Biochemical and Biophysical Research Communications 431 (2013) 225–231

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Heterochromatin protein 1γ overexpression in P19 embryonal carcinoma cells elicits spontaneous differentiation into the three germ layers

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ARTICLE INFO

Article history:
Received 27 December 2012
Available online 9 January 2013

Keywords:
HP1γ
Embryonal carcinoma cells
Pluripotency
Differentiation
Chromatin

ABSTRACT

P19 embryonal carcinoma (EC) cells are pluripotent stem cells and have numerous morphological and biochemical properties in common with embryonic stem (ES) cells. However, P19 cells differentiate very ineffectively as embryoid bodies (EBs) without the specific chemical inducers whereas ES cells exhibit spontaneous differentiation to the three germ layers. Recently the heterochromatin protein 1 (HP1) family protein HP1γ, which is an epigenetic modulator that binds histone H3 methylated at lysine 9, is shown to be associated with the progression from pluripotent to differentiated status in ES cells. Therefore, to study the role of HP1γ in the differentiation capacity of P19 cells, we have established a HP1γ-overexpressing P19 cell line (HPlγ-P19). Similar to the parental P19 cells, undifferentiated HP1γ-P19 cells continued to express pluripotency marker genes. However, HP1γ-P19 cells exhibited significant morphological differentiation including beating cardiomyocytes, as well as Tuj1-positive neuronal cells and Sox17-positive endodermal cells after EB formation under a normal culture condition. Moreover, real-time RT-qPCR analysis revealed that HP1γ-P19 EB cells expressed various differentiation marker genes. Thus, HP1γ-P19 cells could give rise to all three germ layers in EBs without any drug treatment. Therefore, HP1γ affects the spontaneous differentiation potential of P19 cells, and might play major roles in the decision of cell fates in pluripotent stem cells.

1. Introduction

Embryonic stem (ES) cells give rise to differentiated cells of the three germ layers [1]. The progression from pluripotent to differentiated status in ES cells is correlated with chromatin condensation [2,3]. Mouse ES cells have an open chromatin structure, which is accessible to a globally permissive transcription state, and accumulate regions of more rigid heterochromatin after differentiation. The formation of heterochromatin requires methylation of histone H3 at lysine 9 (H3K9me3), which increases with the differentiation of ES cells and the subsequent recruitment of chromodomain proteins such as heterochromatin protein 1 (HP1) [4,5]. Mammals have three HP1 proteins, namely HP1α, HP1β, and HP1γ [6]. Among the three HP1 proteins, HP1γ localizes to both heterochromatin and euchromatin for repression of gene transcription [7]. However, mammalian HP1γ and H3K9me3 have been found to play an unexpected role in the transcriptional activation of direct target genes [8,9]. Moreover, localization of HP1γ proteins is dramatically changed during differentiation, which is indicated by the transition of chromatin structure during ES cell differentiation [10,11]. The complexities of HP1γ function and localization may provide flexibility for the balance between the pluripotent state and differentiation.

P19 embryonal carcinoma (EC) cells were derived from a teratocarcinoma formed by transplantation of E7.5 embryos into the testis [12]. In an undifferentiated state, P19 cells exhibit typical compact colonies, strong activity of alkaline phosphatase (ALP), and express the pluripotency related gene Oct4. However, P19 cells do not show Rex1 or Nanog expression [13,14], indicating that P19 cells exhibit the properties of epiblast-like stem cells or primitive ectodermal cells of post-implanted embryos [15]. In contrast to ES cells, the self-renewal activity of P19 cells is sustained in the presence of serum without leukemia inhibitory factor (LIF) and/or feeder cells. P19 cells are pluripotent stem cells like ES cells but they differentiate very inefficiently as embryoid bodies (EBs) under a normal culture condition with serum [16], whereas ES cells exhibit spontaneous differentiation to the three germ layers. However, differentiation of P19 cells can be effectively induced when EBs are exposed to non-toxic concentrations of numerous drugs [17]. RA induces P19 cells to differentiate into neuronal cells, and DMSO induces the differentiation of beating cardiomyocytes. Although P19 cells show a differentiation capacity for various cell types, the induced differentiation is limited to a restricted direction

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0006-291X/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2012.12.128

corresponding to the specific chemical inducer. The difference between P19 and ES cells in terms of their pluripotency is not well documented. To investigate the functional role of heterochromatin factor HP1γ in the cellular differentiation and reprogramming of P19 cells, we have established P19 cells overexpressing HP1γ (HP1γ-P19 cells) and analyzed the differentiation potential of HP1γ-P19 cells.

2. Materials and methods

2.1. Cell culture

P19 cells and their derivatives were cultured as monolayers as described previously [14]. ALP staining was carried out using NAPHTHOL AS-BI PHOSOHATE and FAST RED VIOLET LB SALT solutions (Sigma, CA, USA).

2.2. Establishment of HP1γ-overexpressing cells

To construct an overexpression vector, full-length mouse HP1γ cDNA was amplified by polymerase chain reaction (PCR) and subcloned into a pCAG-IP (IRES Puro) vector. Transfections were performed using Lipofectamine LTX (Life Technologies, CA, USA). DNA constructions and transfections detailed in Supplementary Materials and methods.

2.3. Induction of P19 cell differentiation

P19 or HP1γ-P19 cells were used to generate EBs for differentiation by placing 500 cells in a 20 μl drop of basic medium [12]. After 2 days, the formed aggregates were re-plated into floating suspension culture on Petri dishes for another 2 days, and then transferred onto gelatin-coated dishes at day 4 of differentiation.

2.4. Western blot analysis

SDS–PAGE and western blotting were performed as previously described [14] and detailed in Supplementary Materials and methods.

2.5. Reverse transcriptase-quantitative PCR (RT-qPCR)

RT-qPCR detailed in Supplementary Materials and methods. All data were obtained from triplicate experiments.

2.6. Flow cytometry (FCM) analysis

P19 and HP1γ-P19 cells from EBs or monolayer cultures were dissociated and subjected to FCM analysis using FACSCantoII flow cytometer (Becton–Dickinson, NJ, USA), which detailed in Supplementary Materials and methods.

2.7. Immunostaining

Fixed cells were incubated with primary antibodies, followed by AlexaFluor conjugated secondary antibodies. Samples were analyzed under a Leica TCS SP2 confocal microscope (Leica, Wetzlar, Germany) or ECLIPSE Ti fluorescence microscope (Nikon, Tokyo, Japan). Detailed protocols were present in Supplementary Materials and methods.

3. Results

3.1. Establishment of HP1γ-overexpressing P19 cells

To study the role of HP1γ in the differentiation capacity of P19 cells, we established P19 cell lines overexpressing HP1γ that was tagged with the influenza hemagglutinin (HA) epitope at the C-terminus. The expression of HP1γ protein was analyzed by western blotting with anti-HA anitibody (Fig. 1A). Among six HA-positive cell lines, cell line #4 of transfected P19 cells showed the highest level of exogenous HP1γ protein. Therefore, we mainly used cell line #4 (HP1γ-P19) in this study because other cell lines gave the essentially same results. The morphology of HP1γ-P19 cells was quite similar to that of P19 cells with a typical large nucleoli and scant cytoplasm (Fig. 1B). The expression of exogenous HP1γ protein in HP1γ-P19 cells was also confirmed by immunostaining with the anti-HA antibody (Fig. 1C). All HP1γ-P19 cells had both HA-tagged and endogenous HP1γ in their nuclei at almost the same intensity.

During differentiation, endogenous HP1γ protein continued to be expressed in wild-type (WT) and HP1γ-P19 cells. Moreover, exogenous HP1γ was expressed in differentiating HP1γ-P19 cells

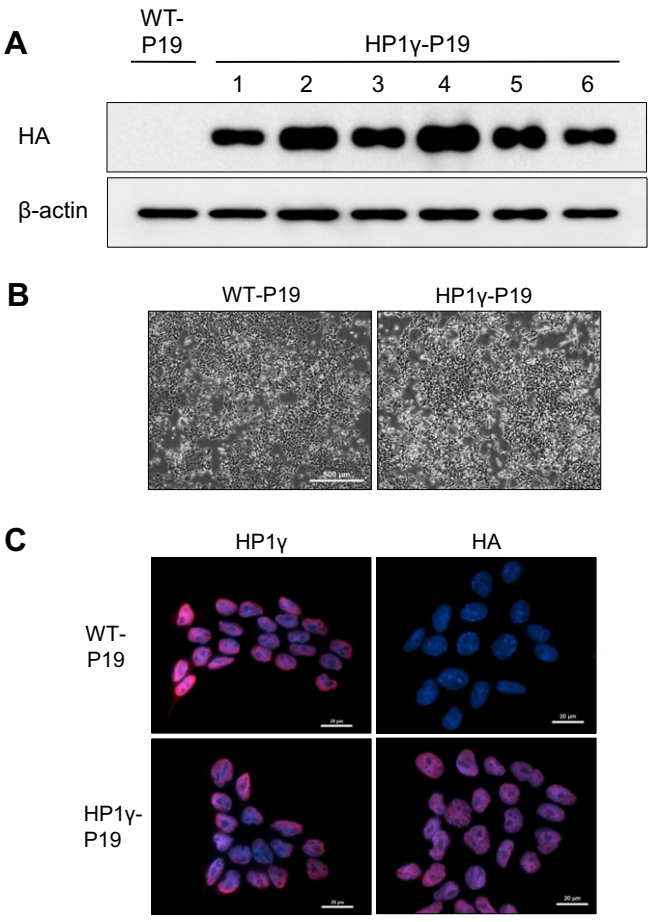

Fig. 1. Establishment of HP1γ-overexpressing P19 EC cells. (A) Exogenous HP1γ overexpression was confirmed by western blotting of six different P19 cell lines using an anti-HA antibody. β-actin was used as a loading control. (B) The morphology of wild-type (WT-) and HP1γ-P19 cells in normal monolayer culture. Scale bar: 500 μm. (C) Immunofluorescence analysis of wild-type (WT-) and HP1γ-P19 cells using anti-HP1γ and -HA antibodies showed the expression of endogenous and exogenous HP1γ under a normal culture condition. The expression of introduced HP1γ was determined by the presence of the HA epitope, which was tagged at the C-terminus. Cells were counterstained with DAPI (blue). Scale bars: 20 μm.

at a similar level as that in undifferentiated cells as shown by western blotting and immunostaining of the HA-tag (Supplementary Fig. S1). Therefore, the expression level of HP1γ was roughly 2–3-fold higher in HP1γ-P19 cells. Thus, HP1γ-P19 cells maintained continuous expression of HP1γ during differentiation. In addition, there was no difference in the expression of HP1α and HP1β proteins between WT- and HP1γ-P19 cells during differentiation (Supplementary Fig. S1A).

3.2. HP1γ-P19 cells maintain pluripotency in monolayer culture

We investigated whether HP1γ overexpression affects the pluripotency of P19 cells in an undifferentiated state. Although overexpression of HP1γ caused a decrease in growth rate, as shown by the formation of small colonies, the colonies of HP1γ-P19 cells were positive for ALP, and both WT- and HP1γ-P19 cells uniformly expressed Oct4 in an undifferentiated state (Fig. 2A, Supplementary Fig. S2). Maintenance of the undifferentiated state in HP1γ-P19 cells was also confirmed by RT-qPCR, which showed expression of Oct4 and the epiblastic cell maker FGF5 (Fig. 3). However, the expression levels of Oct4 and FGF5 were half of those in P19 cells, which was consistent with the weak ALP staining. In FCM analysis, E-cadherin-positive cells, which is expressed in epiblasts, were found to be around 80% of the HP1γ-P19 cell population (Fig. 2B). There was no significant difference between WT- and HP1γ-P19 cells in the expression profile of pluripotency genes, Oct4 and FGF5, and differentiation-related genes, Cdx2, Nestin, Brachyury, Gata4, Flk-1 and AFP, although the expression levels were varied (Fig. 3). Moreover, increased expression of HP1γ could not rescue the expression of pluripotency markers such as Rex1 and Nanog in P19 cells (data not shown). These results indicated that HP1γ overexpression did not affect the undifferentiated state of P19 cells, which maintained their epiblastic nature, and was not the reprogramming factor that directed P19 cells back to their inner cell mass state.

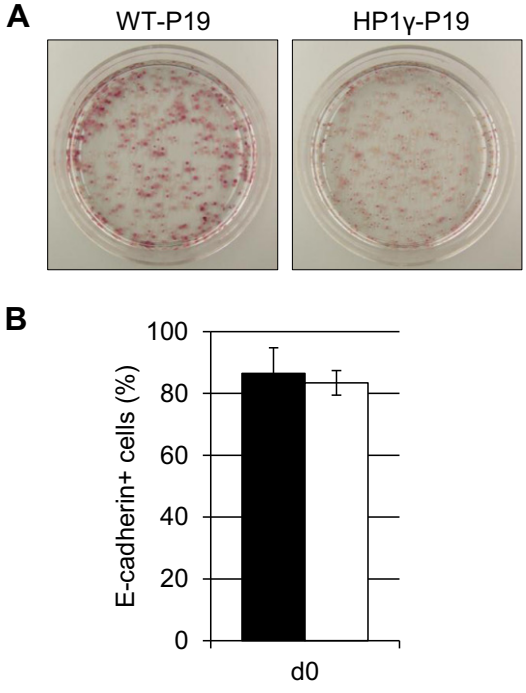

Fig. 2. Maintenance of pluripotency in HP1γ-P19 cells. (A) ALP activity of wild-type (WT-) and HP1γ-P19 cells. HP1γ-P19 cells showed slightly weak ALP activity. (B) The proportion of E-cadherin-positive cells among wild-type- (WT-; closed column) and HP1γ-(open column) P19 cells under a normal culture condition was measured by FCM using an anti-E-cadherin antibody. The proportion of E-cadherin positive cells is expressed as the mean + s.d. of three independent experiments.

3.3. HP1γ induces differentiation during EB formation

P19 EBs showed the morphology of densely packed colonies after an attachment to substrate, which mainly consisted of self-renewing stem cells (Fig. 4A). In contrast, EB formation of HP1γ-P19 cells led to the appearance of a variety of differentiated cells that spread outside of the densely packed colonies, including beating cardiomyocytes (Fig. 4A). Therefore, we compared the expression level of pluripotency and developmental genes between WT- and HP1γ-P19 cells during EB differentiation at different time points (Fig. 3). As suggested by their morphology, RT-qPCR analysis showed that P19 cells continuously expressed Oct4 and FGF5 after differentiation, although the level of expression was slightly reduced. The expression of Brachyury, the earliest mesendodermal marker gene, was highest at day 3 of differentiation in P19 cells. Nestin, a neuroectodermal maker, was constantly expressed in P19 cells before and after differentiation. However, induction of other differentiation-related genes was not detected during EB differentiation. These results suggest that P19 cells are not specified toward cell differentiation after EB formation.

In the differentiating EBs of HP1γ-P19 cells, HP1γ overexpression caused down-regulation of Oct4 and FGF5, and virtually no expression was observed after day 5 of differentiation (Fig. 3). Endodermal cells were clearly induced after EB formation of HP1γ-P19 cells, as shown by the dramatically increased expression (more than 100-fold) of differentiation marker genes Gata4 and AFP. While Brachyury and Nestin showed the same expressional profile as that in wild-type cells, expression of the cardiovascular progenitor marker Flk-1 was highly elevated after differentiation of HP1γ-P19 cells. In addition, expression of the trophectoderm marker gene Cdx2 was also induced to a peak at day 3 of differentiation. These results indicate that HP1γ-P19 cells can give rise to all three germ layers after EB formation.

3.4. Elevated HP1γ expression confers the potential for P19 cells to differentiate into the three germ layers

As mentioned above, HP1γ-P19 cells have the capacity to differentiate into all three germ layers. Outgrowths of EB cultures contained a variety of cell types that we analyzed more closely for differentiation markers by immunostaining (Fig. 4A). P19 cells can be induced to differentiate into neuronal cells by RA treatment. Therefore, we first examined neuronal marker genes. In HP1γ-P19 EBs without RA treatment, neuroectodermal differentiation was confirmed by Tuj1 immunoreactivity and positive cells showed several extended neurites (Fig. 4B). Outgrowing cells outside of the densely packed colonies had a characteristic endodermal morphology. However, only some cell clusters expressed the transcription factors Gata6 and/or Sox17 in loosely packed colonies (Fig. 4C). Far outgrowing cells were widespread and positive for the endodermal marker CK18 (Fig. 4C). Some cells in large densely packed colonies also exhibited marker gene expression of neural and endodermal cells, and few cells were positive for Oct4, suggesting that most cells from HP1γ-P19 EBs were differentiating (data not shown). These results indicate that HP1γ-P19 cells can differentiate into the endodermal lineage as well as ectodermal cells.

We further characterized the mesodermal differentiation of HP1γ-P19 cells partly because the EBs of HP1γ-P19 cells contained beating cardiomyocytes (data not shown). The presence of Flk-1-positive cells, which are the common progenitor cells for cardiovasculogenesis, were analyzed by FCM at days 3, 5, and 7 after EB formation (Supplementary Fig. S3A). In the EBs of HP1γ-P19

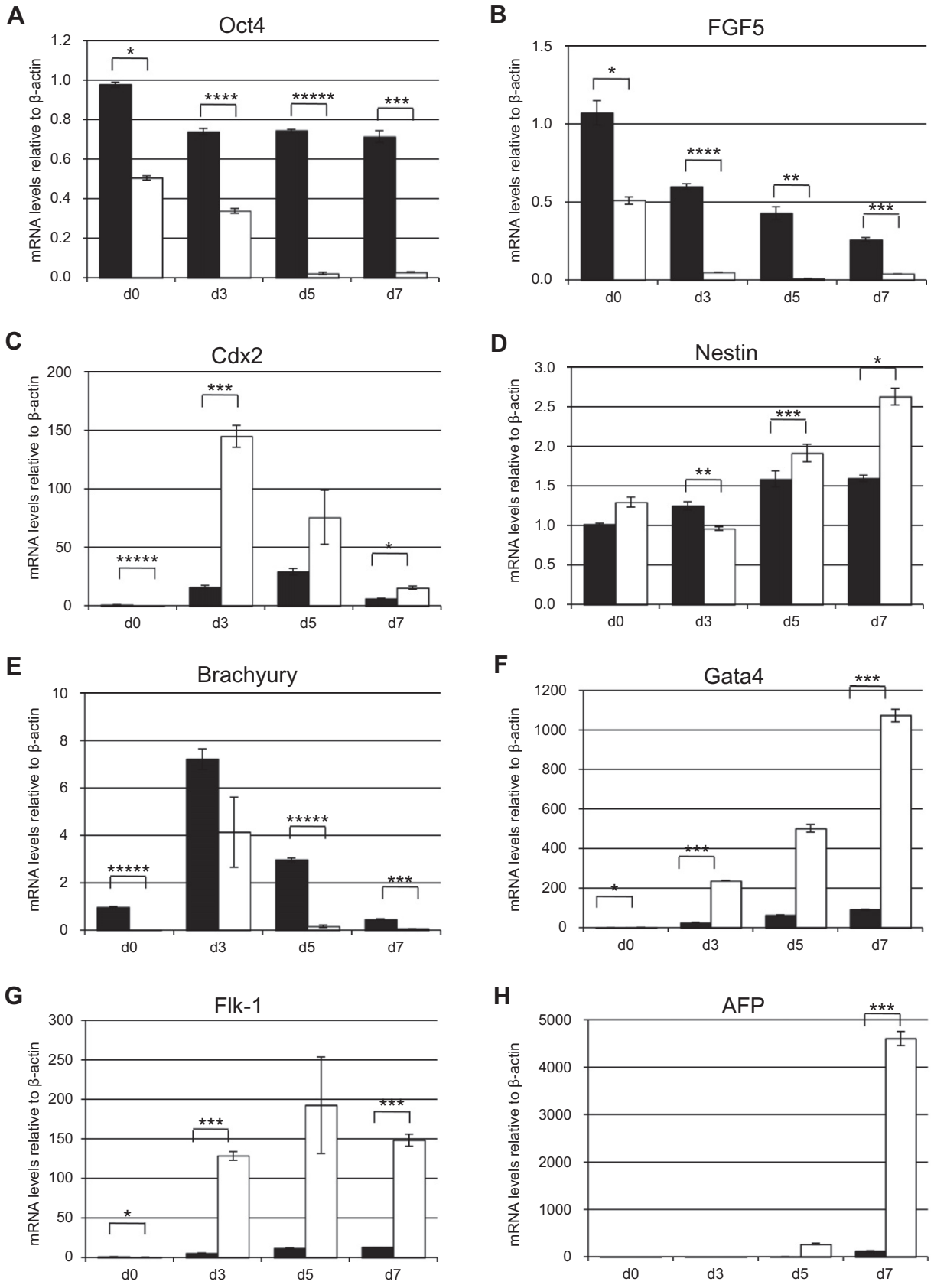

Fig. 3. HP1γ overexpression induces the differentiation of P19 cells into the three germ layers. Expression of pluripotency and lineage markers in wild-type- (WT-; closed column) and HP1γ-(open column) P19 cells in EBs at days 0 (d0), 3 (d3), 5 (d5) and 7 (d7) of differentiation was confirmed by RT-qPCR. Pluripotency markers, Oct4 (A) and FGF5 (B), trophectodermal marker, Cdx2 (C), ectodermal marker, Nestin (D), mesodermal markers, Brachyury (E) and Flk-1 (G), and endodermal markers, Gata4 (F) and AFP (H), were examined. mRNA levels were normalized to those of β-actin and expressed as the mean + s.d. of three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$, $^{****}p < 0.001$, and $^{*****}p < 0.0005$.

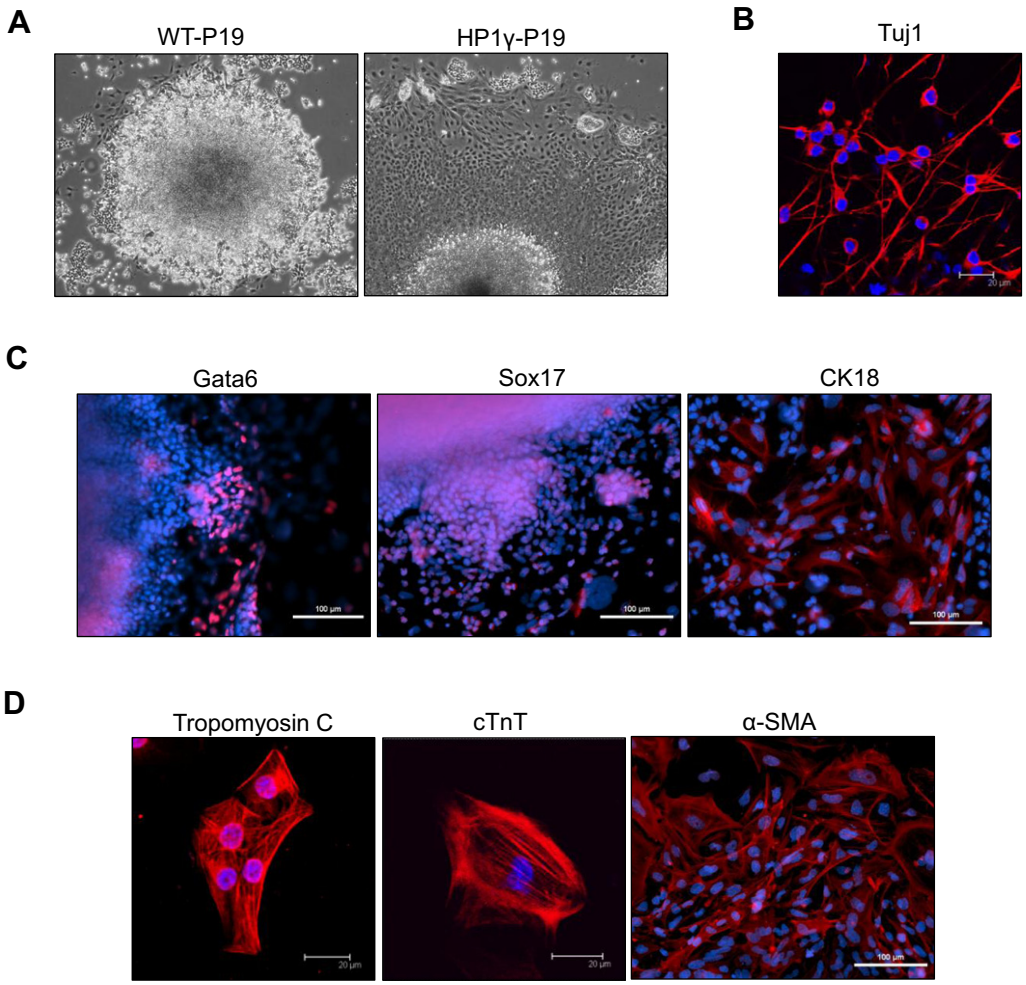

Fig. 4. Cell aggregates of HP1γ-P19 cells under a normal culture medium contain the three germ layers. (A) The morphology of wild-type (WT-) and HP1γ-P19 cells at day 7 after EB formation. Outgrowing cells showed the morphology of differentiating cells. (B) Immunofluorescence analysis of Tuj1 in HP1γ-P19 cells at day 10 after EB formation. Scale bars: 20 μm. (C) Immunofluorescence of Gata6, Sox17, and CK18 in HP1γ-P19 cells at day 6 (Gata6, and CK18) and day 7 (Sox17) during differentiation. Scale bars: 100 μm. (D) Beating cells were dissociated from HP1γ-P19 cell aggregates at day 12 of EB formation and immunostained for Tropomyosin C and cardiac Troponin T. Scale bars: 20 μm. Immunofluorescence analysis of α-SMA was directly performed on outgrowing cells from HP1γ-P19 EBs at day 12 of differentiation. Scale bars: 100 μm. Cells were counterstained with DAPI (blue).

cells, the population of Flk-1 positive cells increased by approximately 4.2-fold compared with those of P19 cells at day 3 of differentiation. Flk-1-positive cells maintained their higher proportion through to day 7 of differentiation. These results were consistent with the RT-qPCR analysis, demonstrating that HP1γ promoted the differentiation of P19 cells into cardiovascular mesodermal cells.

The cardiovascular progenitor cells developed into three types of cells, namely hematopoietic, endothelial and cardiac cells. Immunostaining of HP1γ-P19 cells revealed that the beating cells were positive for cardiac contractile proteins such as Tropomyosin C and cardiac Troponin T as well as smooth muscle cells expressing α-SMA (Fig. 4D). Moreover, EBs derived from HP1γ-P19 cells showed a higher prevalence of endothelial cells expressing CD31 (Supplementary Fig. S3B), indicated that cardiovascular differentiation was induced by HP1γ. Interestingly, CD34- and CD45-positive cells were less than 1% among both WT- and HP1γ-P19 cells, as shown by FCM analysis (Supplementary Fig. S3B), suggesting that overexpression of HP1γ failed to induce P19 cells to differentiate into hematopoietic cells during EB formation. Finally, FCM analysis revealed that the percentage of E-cadherin-positive cells was markedly reduced after EB formation of HP1γ-P19 cells, suggesting that few epiblastic stem cells remained in the culture (Supplementary Fig. S3C). Taken together, HP1γ confers the potential for P19 cells to differentiate to the three germ layers, which is not initiated by drug treatment.

4. Discussion

Many studies have described the multifunctional roles of the HP1 family in heterochromatin formation and euchromatic gene expression associated with developmental processes [8–10,18], but the physiological functions of the HP1 family have remained to be elucidated. Using an overexpression approach to study the role of HP1γ in P19 cells, we show that HP1γ is involved in the triggering of differentiation.

Under proliferating conditions, expanding HP1γ-P19 cells maintained the same stem cell-like morphology as that of the wild-type cells through passages and continued to express Oct4 and FGF5 along with ALP activity. Moreover, among WT- and HP1γ-P19 cells, the proportion of E-cadherin-positive cells was similar and the induction of differentiation markers was completely blocked in monolayer culture without LIF and/or feeder cell. Similar to its parental P19 cells, HP1γ-P19 cells expressed different pluripotency marker genes from those expressed by ES cells, in which neither Rex1 nor Nanog were expressed. These results suggest that HP1γ overexpression does not induce spontaneous

differentiation of P19 cells or affect their pluripotent status. However, the expressional levels of Oct4 and FGF5 were roughly half of that in wild-type cells, and an apparently weak signal of ALP was observed in HP1γ-P19 cells. Because HP1γ-P19 cells formed small colonies compared with those of the wild-type cells, they shared some common features with ES cells in terms of epigenetic regulation. Knockdown of HP1γ in ES cells does not affect the expression of core transcriptional factors for pluripotency, such as Oct4, Nanog and Sox2, but increases the propensity of ES cells to differentiate and reduces their proliferation [19]. Moreover, ES cells lacking the Tip60-p400 histone acetyl transferase complex continue to express pluripotency markers but show defects in the cell cycle and proliferation along with the expression of differentiation markers [20]. Thus the slowing of proliferation in HP1γ-P19 cells may correlate with the decrease in expression of pluripotency markers and the commitment to differentiate. Because Nanog is not expressed in P19 cells, HP1γ overexpression may increase the sensitivity for differentiation as shown in Nanog-knockout ES cells [21].

Although P19 cells expressed a higher level of the earliest mesodermal marker, Brachyury, than that in HP1γ-P19 cells (Fig. 3), P19 cells did not show any sign of mesodermal differentiation even after EB formation. In contrast, the RT-qPCR analysis revealed that HP1γ-P19 cells expressed the lateral plate mesoderm marker Flk-1 in EBs (Fig. 3). The number of Flk-1-positive progenitor cells was also increased at day 3 of differentiation and gradually reduced along with the differentiation to endothelial cells and cardiomyocytes, as revealed by the FCM analysis (Supplementary Fig. S3). These results indicated that HP1γ conferred the actual mesodermal differentiation potential to P19 cells. As shown in the RT-qPCR analysis, strong induction of the endodermal markers Gata4 and AFP was found. Moreover, the EBs of HP1γ-P19 cells also contained endodermal cells that expressed Gata6, CK18 and/or Sox17, indicated that the clear induction of endodermal differentiation was occurred in the EBs of HP1γ-P19 cells. However, the induction of Nestin expression was not as dramatically high as that of the endodermal markers despite the apparent differentiation of neuronal cells (Fig. 4B). HP1γ depletion hampers endodermal differentiation but not mesodermal or ectodermal differentiation in ES cells [19]. Overexpression of HP1γ in P19 cells showed an opposing effect to that in HP1γ-depleted ES cells in terms of endodermal differentiation. These results suggest that HP1γ is involved in the endodermal differentiation of P19 cells. Genetic and proteomic studies in mice have shown that the interaction between transcriptional intermediary factor (TIF)1β and HP1γ proteins sustains endodermal differentiation, and is essential for late endoderm formation of F9 EC cells [22]. Thus, TIF1β may be implicated in the induction of endodermal cells from HP1γ-P19 cells.

During EB formation, overexpression of HP1γ conferred the capacity for spontaneous differentiation of P19 cells without any chemical inducers. This differentiation pattern of HP1γ-P19 cells contrasts to that of wild-type P19 cells in two aspects. First, P19 cells are pluripotent stem cells, but they require factors like RA and DMSO other than simple EB formation to exhibit their differentiation capacity [17]. However, when HP1γ-P19 EBs were formed, differentiation into a variety of cell types was observed without any stimulation by chemical inducers. Thus, overexpression of HP1γ can be a substitute for chemical inducers to differentiate P19 cells in EBs. Epigenetic regulators, such as the histone deacetylase and demethylase Kdm3a, have been found to be involved in RA receptor-dependent differentiation of EC cells [23,24]. These results suggests that the change of epigenetic status is a prerequisite for differentiation of EC cells by inducers, and HP1γ may affect the chromatin structure in P19 cells to sustain the activation of differentiation genes. Second, treatment with RA and DMSO induces the differentiation of P19 cells into the neuronal lineage and mesodermal cells, respectively. P19 cell differentiation occurs *in vitro* in a limited fashion corresponding to the type of specific chemical inducer [16]. In contrast, HP1γ-P19 cells could differentiate simultaneously into ectodermal, mesodermal and endodermal lineages and may behave similarly to ES cells during EB formation, indicating that HP1γ overexpression conferred the ability for spontaneous differentiation of P19 cells into the three germ layers. Although the mechanisms underlying this spontaneous differentiation remain to be elucidated, the change in chromatin state caused by HP1γ overexpression may play a key role on transcriptional activation induced by simple EB formation.

Studies of HP1γ−/− mice have reported that HP1γ has an important role in primordial germ cell (PGC) development [25,26] but not in somatic cell development. The lack of phenotypic alteration in somatic cells may be due to the presence of other HP1 isoforms [6]. Although the three HP1 isoforms have some distinct, non-redundant functions, their structures resemble each other and they show some common features [6]. For example, HP1γ and to a lesser extent HP1β localize to heterochromatin and euchromatin [27]. Knockdown experiments of HP1α and HP1γ by RNA interference in Hela cells show some functional redundancy between the two isoforms in terms of chromosome segregation [28]. Our results showed that HP1α, β and γ were expressed constitutively during differentiation of EBs (Supplementary Fig. S1A). Therefore, we may not be able to delineate any physiological roles of HP1 proteins in P19 cells and the associated *in vivo* developmental processes by simple HP1γ depletion.

The origin and gene expression profile of P19 cells resembles those of epiblastic cells. Therefore, the study of HP1γ in P19 cells helps us to understand the epigenetic regulation of pluripotency in epiblastic cells, and especially the role of chromatin status in triggering differentiation.

Acknowledgments

This study was supported by JSPS KAKENHI, Grant Numbers 18570201 (Y.S.) and 20590866 (I.H.), a Grant-in-Aid for JSPS Fellows (K.M.), and the Cooperative Research Program of Institute for Frontier Medical Sciences, Kyoto University, Japan.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.12.128.

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