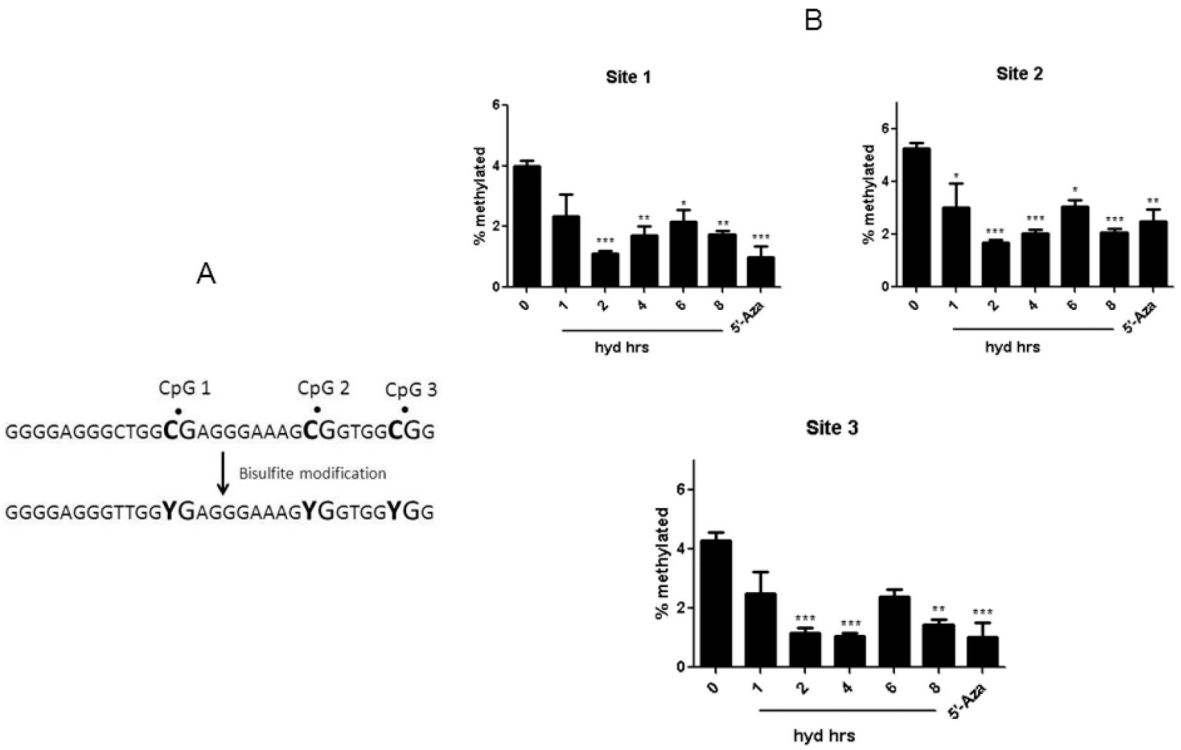

Figure 3. Hyd decreases DNA methylation at the TEAD4 promoter

Pyrosequencing was used to analyse the methylation status of three cytosines in the Tead4 promoter. DNA isolated from untreated cultures, and cultures treated with Hyd for up to 8 hrs, 5′Azacytidine (5′Aza) was used as a positive control. The three sites of interest are illustrated in A. The data for each site (B) is expressed as the percentage of methylation and presented as a mean ± S.E.M. * $p<0.05$ and ** $p<0.01$, *** $p<0.001$ using ANOVA with Dunnett's multiple comparisons post hoc test.

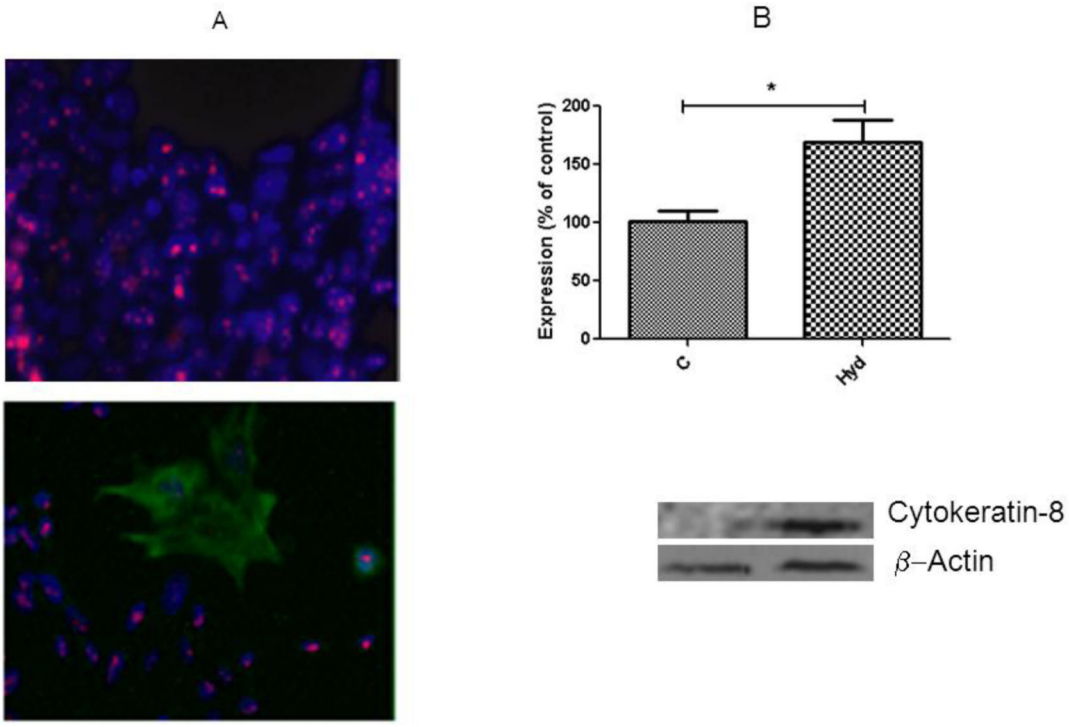

Figure 4. Hyd increases expression of Troma-1/cytokeratin-8 in P19 cells

P19 cells cultured for 5 d in the presence or absence of 10 uM Hyd and stained with a 1:25 dilution of antibody against Troma-1/cytokeratin 8 (green) and a 1:100 Oct-4 (red) diluted antibody and then incubated with DAPI to stain nuclei blue (A). Western blots were conducted on cultures treated with 10 μM Hyd for 5 d. The intensities of the bands in the blots were measured and normalized to actin. The data are expressed as a percentage of the control (absence of Hyd) and presented as a mean of 3 separate experiments± S.E.M. Representative blots are shown below the graphs. * $p<0.05$ using ANOVA with Dunnett's multiple comparisons post hoc test.

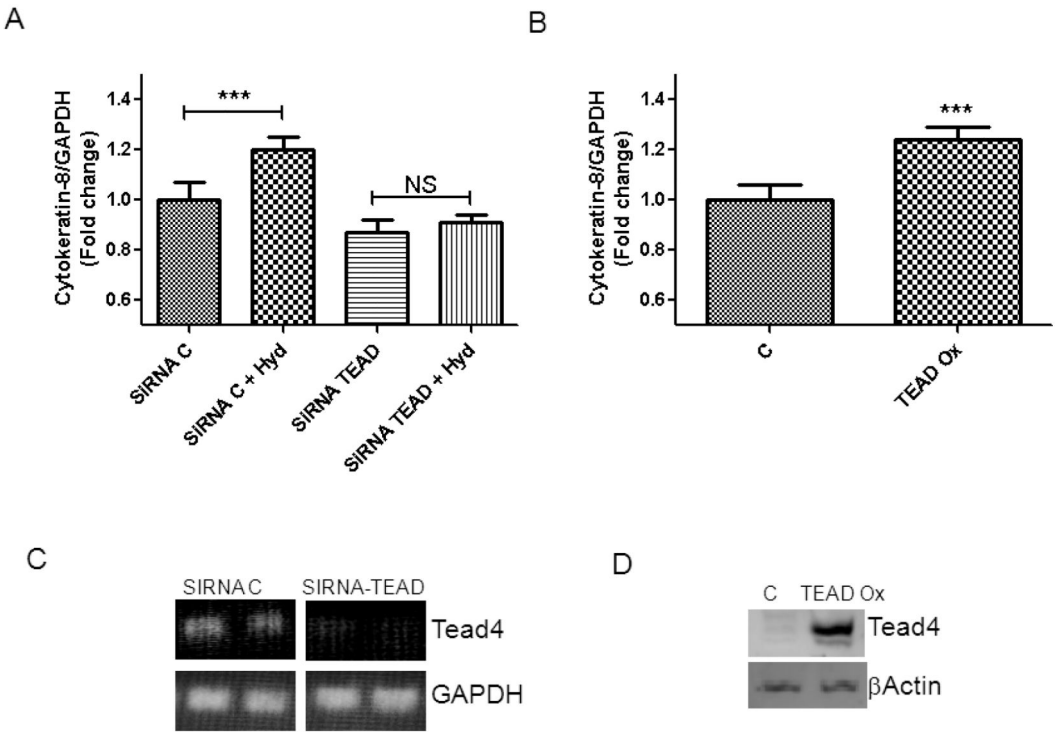

Figure 5. Involvement of Tead4 in expression of Troma-1/cytokeratin 8

Troma-1/cytokeratin 8 and GAPDH mRNA were measured by real time-PCR in RNA isolated from P19 cells treated with Hyd for 24 hrs after transfection with either siRNA scramble (siRNA C) or siRNA Tead4 (A). A representative rt-PCR of the knockdown with Tead4 siRNA is also shown (C). Troma-1/cytokeratin 8 and GAPDH mRNA was also measured by real time-PCR in RNA isolated from P19 cells transfected expressing the CMV-driven Tead4 expression vector (B). Below the graph is a western blot of P19 cells transfected with the empty pCMV-sport6 plasmid or pCMV-sport6 TEAD4 (D)*** $p<0.001$ using ANOVA with Dunnett's multiple comparisons post hoc test.

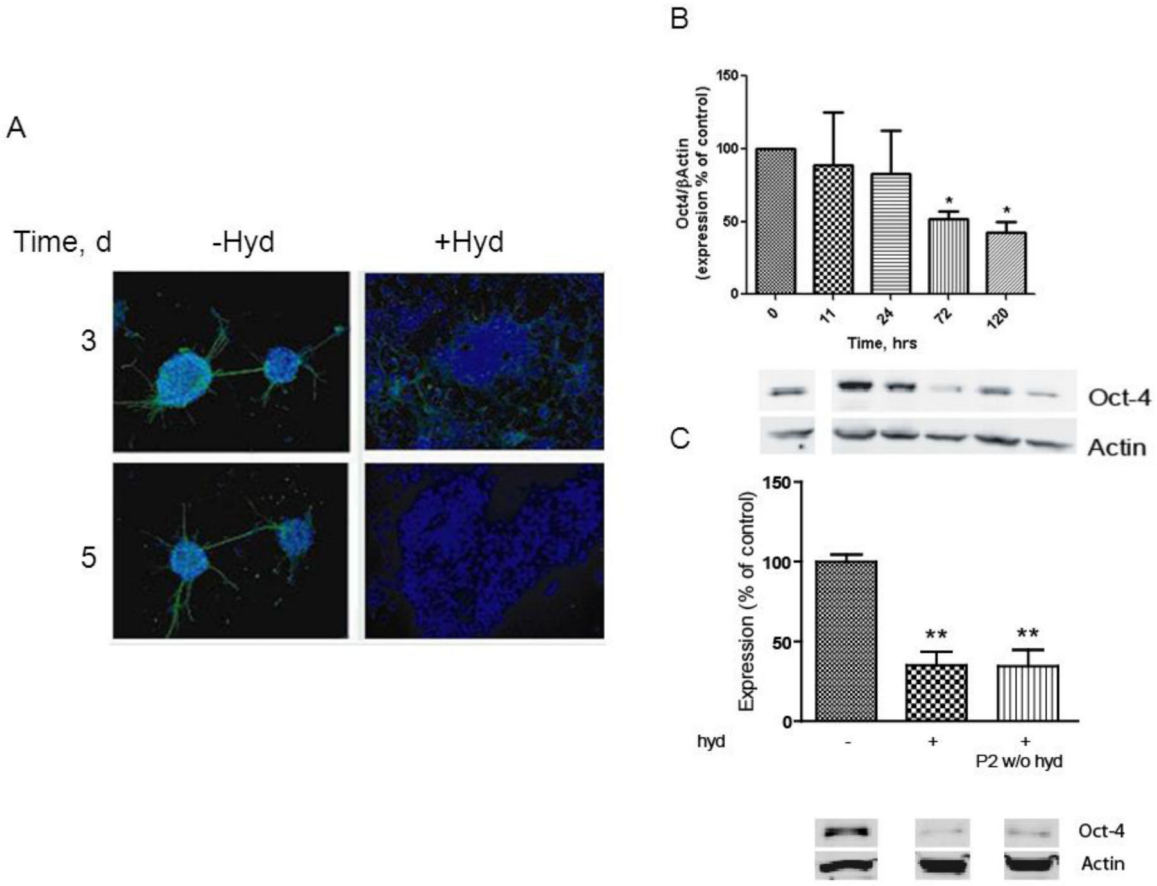

Figure 6. Hyd decreases the multipotency of P19 cells

P19 cells were exposed to retinoic acid to initiate neuronal differentiation following a 5 day treatment with 10 μM Hyd or an equivalent volume of PBS. Cells were allowed to differentiate for either 3 or 5 d and stained with mouse monoclonal antibody against β-IIII tubulin and a FITC labelled secondary and mounted in Prolong with DAPI (A). Levels of Oct4 were measured by Western blots in P19 cells treated for up to 120 hrs with 10 μM Hyd (B). Levels of Oct4 were also measured in P19 cells treated for 4 days with Hyd (+), or 4 days with Hyd followed by 5 days without Hyd (+/−), or without Hyd (−) (C). The intensities of the bands in the blots were measured and normalized to actin. The data are expressed as percentage of control and presented as a mean of triplicate experiments ± S.E.M. Representative blots are shown below the graphs. * $p<0.05$ and ** $p<0.01$, using ANOVA with Dunnetts multiple comparisons post hoc test.