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RNA Polymerase II Associated Factor 1/PD2 Maintains Self-Renewal by Its Interaction with Oct3/4 in Mouse Embryonic Stem Cells

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ABSTRACT

Embryonic stem cells (ESCs) maintain self-renewal while ensuring a rapid response to differentiation signals, but the exact mechanism of this process remains unknown. PD2 is the human homolog of the RNA polymerase II-associated factor 1 (Paf1). The Paf1/PD2 is a member of the human PAF complex that consists of four other subunits, hCdc73, hLeo1, hCtr9, and hSki8, and is involved in the regulation of transcriptional elongation and further downstream events. Here, we show that Paf1/PD2 is overexpressed in mouse ESCs and is involved in the maintenance of mouse ESCs. The Paf1/PD2 knockdown and knockout ESCs grown under self-renewal conditions express substantially reduced levels of self-renewal regulators, including Oct3/4, SOX2, Nanog, and Shh. We observed that the level of Paf1/PD2 expression is much higher in self-renewing mouse embryonic carcinoma cells than in the differentiating cells. Knockout of Paf1/PD2 altered ESC phenotype by increasing apoptosis and decreasing the percentage of cells in S-phase of the cell cycle. Interestingly, we found that the key genes that regulate endodermal differentiation (Gata4, Gata6, and Fgf8) are induced in the Paf1/PD2 heterozygous knockout ESCs. This suggests that Paf1/PD2 plays a specific role in regulating early commitment of ESCs to endodermal differentiation. Furthermore, for the first time, we showed that Paf1/PD2 protein interacts with Oct3/4 and RNA polymerase II, and through this interaction Paf1/PD2 may regulate Oct3/4-mediated gene expression. Thus, the Paf1/PD2 protein is a newly discovered element of the interconnected regulatory network that maintains the self-renewal of mouse ESCs. STEM CELLS *2009;27:3001–3011*

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

The development and function of multicellular organisms is based on the activity of stem cells. During early embryonic development, pluripotent stem cells give rise to all cell types in the body, including the germline [1]. Since this property can be exploited for genetic engineering and holds great promise for applications in regenerative medicine, an important goal is to understand the molecular pathways unique to pluripotent and self-renewal cells. Although the precise differentiation potential varies between different stem cell types, all stem cells share the fundamental property of self-renewal, that is, the maintenance of their population and existing in an undifferentiated state [2, 3]. Embryonic stem cells (ESCs), derived from the inner cell mass of the blastocyst, are the most commonly used cell types in studies of early embryonic development and the pluripotent state [4–6], largely because of their ability to self-renew in tissue culture for extended periods.

The pluripotency of ESCs is regulated by a unique network of ESC-specific transcription factors, including Oct3/4, Nanog, SOX2, and their binding partners [7–9]. Recent reports show a defined combination of Oct3/4, SOX2, and other transcription factors is sufficient to induce mouse fibroblast cells to take on an ESC-like phenotype, with the resulting cells labeled as induced pluripotent stem cells (iPSs) [10]. The ESC lines are capable of unlimited growth in culture while retaining their undifferentiated state and full developmental potential. Murine ESCs are typically cultured on fibroblast feeder cells with serum and the addition of leukemia inhibitory factor (LIF), a cytokine that activates the gp130/Stat3 signaling pathway essential for self-renewal [11]. In addition, there is a distinct genetic pathway regulating ESC self-renewal, which involves the transcription factors Tbx3 and Esrrb and the signaling cofactor Tcl1 [12].

The 19q13 amplicon in pancreatic cancer cells contains a novel pancreatic differentiation 2 (PD2) gene, which was identified by differential screening analysis [13]. PD2 is the human homolog of the yeast RNA polymerase II-associated factor 1 (yPaf1) and is part of the human (h) PAF complex. hPAF is composed of five subunits that include PD2/hPaf1, parafibromin/Cdc73, hLeo1, hCtr9, and hSki8 [13, 14]. This

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multifaceted complex was first identified in yeast (y) PAF and subsequently in *Drosophila* and humans. Recent advances in the study of the hPAF complex have revealed various functions of this complex in humans are similar to those of the yPAF complex, including efficient transcription elongation, mRNA quality control, and cell cycle regulation. We have previously shown that PD2/hPaf1 is overexpressed in poorly differentiated pancreatic cancer cells compared with well-differentiated cells. In addition, a recent study showed that one of the PAF complex subunits, parafibromin, plays a role in mammalian development and survival in adults [15].

In the present study, we elucidate the role of Paf1/PD2 in the maintenance of ESCs. We show that the knockout of Paf1/PD2 in ESCs loses its self-renewal function and leads to the induction of key endoderm genes, indicating a specific role of Paf1/PD2 in the early commitment to an endodermal lineage. Our result demonstrates, for the first time, the association of Paf1/PD2 with Oct3/4 for self-renewal maintenance.

Materials and Methods

ESC Culture

E-14 ESCs, PD2 heterozygous knockout ($PD2^{+/-}$) ESCs, J1 ESCs, and F9 embryonic carcinoma (EC) cells were cultured in gelatinized tissue culture dishes in ESC medium containing Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY, http://www.invitrogen.com) supplemented with 15% ESC-specific fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, http://www.invitrogen.com), L-glutamine (Gibco), 100 nM nonessential amino acids (Gibco), 1000 U/ml LIF (Sigma-Aldrich, St. Louis, http://www.sigmaaldrich.com), and 100 μM β-mercaptoethanol (Sigma-Aldrich).

Generation of Paf1/PD2 Heterozygous Knockout ESCs by Gene Trap Method

The Paf1/PD2 knockout mouse ESCs were established using the ESC line RRO233 from the BayGenomics Gene trap resource (Los Angeles, http://baygenomics.ucsf.edu) [16]. The linearized Gene trap vector was inserted in intron 1 of the Paf1/PD2 gene, resulting in a Paf1/PD2-β-galactosidase fusion protein that lacked 512 amino acids from the C-terminal of the full-length protein (535 amino acids). These RRO233 ESCs were used for further experiments to elucidate the role of Paf1/PD2 in ESCs.

RNA Isolation and Quantitative Reverse-Transcription Polymerase Chain Reaction

Total cellular RNA was extracted from ESCs using the RNAeasy kit (Qiagen, Hilden, Germany, http://www1.qiagen.com) and processed for reverse transcription. The initial polymerase chain reaction (PCR) activation step was at 94°C for 4 minutes, followed by the denaturation step at 94°C for 1 minute, primer-annealing step at 58°C for 30 seconds, extension step at 72°C for 1 minute, and the final extension step at 72°C for 10 minutes. PCR reaction products were then separated by electrophoresis using a 2% agarose gel. Gels were stained using 0.5 μg/ml of ethidium bromide, illuminated with UV light. Total cell RNA was reverse-transcribed and assayed by quantitative real-time PCR using SYBR Green incorporation. The expression of all genes was normalized to that of internal control β-actin and expressed relative to the indicated reference sample (average $\pm$ SD of triplicate reactions). The expressions of lineage-specific genes were compared between the ESCs and $PD2^{+/-}$ ESCs by the two-tailed Student's t test. A p value of $<.05$ was considered statistically significant.

RNA Interference

The region of mouse Paf1/PD2 was targeted with specific siRNA (sequence 5′-CGAGTCAAGTACTGCAATA-3′). Synthetic sense and antisense oligonucleotides (Dharmacon, Lafayette, CO, http://www.dharmacon.com) were annealed in 100 mM potassium acetate, 30 mM HEPES-KOH (pH 7.4), and 2 mM magnesium acetate for 1 minute at 90°C and 1 hour at 37°C, and frozen. Oligonucleotides were transfected into cells with *Trans*IT-TKO (Mirus Bio LLC, Madison, WI, http://www.mirusbio.com) in accordance with the supplier's recommendations.

Immunoblot Assay

ESC and F9 EC cell lines were processed for protein extraction and Western blotting using standard procedures. Briefly, the cells were washed twice in phosphate-buffered saline (PBS) and lysed in RIPA buffer (100 mM Tris, 5 mM EDTA, 5% Nonidet P40; pH 8.0) containing protease inhibitors (1 mM phenyl-methyl sulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin) and kept at 4°C and supernatants were collected. Resolved proteins were transferred onto the polyvinylidene fluoride (PVDF) membrane. After quick washing in phosphate-buffered saline and 0.1% Tween 20 (PBST), the membranes were blocked in 5% nonfat dry milk in PBS for at least 2 hours and then incubated with primary antibodies (anti-Paf1/PD2, anti-Leo1, anti-Cdc73, anti-Ski8, anti-Oct3/4, anti-SOX2, anti-β-actin) (diluted in 3% BSA in PBS) overnight at 4°C. Then the membrane was washed (3 $\times$ 10 minutes) in PBST at room temperature and probed with 1:2,000 diluted horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies for 1 hour at room temperature and washed 5 $\times$ 10 minutes with PBST. The signal was detected with an enhanced chemiluminescence (ECL) chemiluminescence kit (Amersham Biosciences, Buckinghamshire, U.K., http://www.amersham.com).

Alkaline Phosphatase Staining

Alkaline phosphatase was visualized using stemTAGTM alkaline phosphatase staining kit (Cell Biolabs, Inc., San Diego, http://www.cellbiolabs.com). The kit was used according to the manufacturer's specifications with the following modification: cell layers were fixed in 4.5 mM citric acid, 2.25 mM sodium citrate, 3 mM sodium chloride, 65% methanol, and 4% paraformaldehyde prior to washing and staining.

Confocal Microscopy

Cells were plated onto sterile round coverslips (CIR 18-1 Fisher brand 12-545-10; Fisher Scientific International, Hampton, NH, http://www.fisherscientific.com) and grown in 12-well plates for 24 hours. Cells were fixed in acetone/methanol (1:1; prechilled to −20°C) and permeabilized with 0.1% Triton X-100 in PBS. Then the cells were washed in PBS and incubated with primary (for 2 hours) and fluorescent-tagged secondary (for 30 minutes) antibodies at room temperature. Antibodies were diluted in 5% goat serum. Finally, coverslips were mounted with vectashell containing 4′,6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA, http://www.vectorlabs.com).

Immunoprecipitation Analysis

Equal amounts of protein (20 μg) were incubated overnight with anti-PD2 (rabbit polyclonal), anti-Oct3/4 (mouse monoclonal), and anti-RNA polymerase II (Pol II; mouse monoclonal) antibodies in a 500-μl total volume. Protein A^+ G-Sepharose beads (Sigma-Aldrich Corp. St. Louis, MO, USA, http://www.sigmaaldrich.com) were added to the lysate-antibody mix and incubated on a rotating platform for 3 hours at 4°C and then washed four times with lysis buffer. The immunoprecipitates or total cell lysates were electrophoretically resolved on SDS-polyacrylamide gel electrophoresis (10%). Resolved proteins were transferred onto the PVDF membrane. After quick washing in PBST, the membranes were blocked in 5% nonfat dry milk in PBS for at least 2 hours and

then incubated with primary antibodies (anti-Paf1/PD2, Oct3/4, and RNA Pol II). The immunoblots were washed five times (5 × 10 minutes), incubated for 1 hour with horseradish peroxidase-conjugated secondary antibodies, washed five times (5× 10 minutes), reacted with enhanced chemiluminescence ECL reagent (Amersham Biosciences), and exposed to x-ray film to detect the signal.

Colony-Forming Analysis

PD2 heterozygous knockout and wild-type (WT) ESCs were seeded in triplicate at 200, 400, 800, and 2,000 cells per 10-cm dishes in complete ESC medium. After 2 weeks of growth, the cells were fixed and stained with crystal violet stain (0.1%, wt/vol) in 20 nm 4-morpholinepropanesulfonic acid (Sigma-Aldrich), and the grossly visible colonies were counted; all experiments were repeated at least three times. Plating efficiency was determined as the number of colonies formed, divided by the total number of cells plated.

Apoptosis Assay

Apoptosis was measured using the annexin V-fluorescein isothiocyanate apoptosis detection kit (Roche Diagnostics, Indianapolis, IN, http://www.roche-diagnostics.us). Apoptosis was detected by staining the cells with annexin V and propidium iodide solution followed by flow cytometry.

Cell Cycle Analysis

Confluent cells were detached from the flask with trypsin, washed in PBS, and fixed in 2 mM glycine (pH 2)/70% ethanol. Fixed cells were then washed and resuspended in 1 ml of Telford reagent (90 mM EDTA, 2.5 mU of RNase A/ml, 50 μg of propidium iodide/ml, and 0.1% Triton X-100 in PBS). The total DNA content was analyzed in a FACSCalibur analyzer using ModFit LT software (Becton, Dickinson and Company, Franklin Lakes, NJ, http://www.bd.com).

Embryoid Body Formation

Embryoid bodies (EBs) were made as described elsewhere [17], with minor modifications. Cells were plated in a V-bottom 96-well plate at a final concentration of 5,000 cells per well in EB medium (DMEM; Gibco) supplemented with 15% ESC-specific FBS (Invitrogen), L-glutamine (Gibco), 100 nM nonessential amino acids (Gibco), and 100 μM β-mercaptoethanol (Sigma-Aldrich). After 2 days, EBs were transferred with DMEM medium to an ultra-low-binding 6-well plate. Then EBs were photographed using a phase-contrast microscope and collected for RNA extraction to analyze the lineage-specific genes.

Teratoma Formation

To induce teratoma formation, 1×10^6 cells from each cell population were injected into the quadriceps muscle of the hind legs of immunocompromised mice. Teratomas were dissected after 16 days. A small piece of each tumor was processed in PCR analyses to confirm genotypes. Half of the remaining tissue was fixed in 0.4% paraformaldehyde and embedded in optimal cutting temperature medium (Sakura Finetek Inc., Zoeterwoude, The Netherlands, http://www.sakuraeu.com) after incubating overnight in 30% sucrose; the remaining half was fixed in 10% formalin overnight, transferred to 70% ethanol, and paraffin embedded. Paraffin-embedded tissue sections were analyzed by hematoxylin and eosin staining.

Histological Analysis

Isolated teratomas were fixed in 4% paraformaldehyde in phosphate-buffered saline overnight and subsequently embedded in paraffin wax. Sections were cut at a thickness of 2 μm and stained with hematoxylin and eosin. The different structures were photographed and analyzed for structural variation.

ChIP-Re-ChIP Assay

ChIP-Re-ChIP experiment indicates sequential chromatin immunoprecipitations with two antibodies to study the simultaneous presence of two proteins in the genome sequence of interest [18]. ESCs were fixed with 1% formaldehyde, washed, harvested, and resuspended in 200 μl SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl [pH 8.1], 1 mM PMSF, and 1 μg/ml aprotinin). Samples were sonicated and diluted in ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl [pH 8.1], 167 mM NaCl, 1 mM PMSF, and 1 μg/ml aprotinin). For input control, 14 μl of sonicated samples was separated. Immunoprecipitation was performed with ChIP-specific Oct3/4 antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, http://www.scbt.com) as previously described. Chromatin extracts were pulled down with a protein G bead. The samples were washed extensively with wash buffers (low salt, high salt, LiCl, and Tris/EDTA buffers) and incubated at 65°C for 4 hours in 0.3 M NaCl. The eluted Oct3/4 pull-down chromatin extract was used for Re-ChIP assay. For the Re-ChIP assay, PD2 ChIP-specific antibody (Abcam, Cambridge, U.K., http://www.abcam.com) was used for immunoprecipitation and was washed using the above-mentioned method. MUC4 antibody was used as a control for the ChIP-Re-ChIP assay. The eluted Re-ChIP DNA and input samples were amplified by SYBR Green quantitative PCR (QPCR) with specific promoter primers of FGF-4, Nanog, and Lefty-1. All the nonspecific primers were chosen from the 3′ region of these genes. All primers used in this study are available in supporting information Table 1.

Results

Paf1/PD2 Expression in Mouse Embryonic Stem Cells

Paf1/PD2 is a member of the hPAF complex that comprises four other subunits, Cdc73/parafibromin, hLeo1, hCtr9, and hSki8. This complex is involved in the regulation of RNA elongation and the events downstream of transcription [13]. We began by assessing the levels of Paf1/PD2 in J1 and E-14 mouse ESCs and F9 EC cells under self-renewal conditions. Western blot analysis showed that the ESCs and EC cells had higher levels of Paf1/PD2 expression compared with the differentiated cells (Fig. 1A). Furthermore, we sought to determine the function of Paf1/PD2 in ESC and EC cell maintenance. For these studies we have obtained Paf1/PD2 heterozygous knockout ($PD2^{+/-}$) mouse ESC line (RRO233) from BayGenomics. The $PD2^{+/-}$ ESC was developed by gene trap mutagenesis method, wherein gene trap vector (pGT2Lxf) was inserted in-frame into the Paf1/PD2 gene immediately after exon 1, leading to the formation of a truncated protein having 23 amino acids from the N-terminal (Fig. 1B). The WT ESCs and $PD2^{+/-}$ ESCs were grown with LIF in ESC-specific culture condition. We performed Western blot and its densitometry (*, $p = .01$) (Fig. 1C), reverse-transcription (RT)-PCR (Fig. 1C), and quantitative RT-PCR (*, $p = .004$) (Fig. 1C) to examine the Paf1/PD2 expression in both WT ESCs and $PD2^{+/-}$ ESCs. The $PD2^{+/-}$ ESC line had a substantially lower level of Paf1/PD2 transcript and protein than the control mouse ESCs.

Unique Expression of the Paf1/PD2 Subunit in Mouse ESCs

The hPaf1 is part of the PAF complex composed of four other subunits: Cdc73, Leo1, Ctr9, and Ski8. Previously established functions of the PAF complex revealed a coordinated role of all the members of the complex. The PAF complex

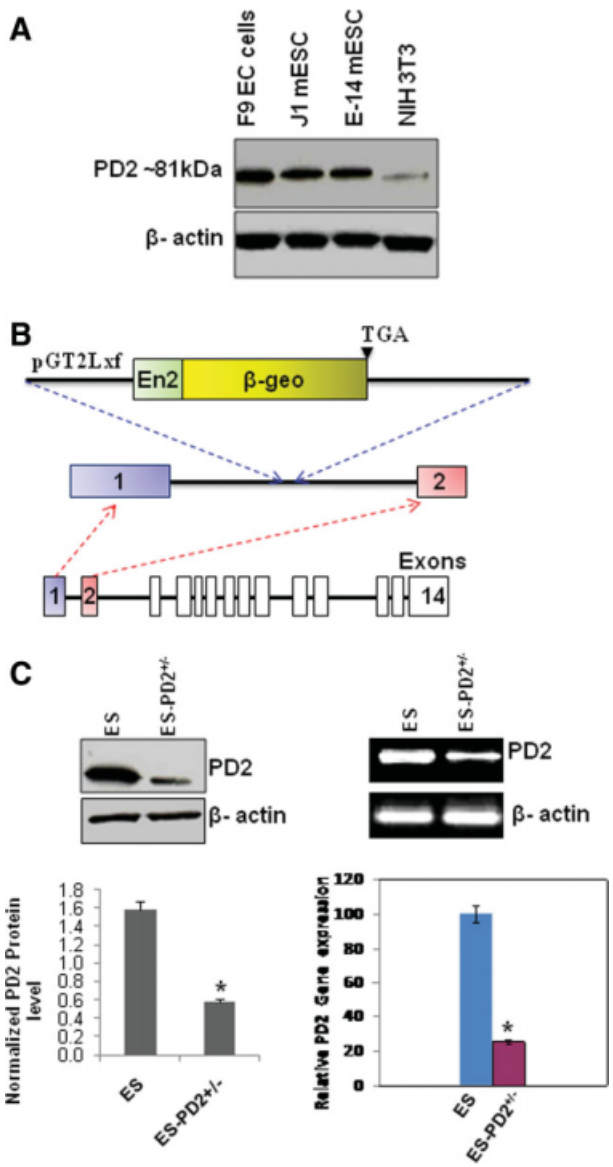

Figure 1. Expression of Paf1/PD2 in ESCs and heterozygous knockout ESCs. (**A**): Western blot analysis showed that Paf1/PD2 was overexpressed in F9 embryonic carcinoma cells, and J1 and E-14 mouse embryonic stem cells compared with differentiated NIH3T3 cells. (**B**): Generation of Paf1$^{-/-}$ mice. Schematic diagrams of the Gene trap vector pGT2Lxf insertion between exons 1 and 2. pGT2Lxf contains En2, the splice acceptor/Engrailed-2 exon, and *β-geo*. *β-geo* was inserted downstream of exon 1 of the *Paf1* gene, producing a chimerical mRNA of Paf1 and *β*-geo mRNAs. Paf1 mRNA encodes a fusion protein containing the N-terminal 23 a residues and *β*-gal. (**C**): Expression of Paf1/PD2 (~81 kDa) in heterozygous knockout (PD2$^{+/-}$) ESCs and wild-type ESCs by Western blot method with densitometry (left lower panel: *, $p = .01$) and mRNA expression by reverse-transcription polymerase chain reaction (RT-PCR) and quantitative RT-PCR analysis (right lower panel: *, $p = .004$). Abbreviations: *β*-geo, *β*-gal gene; EC, embryonic carcinoma; mESC, mouse ESC; PD2$^{+/-}$, PD2 heterozygous knockout.

coordinates events during transcription and RNA quality control [13]. In light of the previous study, our aim was to analyze the expression of other PAF complex subunits (Cdc73, Leo1, Ski8, and Ctr9) in Paf1/PD2 heterozygous knockout ESCs and PD2 RNAi (short interfering RNA [siRNA] against PD2 mRNA)-treated J1 ESCs. Interestingly, in contrast to the previous report [13, 19], we did not observe any coordinated expression of the other PAF complex subunits in the mouse ESCs. Western blot analysis of Cdc73 (parafibromin), Leo1, Ski8, and Ctr9 proteins showed no variation in their expression levels in the PD2$^{+/-}$ ESCs compared with the control ESCs (Fig. 2A). In addition, the transient knockdown of Paf1/PD2 in the J1 mouse ESCs also showed no decrease in the expression of other PAF complex members: Cdc73, Leo1, Ski8, and Ctr9 (Fig. 2B). Interestingly, the knockdown of Paf1/PD2 in the NIH3T3 mouse fibroblast cell line (a differentiated cell line) showed a decrease in the expression of other PAF complex members such as Cdc73, Leo1, Ski8, and Ctr9 (Fig. 2C). Thus, our results suggest that the expression of the PAF complex subunits is coordinated in differentiated mouse fibroblast cells but not in the undifferentiated ESCs. These findings suggest that Paf1/PD2 has a unique role in embryonic stem cell maintenance.

Paf1/PD2 Regulates Self-Renewal in Mouse ESCs

ESCs are pluripotent cells that have the potential for both indefinite self-renewal and differentiation into the three germ layers of the body [20]. Oct3/4, SOX2, and Nanog are considered a part of the core set of factors associated with the maintenance of pluripotency and self-renewal character in ESCs [21–23]. In our study, we have analyzed the level of the self-renewal markers Oct3/4, SOX2, and Nanog in PD2$^{+/-}$ ESCs and compared it with their expression in WT ESCs. Western blot and RT-PCR results for all these self-renewal proteins and mRNA showed a decreased level of expression in the PD2$^{+/-}$ ESCs compared with WT ESCs (Fig. 3A). The expression of sonic hedgehog (Shh), a marker of self-renewal property, was almost completely lost in the PD2$^{+/-}$ ESCs (Fig. 3A). Confocal analysis also showed a decreased expression of PD2 and Oct3/4 in the PD2$^{+/-}$ ESCs compared with the WT ESCs (Fig. 3B). To determine any changes in the cell morphology due to reduced Paf1/PD2 expression, cells were seeded at low density and observed under an optical microscope (×100 magnification). A vast majority of colonies in the PD2$^{+/-}$ ESCs appeared differentiated compared with the WT ESCs (Fig. 3C). To confirm this result, we performed alkaline phosphatase staining [24], which is indicative of the self-renewal propensity of ESCs. As expected, in the PD2$^{+/-}$ ESCs, almost 50% of the colonies did not stain with alkaline phosphatase, whereas all the WT ESC colonies were completely stained with alkaline phosphatase (×100 magnification) (Fig. 3D).

We have also analyzed the functional consequences of Paf1/PD2 heterozygous knockout in ESCs by assessing its effect on circular colony formation, cell cycle, and apoptosis. Colony-forming assay was performed to investigate the ESC potency of PD2$^{+/-}$. The PD2$^{+/-}$ ESCs showed a substantial reduction in short-term colony formation compared with WT ESCs with a differential concentration of cells (×40 magnification) (Fig. 4A). Analysis of propidium iodide and annexin V-positive cells by flow cytometry indicated a significant (*, $p = .005$) increase in early apoptosis in PD2$^{+/-}$ ESCs compared with WT ESCs (Fig. 4B). Late apoptosis or necrosis did not show any significant variation in either cell line (Fig. 4B). A comparison of cell cycle distribution in PD2$^{+/-}$ and WT ESCs revealed a decreased S-phase population in the PD2 knockout ESCs (52.67%) compared with WT ESCs (60.52%) (Fig. 4C). These results indicate that Paf1/PD2 knockout causes a growth pattern of ESCs.

To confirm the aforementioned results, we transiently knocked down Paf1/PD2 in J1 mouse embryonic stem cells (supporting information Fig. 1A). Similar to PD2$^{+/-}$ ESCs, the expression of self-renewal factors Oct3/4, SOX2, and

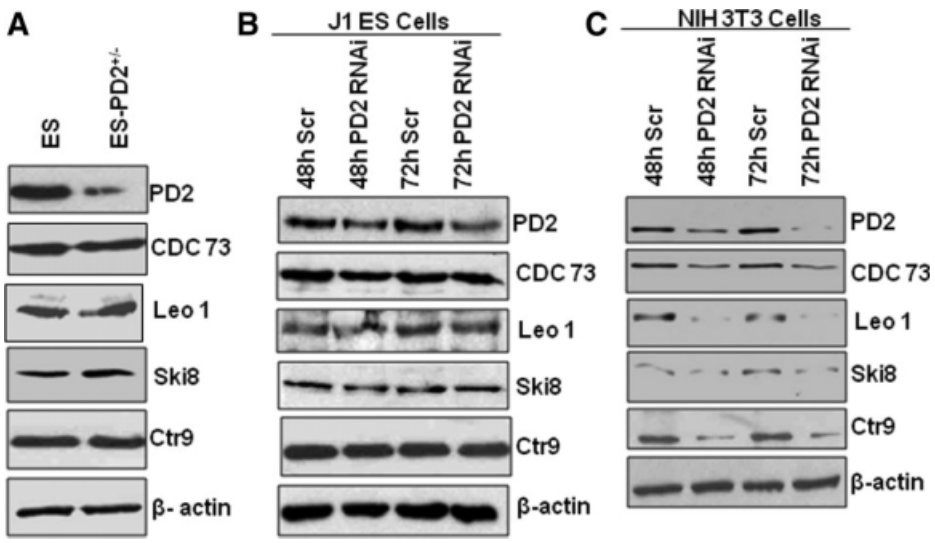

Figure 2. Expression of PAF complex molecules in Paf1/PD2 knockout and RNAi-treated ESCs. (**A**): Western blot analysis showed an expression of other PAF complex molecules (Cdc73, Leo1, sKi8, and Ctr9) in wild-type ESCs and Paf1/PD2 heterozygous knockout ESCs. (**B**): Transient knockdown of Paf1/PD2 protein using specific RNAi at 48 and 72 hours in J1 ESCs. Expression of Paf1/PD2 protein and other PAF complex proteins (Cdc73, Leo1, sKi8, and Ctr9) in Paf1/PD2 RNAi-treated J1 ESCs by Western blot. β-actin served as a loading control. (**C**): Transient knockdown of Paf1/PD2 protein (NIH3T3 cells) using specific RNAi for both 48 and 72 hours. Expression of Paf1/PD2 protein and other PAF complex proteins (Cdc73, Leo1, sKi8, and Ctr9) in Paf1/PD2 RNAi-treated NIH3T3 cells by Western blot. β-actin served as a loading control. Abbreviations: h, hour; $PD2^{+/-}$, PD2 heterozygous knockout, Scr, Scrambled RNAi.

Nanog was reduced in the Paf1/PD2 knockdown cells (supporting information Fig. 1A). An examination of the morphology of the Paf1/PD2 RNAi-treated cells showed features of differentiation compared with scrambled RNAi-treated cells, which retained their undifferentiated character (original magnification ×200) (supporting information Fig. 1B). These results confirm that Paf1/PD2 is essential for self-renewal of embryonic stem cells.

Paf1/PD2 Expression Decreases During the Differentiation of F9 Cells Along with Self-Renewal Markers

The F9 cells were treated with 9-cis retinoic acid (1 μM) for 5 days to investigate the effect of differentiation on the level of Paf1/PD2 expression. Retinoic acid (RA) treatment in vitro induces F9 EC cells to differentiate into the primitive endoderm layer [25]. The F9 EC cells and ESCs are developmentally pluripotent cells derived from murine embryos. In RA-treated F9 cells, a gradual change in cell morphology was observed, characterized by less tightly packed cells within the colonies and the generation of long filamentous structures compared with the untreated cells (original magnification ×100) (supporting information Fig. 2). Similarly, the J1 mESCs also showed the same morphological variation in RA-treated cells (supporting information Fig. 3). F9 cell differentiation by retinoic acid treatment led to decreased levels of Oct4 and Oct5 proteins [26]. In our study, we analyzed the expression of Paf1/PD2, the other PAF complex molecules, and self-renewal markers in both retinoic acid-treated and untreated F9 cells. As expected, expression of the Paf1/PD2 protein progressively decreased along with that of the self-renewal proteins Oct3/4, SOX2, and Nanog in the differentiating F9 cells following RA treatment (Fig. 5A). However, other PAF complex molecules, Cdc73, Leo1, Ski8, and Ctr9 proteins, did not show any change in either the differentiated or the undifferentiated F9 cells (Fig. 5A, 5B). Furthermore, J1 mESCs also confirmed the same observation (supporting information Fig. 4). The differentiation of EC cells and ESCs leads to the loss of Paf1/PD2 expression similarly like self-renewal markers. Furthermore, it appears that there is no coordinated expression of the PAF complex molecules during the differentiation of the primitive endoderm layer from EC cells. The retinoic acid-treated cells were also analyzed by confocal microscopy to look at the pattern of Paf1/PD2 and Oct3/4 expression. Paf1/PD2 (red) and Oct3/4 (green) expression gradually decreased in differentiating F9 EC cells (original magnification ×630) (supporting information Fig. 4).

Paf1/PD2 Deficiency Results in Increased Expression of Lineage-Specific Markers in ESCs

Cell fate decision of pluripotent embryonic stem cells is dictated by the activation and repression of specific sets of lineage-specific genes. To determine the lineages into which $PD2^{+/-}$ ESCs differentiate upon their exit from Paf1/PD2-mediated self-renewal, we performed quantitative RT-PCR (QRT-PCR) assays for lineage-specific genes in $PD2^{+/-}$ ESCs and WT ESCs growing under self-renewal conditions (in the presence of LIF). $PD2^{+/-}$ ESCs showed a significantly increased expression of endoderm (Gata4 [**, $p < .0001$] and Gata6 [**, $p < .0001$])-specific markers and little to no variation in mesoderm (Brachyury and Sox18)-, ectoderm (Calbindin and Snail)-, and trophoectoderm (Cdx2)-specific markers in comparison to WT ESCs (Fig. 6A). Expression of all of these differentiation markers was either absent or detectable at very low levels in the self-renewing WT ESCs (Fig. 6A). We carried out EB formation analysis to identify the Paf1/PD2 and its role in ESC potency. Embryoid bodies are aggregates of cells derived from ESCs and EB culture is used to examine the differentiation potential of the ESC line. $PD2^{+/-}$ ESCs and WT ESCs grew normally in short-term differentiation cultures without LIF. However, deficient EB formation was observed in the $PD2^{+/-}$ ESCs compared with WT ESCs (Fig. 6B). The differentiation of $PD2^{+/-}$ ESCs into multiple lineages while growing under self-renewal conditions prompted us to determine the differentiation characteristics of EB cells derived from wild-type ESCs. QRT-PCR of $PD2^{+/-}$ ESC line-derived EBs showed a significantly increased

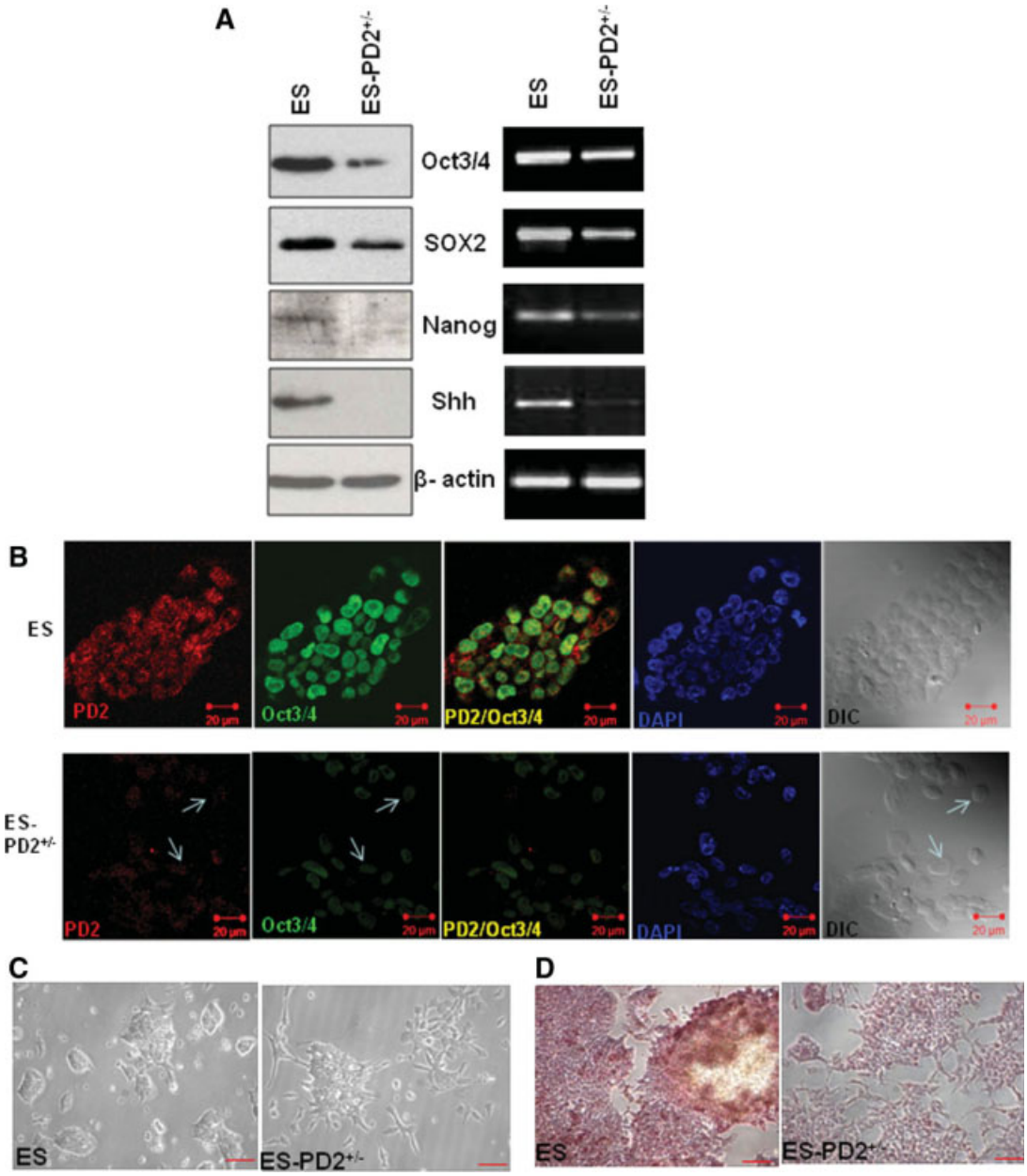

Figure 3. Expression of self-renewal markers and morphological variation of Paf1/PD2 knockout and wild type ES cells. (**A**): Expression of Oct3/4, SOX2, Nanog, and Shh proteins and mRNA in Paf1/PD2 knockout ESCs and wild-type (WT) ESCs by Western blot and reverse-transcription polymerase chain reaction analysis. (**B**): Confocal analysis to show Paf1/PD2 and Oct3/4 to localization in WT ESCs and Paf1/PD2 knockout ESCs. The arrows indicate the decreased expression of PD2 and Oct3/4 in Paf1/PD2 knockout ESCs. (**C**): Phase-contrast images of control and Paf1/PD2 knockout ESCs (scale bar = 0.8 mm). (**D**): Alkaline phosphatase analysis of WT ESCs and Paf1/PD2 knockout ESCs (scale bar = 0.8 mm). Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; DIC, differential interference contrast; $PD2^{+/-}$, PD2 heterozygous knockout.

expression of endoderm (Gata4 [*, $p = .01$], Fgf8 [**, $p < .0001$], and Gata6 [**, $p < .0001$])-specific markers and little variation in mesoderm (Brachyury, PitX2, and Sox18)-, ectoderm (Calbindin and Snail)-, and trophoectoderm (Cdx2)-specific markers compared with WT ESC-derived EBs (Fig. 6C). Some of these markers were either absent or markedly reduced in WT ESC-derived EBs (Fig. 6C). Therefore, although EBs derived from both wild-type and $PD2^{+/-}$ ESCs started expressing multiple differentiation markers, the $PD2^{+/-}$ ESC-derived EB cells expressed a greater number of endoderm-specific lineage markers than the EB cells derived from WT mouse ESCs. This suggests that $PD2^{+/-}$ predisposes mouse ESCs to endodermal differentiation. Furthermore, we induced teratoma formation to analyze the differentiation potential of $PD2^{+/-}$ ESCs. This teratoma formation was to test whether the embryonic stem cells are pluripotent by allowing the cells to differentiate spontaneously in cell culture, manipulating the cells so they will differentiate to form ESCs characteristic of the three germ layers. Both ESCs and $PD2^{+/-}$ ESCs (1×10^6) were injected into the immunocompromised mice and teratomas were dissected after 16 days. The hematoxylin and eosin staining of WT ESC-derived teratoma sections showed mixture of many differentiated or partly differentiated cell types, an indication that the WT ESCs are capable of differentiating into multiple cell types (supporting information Fig. 5). In contrast $PD2^{+/-}$ ESC-derived teratoma

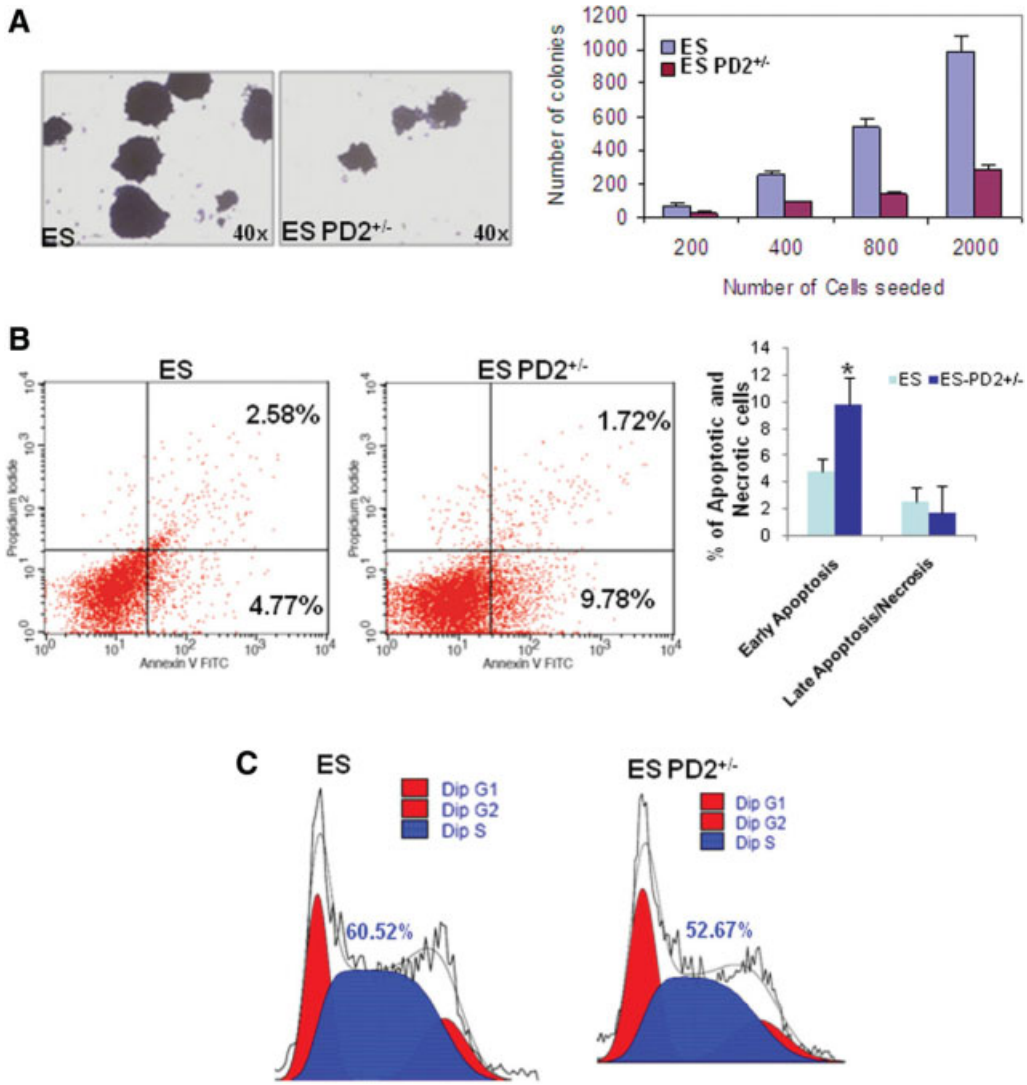

Figure 4. Loss of Paf1/PD2 abolishes ESC functions. (**A**): Qualitative and quantitative analysis for short-term colony formation in WT and Paf1/PD2 knockout ESCs. (**B**): Annexin V and propidium iodide staining analysis by flow cytometry to identify apoptosis in WT ESCs and Paf1/PD2 knockout ESCs. The statistical analysis showed significant (*, $p = .05$) variation in early apoptosis but not in late apoptosis or necrosis in Paf1/PD2 knockout ESCs. (**C**): Cell cycle analysis of WT ESCs and Paf1/PD2 knockout ESCs by flow cytometry using propidium iodide. Abbreviations: FITC, fluorescein isothiocyanate; $PD2^{+/-}$, PD2 heterozygous knockout.

showed defective germ layer formation (neural epithelium, skeletal muscle cells, and gut epithelium) compared with WT ESCs (supporting information Fig. 5).

Paf1/PD2 Interacts with Oct3/4 and RNA Polymerase II in ESCs

To investigate the molecular mechanism underlying the role of Paf1/PD2 in the self-renewal of embryonic stem cells, we looked for any protein-protein interaction between Paf1/PD2 and Oct3/4 in ESCs. Protein-protein interaction is of fundamental importance for transcriptional regulation [27–29]. We performed reciprocal immunoprecipitation studies, which revealed a clear interaction between Paf1/PD2, Oct3/4, and RNA Pol II in mouse ESCs (Fig. 7A). Our previous study showed that Paf1/PD2 is part of the transcription machinery, acting as a docking protein in between the complexes and is directly involved in transcription initiation and elongation [14]. Our current results show that Paf1/PD2 interacts directly with RNA Pol II as well as Oct3/4 in ESCs. These results suggest that Paf1/PD2 may be an interacting partner for Oct3/4 to regulate the transcription machinery of Oct3/4 in ESC maintenance.

Paf1/PD2 and Oct3/4 Regulate FGF-4, Nanog, and Lefty-1 in ESCs

ChIP-re-ChIP assay represents a direct strategy to determine the in vivo colocalization of proteins interacting with or in close contact to a chromatinized template on the basis of double and independent rounds of immunoprecipitations with high-quality ChIP-grade antibodies [18]. We have performed the ChIP-Re-ChIP assay to determine the role of Paf1/PD2 in ESC maintenance. Oct3/4 and PD2 ChIP-specific antibodies were used to pull down chromatin extracts of ESCs, and their binding to the promoter regions of FGF-4, Nanog, and Lefty-1 genes was examined by QPCR using promoter-specific primers. QPCR analysis showed a significant enrichment of FGF-4, Nanog, and Lefty-1 promoters in the Oct3/4-PD2 pull-down extracts relative to those pulled down with the control antibody (Fig. 7B). The nonspecific primers for FGF-4, Nanog, and Lefty-1 were not amplified in ChIP-Re-ChIP

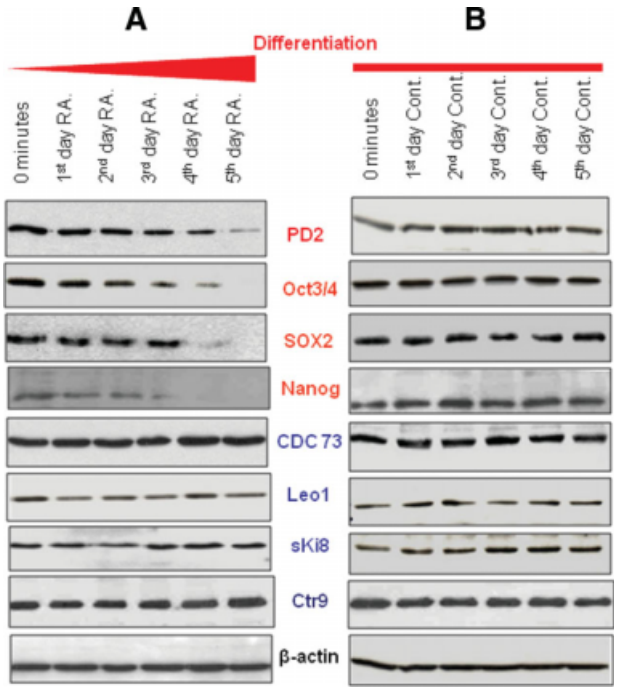

Figure 5. Expression of PAF complex and self-renewal proteins in retinoic acid-treated F9 embryonic carcinoma (EC) cells. (**A**): Expression of Paf1/PD2, Cdc73, Leo1, sKi8, and Ctr9, and self-renewal proteins Oct3/4, SOX2, and Nanog in differentiated F9 EC cells by Western blot analysis. (**B**): Western blot analysis showed the expression of Paf1/PD2, Cdc73, Leo1, sKi8, and Ctr9 and self-renewal proteins Oct3/4, SOX2, and Nanog in undifferentiated F9 EC cells. Abbreviations: Cont, control; RA, retinoic acid.

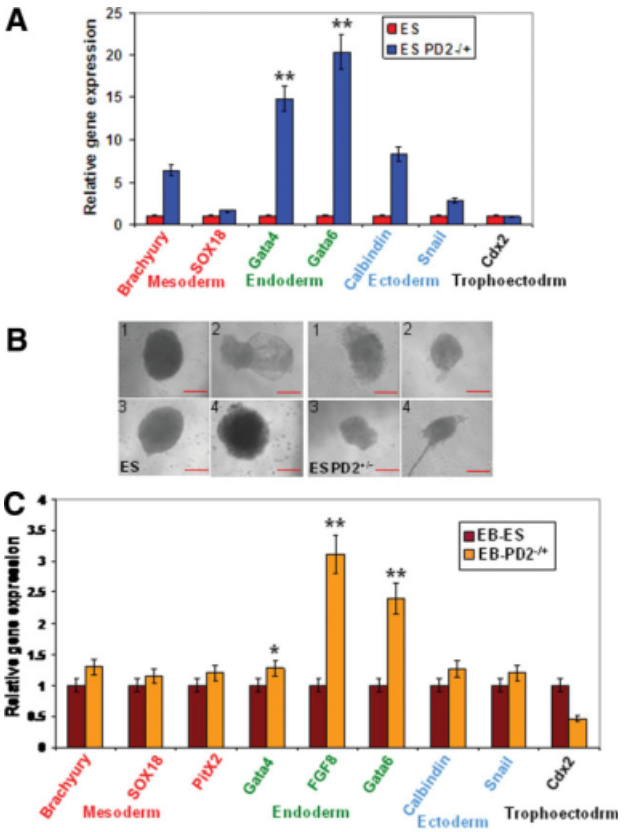

Figure 6. Paf1/PD2 affects lineage-specific genes in ESCs. (**A**): Analysis of lineage-specific markers for ectoderm (Calbindin and Snail), endoderm (Gata4 [**, $p < .0001$] and Gata6 [**, $p < .0001$]), mesoderm (Brachyury and Sox18), and trophoectoderm (Cdx2) layers in WT ESCs and $PD2^{+/-}$ ESCs by QRT-PCR. (**B**): Induced embryoid body (EB) formation in both WT ESCs and $PD2^{+/-}$ ESCs (scale bar = 0.8 mm). Defective and smaller embryoid body formation in Paf1/PD2 knockout ESCs compared with ESCs. (**C**): QRT-PCR analysis showed the expression of lineage-specific markers for ectoderm (Calbindin, Snail, and PitX2), endoderm (Gata4 [*, $p = .01$], Fgf8 [**, $p < .0001$], and Gata6 [**, $p < .0001$]), mesoderm (Brachyury and Sox18), and trophoectoderm (Cdx2) layers in embryoid body of WT ESCs and $PD2^{+/-}$ ESCs. Abbreviations: EB, embryoid body; $PD2^{+/-}$, PD2 heterozygous knockout.

DNA. This result suggests that the complex of Oct3/4 and PD2 together regulates self-renewal genes such as FGF-4, Nanog, and Lefty-1. It is possible that Oct3/4 may interact indirectly with RNA pol II via Paf1/PD2, and thereby regulate the expression of a subset of self-renewal and pluripotency genes.

Discussion

A detailed molecular understanding of how specific factors influence the balance between pluripotency and differentiation of mammalian cells is crucial to develop ESCs for their potential therapeutic use. The importance of this fact is becoming increasingly evident as it is now possible to generate ESC-like cells from somatic cells via direct reprogramming [30–35], avoiding many ethical issues. Induced nuclear reprogramming through iPS cell technology is a pioneering achievement in this direction [33, 34]. The repercussions of iPS technology are vast: it provides a way to create patient-specific stem cells that bypasses ethical and technical issues surrounding human ESC derivation and somatic cell nuclear transfer [36, 37], a state-of-the-art model for studying genetic diseases in vitro [38, 39].

In this study, we have investigated the biological role of Paf1/PD2 in mouse ESCs. The human homolog of the yeast transcription elongation factor Paf1 was recently identified by our laboratory and others [13, 14, 19]. The hPaf1/PD2 is part of the human PAF complex along with hCdc73 (parafibromin), hCtr9, hLeo1, and Ski8 [13]. The human PAF complex lacks the Rtf1 protein, a component of the yeast core complex [13], and mouse Paf1 has 98% homology to the human equivalent. In our study, we found that Paf1/PD2 heterozygous knockout in ESCs affects the maintenance of embryonic stem cells, by downregulating factors such as Oct3/4 and SOX2. Oct3/4 and SOX2 are highly expressed in the early embryonic developmental stage for maintaining the pluripotency and self-renewal in embryonic stem cells [7–9]. Ronin, Zfx, REST, Sall4, and Klf5 are also involved in the maintenance of embryonic stem cells by different regulating mechanisms [40–44]. A recent study has shown that PAF complex proteins coordinate the maintenance of ESCs [45]. Our findings show, for the first time, that PAF complex proteins work individually (and not as a complex) for the maintenance of mouse embryonic stem cells.

Our previous report showed that Paf1/PD2 is overexpressed in poorly differentiated rather than in well-differentiated cells [14]. Similarly, Paf1/PD2 is overexpressed in undifferentiated mouse ESCs and EC cells compared with the well-differentiated cells. It is known that the hPAF complex coordinates events during transcription (initiation, promoter clearance, and elongation) and RNA quality control in HEK293F cells [13]. In contrast to the previous reports, we observed that the PAF complex molecules Cdc73, Leo1, and

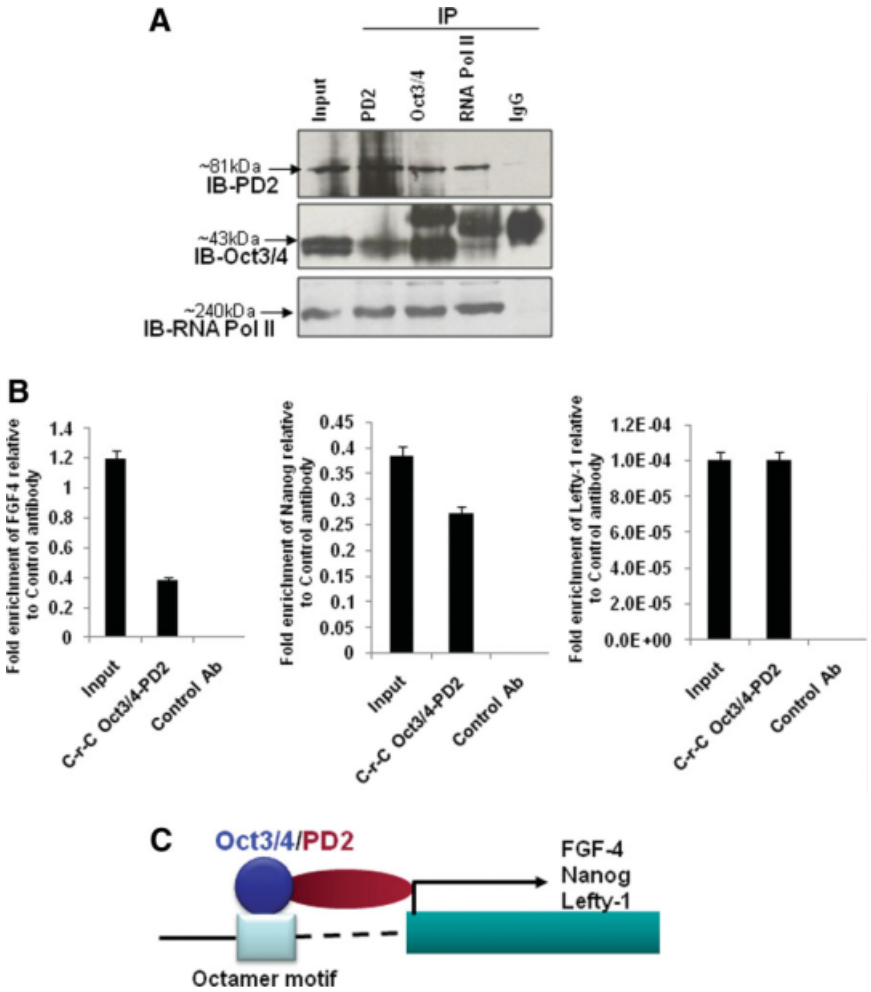

Figure 7. Mechanism of Paf1/PD2 in the maintenance of ESCs. (**A**): Reciprocal immunoprecipitation of Paf1/PD2, Oct3/4, and RNA Pol II in mouse ESCs. (**B**): The ChIP-Re-ChIP analysis for Oct3/4 and PD2 in embryonic stem cells. ChIP-Re-ChIP DNA was used to amplify FGF-4, Nanog, and Lefty-1 promoter fragment by SYBR Green QPCR analysis. The nonspecific primers were not amplified in any of the three genes. MUC4 antibody was used as a control antibody. The fold enrichment of FGF-4, Nanog, and Lefty-1 is related to MUC4 control antibody. (**C**): Mechanisms through which Oct3/4-PD2 interaction can regulate Oct3/4-mediated gene expression. A protein-protein interaction between Oct3/4 and Paf1/PD2 may have different possible implications through which Oct3/4-mediated gene expression can be regulated. An interaction between Oct3/4 with Paf1/PD2 may help to regulate expression of a subset Oct3/4 target genes such as FGF-4, Nanog, and Lefty-1 for the maintenance of ESCs. Abbreviations: Ab, antibody; C-r-C, sequential chromatin immunoprecipitations with two antibodies to study the simultaneous presence of two proteins in the genome sequence of interest; IB, immunoblot; IP, immunoprecipitation; Pol II, polymerase II.

sKi8 did not show any coordinated expression in the Paf1/PD2 heterozygous knockout and knockdown ESCs. A recent study shows that knockdown of all PAF complex molecules is required for maintenance of ESC identity [45] but did not clarify whether these molecules work as a complex or independently. Interestingly, in our study we found that knockout of Paf1/PD2 does not affect the expression of other PAF complex molecules. This uncoordinated effect of PAF complex was observed only in ESCs and not in mouse fibroblast NIH3T3 cells [14]. These results suggest that coordinated expression of PAF complex molecules may play a role only in differentiated cells and not in the undifferentiated ESCs.

The PD2$^{+/-}$ ESCs and Paf1/PD2 siRNA-treated J1 ESCs showed a decreased expression of the self-renewal markers Oct3/4, SOX2, and Nanog compared with the WT ESCs. As expected, a reduced expression of Paf1/PD2 resulted in ESC differentiation as shown by morphological examination and alkaline phosphatase staining. These results strongly suggest that Paf1/PD2 plays an important role in the self-renewal of mouse ESCs. Other studies have reported that Ronin, Zfx, REST, Sall4, and Klf5 are also involved in the maintenance of embryonic stem cells by different regulating mechanisms [40–44]. We also found that Paf1/PD2 downregulation in ESCs drastically reduced the number of ESC colonies compared with WT ESCs. Similar phenotypes have also been observed after the inducible deletion of Sall4, Ronin, and Klf5 in ESCs [40, 41, 44]. furthermore, Paf1/PD2 knockout ESCs showed an increase in apoptosis, suggesting a defect in long-term self-renewal due to impaired survival. A previous report showed that deficiency of Zfx, an ESC maintaining gene, resulted in increased apoptosis in ESCs [42]. Paf1/PD2 deficiency also leads to a reduction in the percentage of ESCs in the S-phase, consistent with an exit of these cells from self renewal. This suggests a role for Paf1/PD2 in cell cycle regulation in ESCs. Consistent with this idea, a previous report showed that forced G1-to-S-phase cell cycle progression by Akt1 kinase overexpression induces a LIF-independent self-renewal in ESCs, suggesting a possible link between cell cycle progression and the maintenance of pluripotency [41].

An interesting observation in our study was the progressive loss of Paf1/PD2, but not other PAF complex molecules, upon differentiation of F9 EC cells (induced by RA). This pattern of decrease in Paf1/PD2 expression is similar to that observed for the self-renewal markers Oct3/4 and SOX2. It has been reported that RA treatment induces F9 cells to differentiate into the primitive endoderm layer [25]. Our result suggests that Paf1/PD2 alone may be involved in the differentiation of ESCs into an endodermal lineage. Interestingly, the endoderm differentiation markers showed increased expression in the embryoid body derived from PD2$^{+/-}$ ESCs. Based on these findings, we speculate that the absence of Paf1/PD2 leads to the induction of key endoderm genes, indicating a specific role of Paf1/PD2 in early commitment to an endodermal lineage. The role of other PAF complex molecules in ESC maintenance remains to be explored. furthermore, the development of teratomas with defective germ layers in

PD2$^{+/-}$ ESCs suggests that deficiency of Paf1/PD2 leads to a loss of ESC potency.

Although transcriptional regulation of Oct3/4 gene expression by PAF complex proteins has been delineated [45], the other possible mechanisms through which Paf1/PD2 itself can regulate Oct3/4 protein function remained unexplored. Depending on the cellular context, Oct3/4 can either activate or repress target gene expression. To mediate its function, in addition to its binding to DNA, Oct3/4 interacts with other proteins to form homo- or heterodimers [20, 23, 26, 46–48]. Experimental evidence has shown that due to presence of two distinct DNA binding domains, Oct3/4 has the flexibility to interact with other transcription factors [49]. In the present study, through reciprocal coimmunoprecipitation, we were the first to show a protein-protein interaction between Oct3/4-PD2 and Oct3/4-RNA pol II. Oct3/4 may interact with Paf1/PD2 and RNA pol II individually or through a ternary complex, whereas Paf1/PD2 can act as a cross-linker between Oct3/4 and RNA pol II. Previous studies have shown that the PAF complex interacts with RNA pol II for a transcriptional process [13, 14]. Furthermore, ChIP-Re-ChIP result showed that Oct3/4 and PD2 interact together to regulate several key self-renewal genes including FGF-4, Nanog, and Lefty-1. Therefore, it is possible that Oct3/4 may interact indirectly with RNA pol II via Paf1/PD2, and regulate the expression of a subset of self-renewal and pluripotency genes. As mentioned previously, it is also equally possible that an independent interaction between Oct3/4 and Paf1/PD2 (without RNA pol II) may regulate expression of some genes, whereas Paf1/PD2 will act as a coactivator/corepressor. The coactivator/corepressor activity of Paf1/PD2 may be due to its possible role in chromatin modification and/or the inhibition of interaction between Oct3/4 and other proteins. The specific domains involved in the interaction of Paf1/PD2 with Oct3/4 need to be explored in ESCs.

Conclusion

We have found that Paf1/PD2 can regulate the function of Oct3/4 partly by regulating its interaction with the Oct3/4 protein. Overall, our finding identifies Paf1/PD2 as a novel self-renewal protein that interacts with Oct3/4 and thereby helps in the maintenance of ESCs by regulating self-renewal genes such as FGF-4, Nanog, and Lefty-1 (Fig. 7C). Furthermore, a practical application of our findings could be the development of lineage-specific cell types from ESCs by knockdown of Paf1/PD2. This can be combined with other methods to induce ESC differentiation and thus improve the efficiency of generating specific cell types for experimental and therapeutic stem cell transplantation.

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Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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Review

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Ovarian cancer: emerging concept on cancer stem cells

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Abstract

Emerging evidence suggests that the capacity of a tumor to grow and propagate is dependent on a small subset of cells within a tumor, termed cancer stem cells. In fact, cancer cells, like stem cells, can proliferate indefinitely through a dysregulated cellular self-renewal capacity. Cancer stem cells may originate due to the distribution into self-renewal and differentiation pathways occurring in multi-potential stem cells, tissue-specific stem cells, progenitor cells and cancer cells. Recent studies have shown that ovarian cancer also contains stem cells or tumor-initiating cells. Moreover, ovarian serous adenocarcinomas were disaggregated and subjected to growth conditions to select for self-renewing, non-adherent spheroids previously shown to be derived from tissue stem cells. A recent study showed that epithelial ovarian cancer was derived from a sub population of $CD44^+$, $CD117^+$ and $CD133^+$ cells. The existence of cancer stem cells would explain why only a small minority of cancer cells is capable of extensive proliferation of the tumor. In this review, we have discussed the studies on ovarian cancer stem cells along with the molecular pathways that could be involved in these cancer stem cells.

Introduction

Ovarian cancer is the fifth leading cause of cancer deaths and has the highest mortality rate among gynecologic cancers. It is the most lethal malignancy of the female reproductive system, at the initial stage the five-year survival rate is nearly 45%, which declines to 30% for patients with an advanced disease [1,2]. Greater than 90% of ovarian cancers arise from the surface epithelium [3], and tumorigenesis has been associated with ovulation-associated wound repair and/or inflammation, possibly leading to abnormal stem cell expansion [3,4]. Over the last several years, it has been increasingly evident that a small population (less than 5%) of cancer cells, referred to as "**cancer stem cells (CSCs)**", is responsible for the aggressiveness of the disease, metastasis and resistance to therapy [5-7]. Cancer stem cells, like somatic stem cells, are thought to be capable of self-renewal or unlimited proliferation [7]. The recent discovery that CSCs express certain 'stem cell-specific' markers has renewed interest and provided a rise in the idea that CSCs may arise from somatic stem/progenitor cells. Considerable research efforts have been directed toward the identification of cancer stem cell markers in ovarian cancer.

Stem cells, as classically defined, are cells with a capacity for self-renewal and generation of daughter cells that can differentiate into all the way down different cell lineages found in the mature tissue [8]. Stem cells always undergo asymmetric cell divisions, with each cell generating two cells; one that is identical to itself in stemness and another which is committed to a certain lineage. The daughter cell with stem cell like properties maintains its own compart-

ment over time, while its sister cell undergoes a series of cell divisions [9]. Self-renewal allows stem cells to persist during the entire the lifetime of the organism, while their differentiation potential allows them to perform functions like tissue genesis, tissue maintenance, and regeneration following stress or injury [9].

Of all the types of stem cell, hematopoietic stem cells (HSCs) are the best characterized adult stem cell [10]. HSCs can differentiate to form mature blood cells but can also reproduce themselves, which is known as self-renewal [10]. It is reside in distinct stem-cell niches that vary in location depending on the developmental stages of organism [11]. The human HSCs express high level of CD34 and low or absent level of CD33, CD38, thy-1, and CD71, appears to be enriched for primitive progenitor and HSC activity, while more mature progenitors express one or more of these markers [12]. Furthermore, in therapeutic target hematopoietic stem cells are the only stem cells developed up to therapy for the cancer and other disorders for the blood [11] and following HSC study for other stem cells will lead to improve therapy for other cancers.

Cancer stem cells may arise following transforming mutations that occur in untransformed stem cells, progenitor cells, mature cells, and cancer cells. The genetic program controlling self-renewal and differentiation plays a key role in the genesis of cancer stem cells (Figure 1). Cancer stem cells (CSCs) have been demonstrated to have roles in several cancers, including cancers of the ovaries, breast, brain, prostate, pancreatic, hepatocellular, head and neck cancers and hematological malignancies [5-7,13-27]. According to the CSC model, only a specific subset of the cancer cell population (i.e., the long-lived CSC subset) should be able to sustain *in vivo* tumor growth, whereas all other subsets (i.e., the tumor counterparts of short-lived differentiated cells) should not. Indeed, this assumption has now been repeatedly confirmed in several tumor systems. Three key observations classically define the existence of a CSC population: (i) Only the minority of cancer cells within each tumor are usually endowed with tumorigenic potential when transplanted into immunodeficient mice; (ii) Tumorigenic cancer cells are characterized by a distinctive profile of surface markers and can be differentially and reproducibly isolated from non-tumorigenic ones by flow cytometry or other immunoselection procedures; and (iii) Tumors grown from tumorigenic cells contain mixed populations of tumorigenic and non-tumorigenic cancer cells, thus recreating the full phenotypic heterogeneity of the parent tumor [28]. Furthermore, recent studies have been shown the functions of normal and malignant stem/progenitor cells in tissue regeneration, cancer progression and targeting therapies [29,30]. In this review we aim to provide insight into the evaluation of the evidence that supports the existence of cancer stem cells and the characterization studies that have tried to identify ovarian cancer stem cells. We also discuss how taking this subpopulation of cells into account may affect the way we treat ovarian cancers in the future.

Cancer stem cells

The identification of a reservoir of stem cells within many adult tissues raises the interesting possibility that all adult tissues have stem cells. Stem cell populations within normal tissues are defined by certain common characteristics: self-renewal to maintain the stem cell pool over time; regulation of stem cell number through a strict balance between cell proliferation, cell differentiation and cell death; and the ability to give rise to a broad range of differentiated cells [31,32]. It is observed that like stem cells, cancer cells are widely thought to be able to proliferate indefinitely through a deregulated self-renewal capacity. In fact, cancer stem cells can thus only be defined experimentally by their ability to generate continuously growing tumors. CSCs have the capacity to self-renew, undergoing divisions that allow the generation of more CSCs and ultimately some of them differentiate into the various cell types that compose the tumor mass. To date, the practical translation of this definition, and the gold standard to define the 'stemness' of cancer cells, has been their ability to generate a phenocopy of the original malignancy in immuno-compromised mice [7].

Evidence for the existence of cancer stem cells

To assay the cancer stem cells, a xenograft model for breast cancer was developed that allowed specific cancer tumors isolated directly from a patient to be passaged reliably *in vivo*. In this model, only a subset of cancer cells had the ability to form new tumors [5]. The cancer stem cells isolated from tumors are mostly isolated by flow cytometry as the CD44+ CD24-/^{low} lineage cell population [5]. Furthermore, dilution assays demonstrated that as few as 100 tumorigenic cancer cells were able to form tumors, while tens of thousands of the other (non-CSCs) populations of cancer cells failed to form tumors in nude mice. These tumorigenic cells have been serially generated in new tumors containing additional CD44+ CD24-/low lineage tumorigenic cells as well as the phenotypically mixed population of non-tumorigenic cancer cells [5,7]. In addition, when cultured cells were isolated based on the expression of CD133, a marker expressed by normal CNS stem cells [33], only the CD133+ fraction of cells was capable of forming spheres. These studies suggest that CNS tumors of neural origin contain a stem cell population. Li *et al*. reported that a highly tumorigenic subpopulation of pancreatic cancer cells expresses the cell surface markers CD44, CD24 and epithelial-specific antigen (ESA) [18]. Table 1 summarizes the studies which have

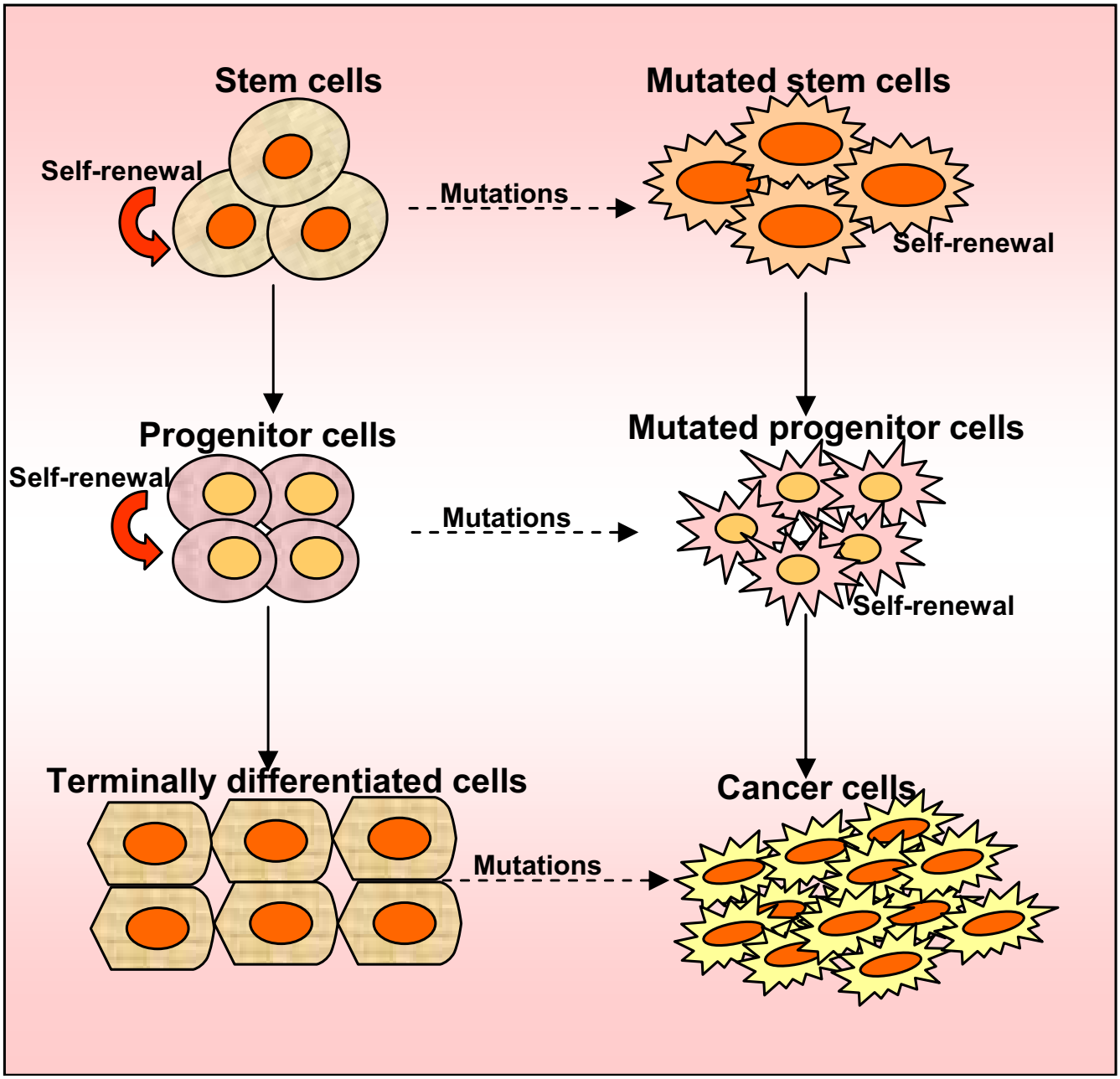

Figure 1
Origin of cancer stem cells. Self-renewal and differentiation potentials are the features of stem cells. Progenitor cells, the product of stem cells that lose the activity of self-renewal, could differentiate into mature cells, which have the feature of differentiation. The hypothesis is that cancer stem cells are caused by transforming mutations occurring in multi-potential stem cells, tissue-specific stem cells, progenitor cells, mature cells, and cancer cells.

described the direct isolation of populations containing cancer stem cells in various malignancies. Another phenotype used to distinguish these cells is their presence within the Side Population fraction as determined by their ability to exclude the Hoechst dye [34].

Therapeutic targets for cancer stem cells

The field of stem cell research has given new hope for the treatment and even a cure for incurable diseases in human. Particularly, the identification of a rare population of adult stem cells in most tissues/organs in humans

Table 1: Cancer type and specific marker for cancer stem cell populations

S. No	Cancer type	Markers for CSC population	References
1.	Brain Tumors	$CD133^+$	[23]
2.	Breast Cancer	$CD24^{-/low}/CD44^+/ESA^+$	[5]
3.	Ovarian Cancer	$CD133^+$/Side population (SP)/$CD44^+$, $CD117^+$	[25,27,59]
4.	Lung Cancer	$CD133^+$	[15]
5.	Prostate Cancer	$CD44^+/\alpha2\beta1^{high}/CD133^+$	[14]
6.	Pancreatic Cancer	$CD44^+/CD24^+$/ESA/$CD133^+$	[16,18]
7.	Hepatocellular Cancer	$CD133^+$	[24,26]
8.	Hematological Malignancies	$CD34^+/CD38^-$	[17]
9.	Colon Cancer	$CD133^+/CD44^+/Lin^-/ESA^+$	[22,28,44]
10.	Head and Neck Cancer	$CD44^+$	[21]

has emerged as an attractive source of multiple stem/progenitor cells for cell replacement-based therapies and tissue engineering in regenerative medicine. Our recent review discussed that cancer stem/progenitor cell research also offers the possibility of targeting these undifferentiated and malignant cells that provide critical function in cancer initiation and relapse for treating patients diagnosed with advanced and metastatic cancer [30,35,36]. Various strategies consisting of molecular targeting of distinct oncogenic signaling elements activated in the cancer progenitor cells and their local microenvironment during cancer progression can be explored [37]. Furthermore, overcoming the intrinsic and acquired resistance of cancer stem/progenitor cells to current clinical treatments represents a major challenge in treating and curing the most aggressive and metastatic cancers [38]. In addition, hematopoitic stem cells are the most characterized stem cells and it has been used for the therapy to cure cancer [11]. In this review we also described that the molecular mechanisms involved in the intrinsic and acquired resistance of cancer cells to current cancer therapies [38].

Pathways of self-renewal and carcinogenesis

Since the cancer stem cells share common properties with normal stem cells, it is reasonable to think that they have overlapping regulatory mechanisms. Indeed, one of the most outstanding questions concerning the biology of stem cells is: how do multi-potent stem cells select a particular differentiation pathway and start to differentiate? Another question is how do stem cells decide to maintain self-renewal properties and continue to proliferate? Recent studies demonstrate that the presence of various genes and signaling pathways are involved in the regulation of the aforementioned processes. Among these, the Sonic Hedgehog (Shh), Notch and Wnt signaling transduction pathways play a major role in the self-renewal of stem cells [39-41]. Recent advances in the understanding of the role of Wnt, Hedgehog, Shh, and Notch signaling pathways in regulating stem cell self-renewal have shed new light on carcinogenesis (Figure 2) [7,42,43]. The next obvious question is the possible connection between tumors and the (Hedgehog) Hh and Wnt pathways and how the activation of these pathways leads, in some cases, to such highly efficient tumorigenesis. Recent genetic evidence suggests that somatic stem cells are the producers of CSCs; that the Wnt and Hh pathways function in the normal regulation of stem-cell number in at least some tissues; and that expansion of the somatic stem-cell population may be the first step in the formation of at least some types of cancers [44-46]. Numerous arguments support a stem-cell origin for human cancer. Foremost is the observation that stem cells possess many of the features that characterize the malignant phenotype, including self-renewal and unlimited replicative potential [47]. Also, the mutations that initiate tumor formation seem to accumulate in cells that persist throughout life, as suggested by the exponential increase of cancer incidence with age. This is thought to reflect a requirement for four to seven mutations in a single cell to effect malignant transformation [47]. Although similar signaling pathways may regulate self-renewal in normal stem cells and cancer stem cells, there are mechanistic differences in some cancers. Interestingly, the mechanistic differences in self-renewal between normal stem cells and cancer stem cells can thus be targeted to deplete cancer stem cells without damaging normal stem cells.

Ovarian tumors

The ovaries contain three main types of cells germ cells, stromal cells and epithelial cells which give rise to germ cell, stromal and epithelial ovarian tumors, respectively. Epithelial ovarian cancers (EOC) were the most common type of ovarian cancers. Comprising nearly 90% of all ovarian cancers EOCs are derived from relatively pluripotent cells of the celomic epithelium or "modified mesothelium". These cells originate from the primitive mesoderm and can undergo metaplasia. Approximately 10% to 20% of epithelial ovarian neoplasms are borderline or low malignant potential tumors and are characterized by a high degree of cellular proliferation in the absence of stromal invasion. Of the invasive epithelial ovarian cancers, about 55–60% are serous, 15% endome-

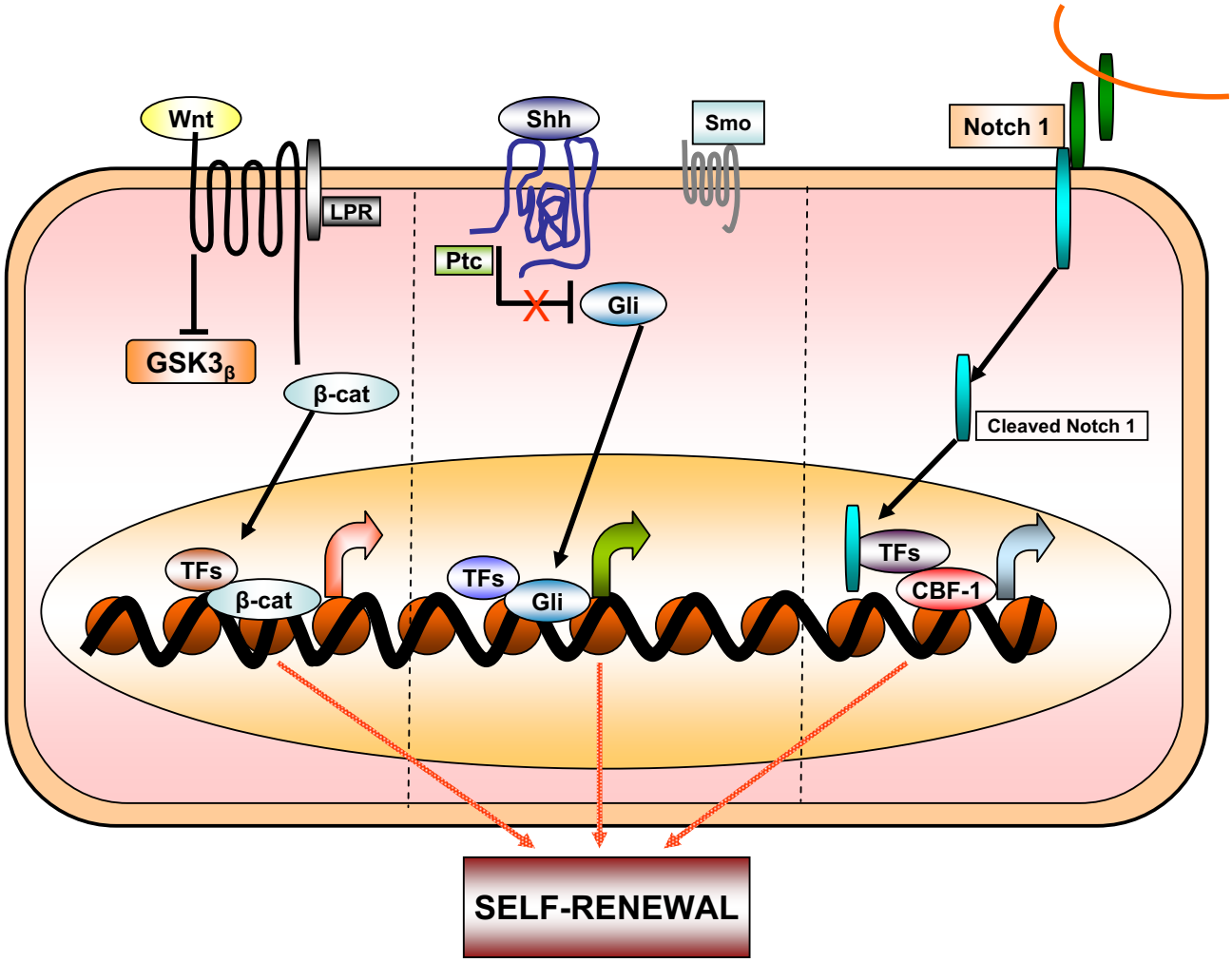

Figure 2
Schematic diagram of signaling pathways that are involved in normal and cancer stem cell biology. Wnt, Shh and Notch1 pathways have been shown to contribute to the self-renewal of stem cells and/or progenitors in a variety of organs, including the ovarian system. When deregulated, these pathways can contribute to oncogenesis. Mutations of these pathways have been associated with a number of carcinomas.

trioid, 5–10% clear cell and <5% mucinous [48] (Figure 3). The various histological subtypes of ovarian carcinoma have identifiable precursor lesions and multiple early genetic alterations. Figure 3 explains the various histological subtypes of ovarian cancer and their associated specific mutations. Mutations may be one of the major factors contributing to the origin of ovarian cancer stem cells. Many of the histological subtypes resemble the epithelial component of the lower genital tract, including papillary serous tumors that have an appearance resembling the glandular epithelium lining the fallopian tube. Mucinous tumors, on the other hand, contain cells resembling endocervical glands, and endometrioid tumors contain cells resembling the endometrium. Non-epithelial types of ovarian cancer include sex cord-stromal tumors (6% of ovarian cancers) and germ cell tumors (3% of all malignant ovarian neoplasms) [49-51]. The histological subtypes of ovarian carcinoma have identifiable precursor lesions and early genetic alterations. Figure 3 explains the histological subtypes and its specific mutations in ovarian carcinoma. Mutations are one of the major alteration factors for the origin of cancer ovarian stem cells.

Markers and their roles in ovarian tumors

In general, tumor markers can be used for one of four purposes: (i) screening a healthy population or a high risk population for the presence of cancer; (ii) making a diagnosis of cancer or of a specific type of cancer; (iii) deter-

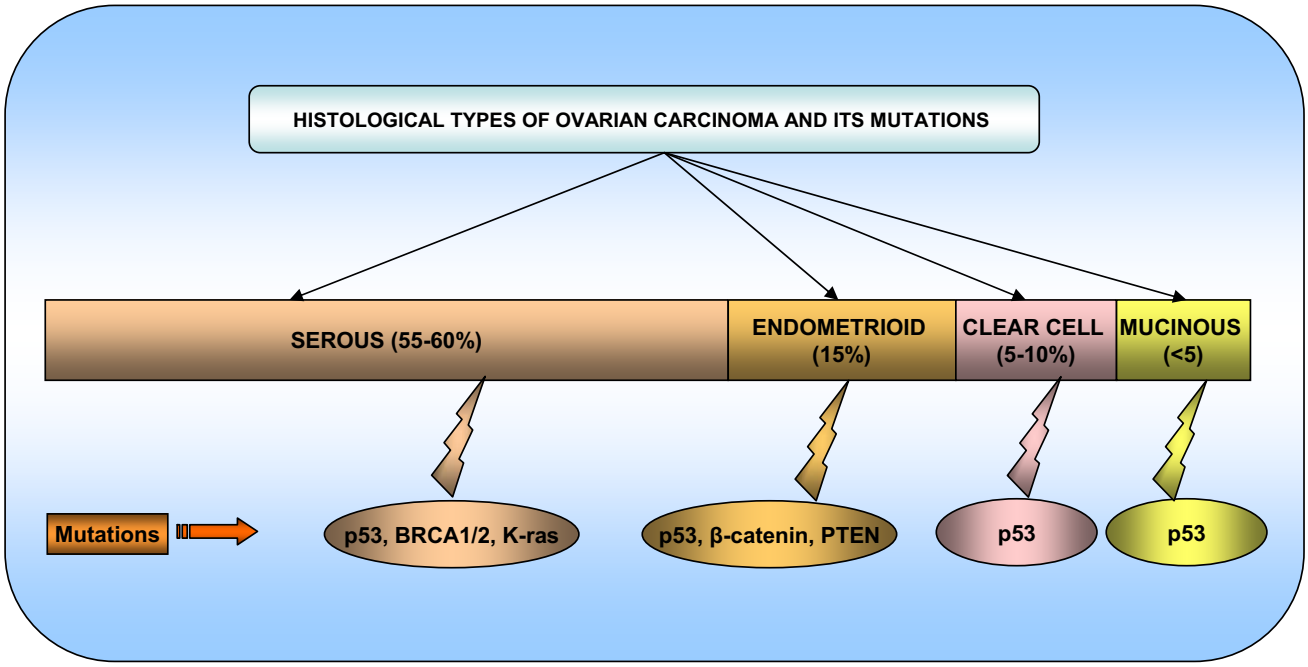

Figure 3
Schematic diagram representing the histological types and its specific mutations in ovarian carcinoma.

mining the prognosis of a patient; and (iv) monitoring the course in a patient in remission or while receiving surgery, radiation, or chemotherapy.

Furthermore, recent studies have identified different prognostic and diagnostic surface markers for ovarian cancer [52] and these markers need to be analyzed for their role in ovarian cancer. One of the well-known tumor antigens is the epithelial cell mucin MUC1, a transmembrane glycoprotein that is differentially expressed on tumor cells compared with normal epithelial cells [53,54]. MUC1 is expressed either not at all or in small amounts on various normal epithelia but aberrantly or neoexpressed at high levels on the majority of adenocarcinomas. Tumor-associated alterations of MUC1 are characterized by hypoglycosylation, increased sialylation, and altered carbohydrate core-type expression [53]. Engelmann *et al* reported that MUC1 molecule is not only expressed on mature cancer cells, but also on tumor cells that have multiple characteristics of stem and progenitor cells [55]. This study demonstrates MUC1 expressed breast cancer cell line MCF7 as a source of a minor population of cells with characteristics of tumor stem/progenitor cells to show for the first time that these cells also express the hypoglycosylated (tumor) form of MUC1, previously described only on mature MCF7 cells and other tumors and tumor cell lines. Moreover, these cells give rise to MUC1$^+$ tumors *in vivo* and that these tumors maintain a small population of MUC1$^+$ cells with the stem/progenitor characteristics [55]. Our recent finding demonstrated the tumor-specific expression of Tumor Associated Glycoprotein-72 (TAG-72) in ovarian cancer and its association with disease stage may serve as a potential marker for effective disease management [56]. In addition, surface marker mucins are overexpressed in many epithelial malignancies including ovarian cancer, suggesting a possible role in the pathogenesis of these cancers. Other studies from our laboratory have provided experimental evidence that the MUC4 mucin interacts with HER2 potentiates its downstream signaling and enhances the motility of ovarian cancer cells. Our findings provide experimental support for the hypothesis that MUC4 mucin expression is associated with a higher metastatic potential and thereby a poor prognosis in ovarian cancer [57]. The future direction of these studies will be to explore the roles of MUC4 and TAG-72 in ovarian cancer stem/progenitor cells.

Ovarian cancer stem cells

A recent study describes that ovarian cancer cell lines were shown to possess "side population" (SP) cells that have been described as cancer stem cells due to their stem-like characteristics including the ability to differentiate into tumors with different histologies. These putative cancer stem cells reflect the various histological subtypes observed in ovarian carcinoma. They also provide a model of cancer metastasis in which these cells are able to

colonize, expand, and differentiate into heterogeneous tumor phenotypes similar to primary tumors. In such a model, both the primary tumors and metastasis would display similar genetic and expression profiles because both populations are supposedly derived from the same lineage of cancer stem cells [58]. Ovarian cancer stem cells, like somatic stem cells, are shown to be capable of unlimited self-renewal and proliferation. In general, multi-potent cancer stem cells may account for the histological heterogeneity often found in tumors [25,27,59]. Moreover, ovarian somatic stem cells would be expected to divide asymmetrically, yielding both a daughter cell that proceeds to terminal differentiation, and an undifferentiated copy capable of self-renewal. Repeated asymmetric self-renewal sets of somatic stem cells or their immediate progenitor's stem cells lead to the accrual of mutations over time, which might ultimately lead to their transformation into cancer stem cells and malignant progression.

Furthermore, another study describes that two mouse ovarian cancer cell lines such as MOVCAR7 and 4306 contain candidate cancer stem cells [25]. These two murine ovarian cancer cells have large SP, making them suitable to study ovarian cancer stem cell biology. A similar, albeit very small, SP was also identified in the human ovarian cancer stem cell lines IGROV-1, SKOV-3 and OVCAR-3 and also in cells claimed from patient ascetic fluid [25]. Further, a study proved that isolated and characterized ovarian cancer-initiating cells (OCICs) are fully capable of reestablishing their original tumor hierarchy *in vivo*. These cells are very organized self-renewing, anchorage-independent spheres and were reproducibly dividable using antibodies against both CD44 and CD117 [27]. These OCICs were capable of intraperitoneal tumorigenesis and could serially propagate tumors in animals. Consequently, this study fulfills all currently accepted criteria for the existence of a subpopulation of tumor-initiating cells [27], and their specific detection and targeting could be highly valuable for therapy of recurrent, chemo-resistant disease. Whereas advanced ovarian cancer is generally initially responsive to standard chemotherapies (ciaplatin and paclitaxel), that responsive almost inevitably followed by drug resistant phenotype [2,60]. One accepted hypothesis about chemoresistance is standard therapies failed to target tumor progenitors, which are have like normal stem cells because of expression of membrane efflux transporters. Zhang et al showed that OCICs, under stem cell-selective conditions, over express ABCG2 and are more resistant to cisplatin and paclitaxel, suggesting a possible role for these cells in ovarian cancer chemoresistance [27].

Conclusion and perspective

The aforementioned studies showed that a so-called ovarian cancer stem cell, with high-proliferative capacity, self-renewal properties and multi-lineage potential, could be responsible for tumor development and the differentiation of more mature epithelial ovarian cells contributing to tumorigenesis. There are important consequences for cancer treatment if the growth of tumors is at least in part, dependent on a cancer stem cell population. The cancer stem cell hypothesis posits that cancer stem cells are a minor population of self-renewing cancer cells that fuel tumor growth and remain in patients after conventional therapy has been completed. The hypothesis predicts that effective tumor eradication will require obtaining agents that can target cancer stem cells while sparing normal stem cells. Experimental evidence suggests that ovarian cancer stem cells are relatively resistant to conventional chemotherapeutic agents. Current cancer therapies often engender severe toxicity because of their general effects on all rapidly dividing cells. Identification of candidate targets for more specific mechanism-based cancer therapy using techniques such as gene chips could reveal signature patterns of transcriptional output which are characteristic of activated self-renewal pathways.

Emerging evidence suggests that these pathways also control patterning and growth in self-renewing adult tissues by regulating the stem-cell compartment. Thus, pharmacological inhibition of these pathways in the worst case might result in severe toxicity due to a loss of normal stem-cell compartments. Further research will be needed to determine whether continuous pathway activity is required in normal and tumor tissues, and whether these requirements differ sufficiently as to allow therapeutic intervention. Even if pathway inhibition is prohibited by normal physiological requirements, other mechanism-based approaches that exploit aberrant pathway activation might be feasible. It has been proposed that malignancy is determined in all tissues by mis-regulation of a common set of genes that control growth by affecting cell proliferation, apoptosis, invasion and angiogenesis. This hypothesis is supported by the demonstration that multiple types of normal human cells can be made tumorigenic by the expression of a defined set of viral and cellular proteins. Therapeutic agents for the treatment of such tumors might target not only self-renewal pathway components, but also other critical transcriptional targets of the self-renewal pathways, or proteins that co-operate with them to deregulate growth.

It is important that agents directed against cancer stem cells discriminate between cancer stem cells and normal stem cells. This will require the identification of realistic drug targets unique to cancer stem cells. The identification of such targets and the development of anti-cancer agents

will require a deeper understanding of normal stem cell biology as well as cancer biology. More importantly, identification of the ovarian cancer stem cell would provide a critical step in advancing the development of novel therapeutic strategies in the management of ovarian cancer. Furthermore, characterizations of such progenitor or cancer stem cells in drug resistant (Ciaplatin, Paclitaxel and etc) manner for ovarian cancer will likely lead to a greater understanding of early events leading to the genesis of this elusive disease, in addition to providing new therapeutics targets aimed at the cells directly responsible for its propagation.

Abbreviations

CSC: Cancer Stem Cell; CNS: Central Nervous System; ESA: Epithelial-Specific Antigen; Shh: Sonic Hedgehog; Hh: Hedgehog; EOC: Epithelial ovarian Cancer; OCIC: Ovarian Cancer Initiating Cells; SP: Side Population

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

PPM participated in drafting the full manuscript and creating figures. SKB participated in substantial contribution to conception and revising it critically for important intellectual content.

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REVIEW

Human RNA polymerase II-associated factor complex: dysregulation in cancer

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Genetic instabilities are believed to be one of the major causes of developing a cancer phenotype in humans. During the progression of cancer, aberrant expression of proteins, either owing to genetic (amplification, mutation and deletion) or epigenetic modifications (DNA methylation and histone deacetylation), contributes in different ways to the development of cancer. By differential screening analysis, an amplification of the 19q13 locus containing a novel pancreatic differentiation 2 (PD2) gene was identified. PD2 is the human homolog of the yeast RNA polymerase II-associated factor 1 (yPaf1) and is part of the human RNA polymerase II-associated factor (hPAF) complex. hPAF is comprised of five subunits that include PD2/hPaf1, parafibromin, hLeo1, hCtr9 and hSki8. This multifaceted complex was first identified in yeast (yPAF) and subsequently in *Drosophila* and human. Recent advances in the study on PAF have revealed various functions of the complex in human, which are similar to yPAF, including efficient transcription elongation, mRNA quality control and cell-cycle regulation. Although the precise function of this complex in cancer is not clearly known, some of its subunits have been linked to a malignant phenotype. Its core subunit, PD2/hPaf1, is amplified and overexpressed in many cancers. Further, an overexpression of PD2/hPaf1 results in the induction of a transformed phenotype, suggesting its possible involvement in tumorigenesis. The parafibromin subunit of the hPAF complex is a product of the HRPT-2 (hereditary hyperparathyroidism type 2) tumor suppressor gene, which is mutated in the germ line of hyperparathyroidism-jaw tumor patients. This review focuses on the functions of the PAF complex and its individual subunits, the interaction of the subunits with each other and/or with other molecules, and dysregulation of the complex, providing an insight into its potential involvement in the development of cancer.
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Introduction

The dysregulation of critical genes is the crux of cancer development and progression. This results in activation of proto-oncogenes, inactivation of antioncogenes and chromosomal instability (Batra *et al*., 1994; Vogelstein and Kinzler, 2004). Genetic and epigenetic alterations lead to losses of or abnormal function of genes affecting processes that maintain or regulate orderly normal cell function resulting in the phenotypic manifestation of specific types of the cancer (Thiagalingam, 2006). The aberrant expression of molecules that influence or are involved in transcription and cell cycle, like cyclin D1 (Knudsen *et al*., 2006) and Myc oncoprotein (Grandori *et al*., 2004), is primarily responsible for oncogenic signals to cells. These proteins are found to be associated with cancer progression either by overexpression or by downregulation during the course of the disease. We have reported a new candidate oncogene, pancreatic differentiation 2 (*PD2*)/*hPaf1*, a member of the hPAF complex, which is dysregulated in cancer (Moniaux *et al*., 2006). Another component of the hPAF complex with a known role in cancer is the hCdc73 subunit, also called parafibromin (Zhang *et al*., 2006). The RNA polymerase II-associated factor (PAF) complex was initially reported in yeast (yPAF) (Supplementary text – section A) and comprises five subunits, Paf1, Ctr9, Leo1, Rtf1 and Cdc73 (Krogan *et al*., 2002). yPAF is associated with both the promoter and coding regions of transcriptionally active genes and plays a role in transcription elongation. This complex also plays a role in mRNA processing and maturation, being responsible for maintaining proper poly(A) tail lengths (Mueller *et al*., 2004).

Although both the yeast and human PAF (hPAF) complexes have five subunits, the hPAF complex differs from yPAF in having an additional eukaryotic specific hSki8 subunit and lacking the human homolog of yRtf1 subunit (Figure 1). Rtf1 does not appear to be a stable part of the human and *Drosophila* PAF complex. However, Paf1 and Rtf1 associate during active

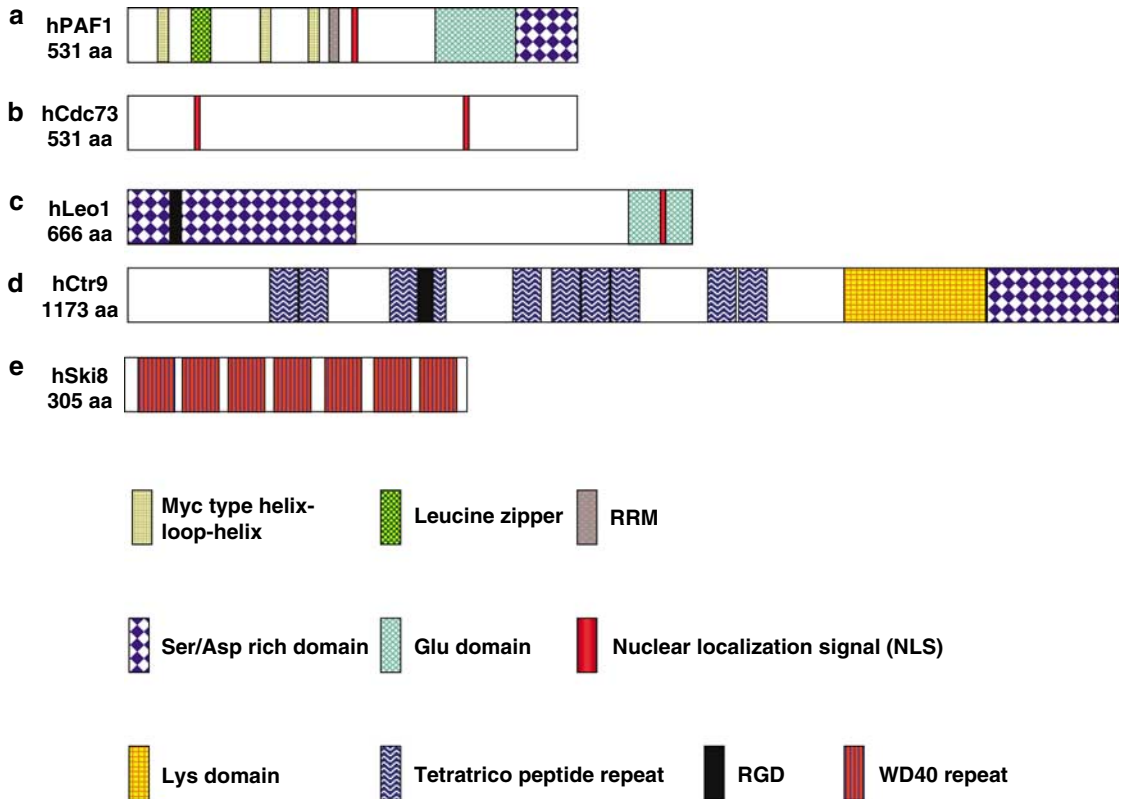

Figure 1 A schematic diagram of the structural domains of various components of human RNA polymerase II-associated factor (hPAF) complex. (**a**) Pancreatic differentiation 2 (PD2)/hPaf1 contains three Myc type helix–loop–helix, a leucine zipper, an RNA recognition motif (RRM), Ser/Asp-rich region with possible catalytic function, a Glu-rich domain and one nuclear localization signal (NLS). (**b**) Parafibromin/hCdc73 has five NLSs, but only two bipartite NLS shown are functional. (**c**) hLeo1 is a Ser/Asp-rich protein containing RGD (Arg-Gly-Asp) sequence that can function for cell adhesion, and an NLS domain. (**d**) hCtr9 is the largest component of the complex containing a tetratrico peptide repeat for protein–protein interactions. (**e**) hSki8 is a WD40-repeat-rich protein that is involved in post-transcriptional processing of RNA. WD40 repeats are found in a number of eukaryotic proteins that cover a wide variety of functions including adaptor/regulatory modules in signal transduction, pre-mRNA processing, cytoskeleton assembly and cell-cycle control.

transcription in *Drosophila* (Supplementary text – section B). Like the yPAF complex, hPAF regulates transcription-associated histone monoubiquitination of H2B, wherein recruitment of histone chaperone FACT (facilitates chromatin transcription), hPAF and the H2B monoubiquitination machinery is a necessary step for elongation by RNAP II (Pavri *et al*., 2006). The hCdc73 subunit of hPAF induces cell-cycle arrest in the G_1 phase by blocking expression of cyclin D1 (Zhang *et al*., 2006). Thus, like its yeast counterpart, the hPAF complex may also have an important role in cell-cycle regulation. The additional subunit, hSki8, is a core protein of the hSKI complex, and the association of hSKI with actively transcribed genes is dependent on the presence of the hPAF complex. This review discusses the information available on hPAF and its individual subunits, describing its role in transcription, cell cycle and other cellular functions. It also examines the amplifications and losses of the genes encoding the complex proteins in various cancers and the ability of hPAF to function individually or as a complex in the initiation and progression of cancer.

Functions of the human PAF complex

The hPAF complex was first identified during characterization of proteins associated with parafibromin (Rozenblatt-Rosen *et al*., 2005). It is well documented that hPAF shares four subunits with yPAF (Ctr9, Paf1, Leo1 and Cdc73) and also contains a novel higher eukaryotic-specific subunit, hSki8 (Zhu *et al*., 2005). Rozenblatt-Rosen *et al*. (2005) identified the hPAF complex proteins by immunoprecipitation experiments in HEK293T cells using polyclonal anti-parafibromin antibody. Subsequently, Zhu *et al*. (2005) determined the hPAF complex components via conventional chromatography, following elution with PD2/hPaf1 antibody, and identified a novel interaction partner, hSki8. The authors suggest that this ambiguity of the result may be due to the difference in purification approaches used by the two groups. Rozenblatt-Rosen *et al*. (2005) used antibodies against hCdc73 followed by elution with acidic glycine, which causes elution of large amounts of IgG. The presence of IgG might have thus masked the low-molecular-weight proteins such as hSki8.

The hPAF complex interacts with the non-phosphorylated and Ser2- and Ser5-phosphorylated forms of the RNAP II large subunit (Rozenblatt-Rosen *et al*., 2005), indicating its involvement in both transcription initiation and elongation. Removal of the nucleosomal barrier, which involves the monoubiquitination of H2B, is a prerequisite for efficient elongation on chromatin by RNAP II. Pavri *et al*. (2006) showed that the establishment of H2B monoubiquitination is dependent on hPAF, the histone chaperone FACT and transcription (Figure 2). Like its yeast counterpart, hPAF has also been implicated in other cellular functions. The parafibromin subunit of the hPAF complex regulates cell cycle by blocking expression of cyclin D1 and causing G1-phase arrest (Zhang *et al*., 2006). In yeast, the SKI complex, which contains the Ski8 subunit, is localized in the cytoplasm and, along with the exosome, is responsible for 3′–5′ mRNA degradation. The localization of the human SKI complex, unlike yPAF, is extended to the nucleus and the cytoplasm. Interestingly, hPAF and hSKI complexes interact, and the association of hSKI with transcriptionally active genes is dependent on the presence of hPAF, suggesting a role of this complex in mRNA quality control (Zhu *et al*., 2005).

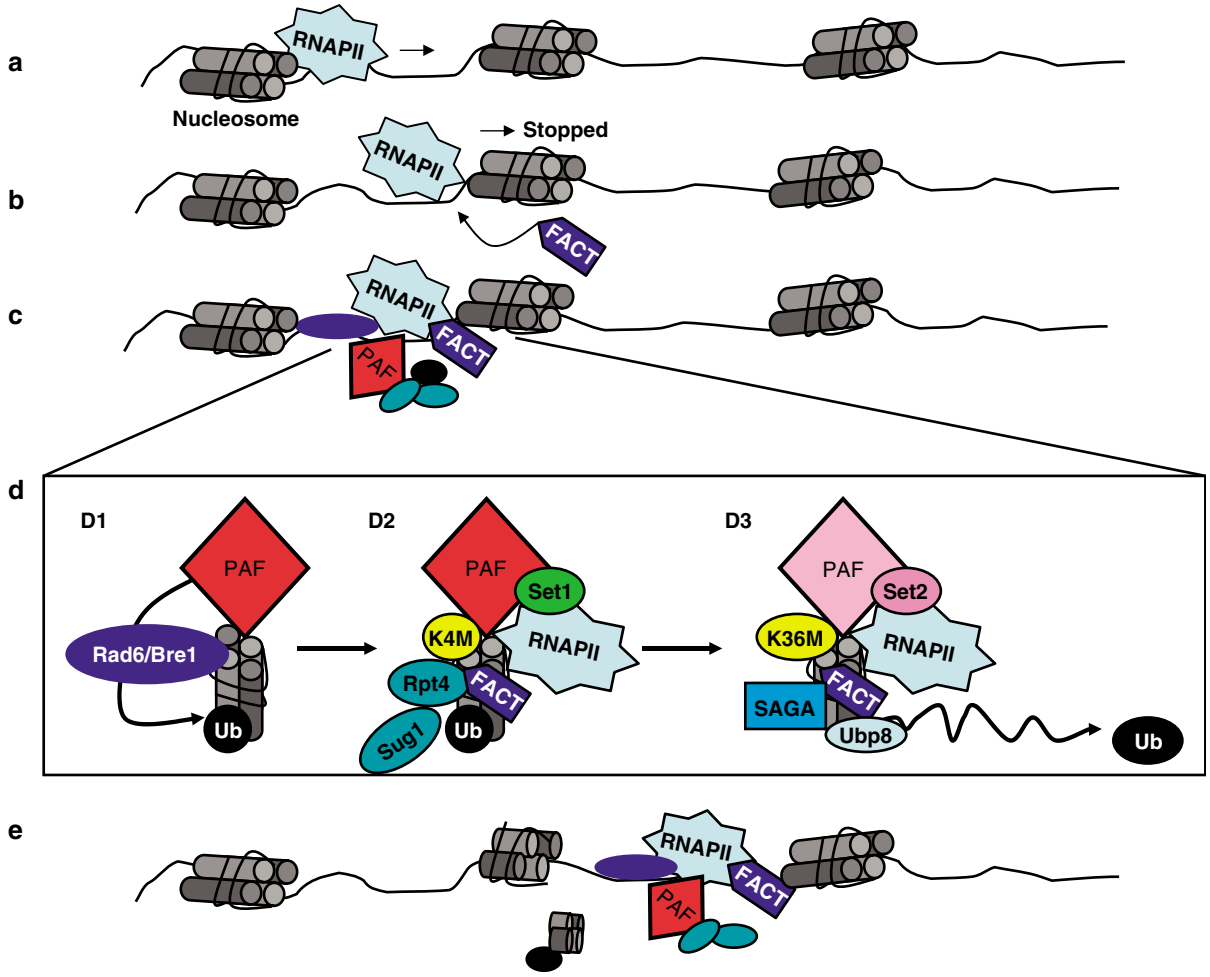

Figure 2 A schematic representation of polymerase II-associated factor (PAF) complex-mediated H2B monoubiquitination for efficient transcription through chromatin. (**a**) Transcription starts with walking of RNAP II over chromatin. (**b**) Efficiency of transcription by RNAP II reduces at the first nucleosome. At this stage, FACT (facilitates chromatin transcription) is recruited over chromatin. (**c**) FACT alone is inefficient in removing the H2A/H2B dimer, resulting in a poor elongation rate. FACT recruits the PAF complex and the ubiquitination machinery. (**d**) This results in the monoubiquitination of H2B and methylation of H3-K36. (**D1**) The PAF complex induces Rad6/Bre1-mediated monoubiquitination of H2B. (**D2**) H2B monoubiquitination is required for the subsequent hiring of the proteasomal ATPases, Sug1 (Rpt6) and Rpt4. This is followed by the methylation of H3-K4 by Set1, which is recruited by the Ser5-phosphorylated RNAP II C-terminal domain (CTD) and PAF. This process requires the presence of Sug1 and Rpt4. (**D3**) Following H3-K4 methylation, H2B is deubiquitinated by Ubp8 subunit of the SAGA (Spt-Ada-Gcn5-acetyltransferase) acetyltransferase complex that allows the Ser2-phosphorylated CTD and PAF recruitment of Set2 and the displacement of Set1. Set2 then methylates H3-K36. H2B monoubiquitination prevents the premature methylation of H3-K36 and serves to regulate these distinct methylation patterns. In the whole process, it is not known why Set1 is replaced by Set2. (**e**) This is followed by efficient displacement of one H2A/H2B dimer from the nucleosome barrier. The dissociated nucleosome is now easily traversed by RNA polymerase II. This process repeats itself on successive nucleosomes resulting in an overall increase in the efficiency of elongation.

PD2/hPaf1 is a new candidate oncogene

During a study to identify differentially expressed genes that may play a role in pancreatic tumor growth and progression, our laboratory identified a double minute amplification corresponding to the chromosomal locus 19q13 (Batra *et al*., 1991). We found that *PD2/hPaf1*, is overexpressed in the poorly differentiated pancreatic cancer cell line, Panc1. Further, the targeted overexpression of this gene in the NIH3T3 cell line led to transformation of the cells (Moniaux *et al*., 2006). These results suggest that the *PD2/hPaf1* gene is a potential candidate of the oncogene family. The basis for overexpression of *PD2/hPaf1* in the Panc1 cell line is a gain in copy number of the gene through an amplification of its locus. We determined the exact location of *PD2/hPaf1* to be on chromosome 19 in the q13.1 region (Moniaux *et al*., 2006), as illustrated in Figure 3a. Interestingly, *PD2/hPaf1* was present on the same amplicon as the potent oncogene, *AKT2*. Activation of *AKT2* triggers various cellular pathways involved in tumorigenesis, such as cell-cycle progression and cell survival. In addition, Cheng *et al*. (1996) reported that the overexpression of *AKT2* in pancreatic cancer cell lines led to an increase in their aggressiveness. These authors also proposed that co-amplification of genes might be responsible for the malfunctioning of this locus. Further, Curtis *et al*. (1998) reported the amplification of the 19q13.1 locus in various pancreatic cancer cell lines, suggesting a potential role of the genes of this locus in the development of pancreatic cancer. A comprehensive list of cancers with a gain in copy number of this locus is given in Table 1. The presence of *PD2/hPaf1* and *AKT2* on the same amplicon and the overexpression of *PD2/hPaf1* in the Panc1 cell line suggest a synergistic role of the two proteins in the stabilization of the 19q13 locus and the development of an oncogenic phenotype in Panc1 cells.

Analysis of the domain structure of PD2/hPaf1 revealed a high degree of similarity between PD2/hPaf1 and the functional domains of DNA- and RNA-binding proteins (Moniaux *et al*., 2006). Among other motifs, it

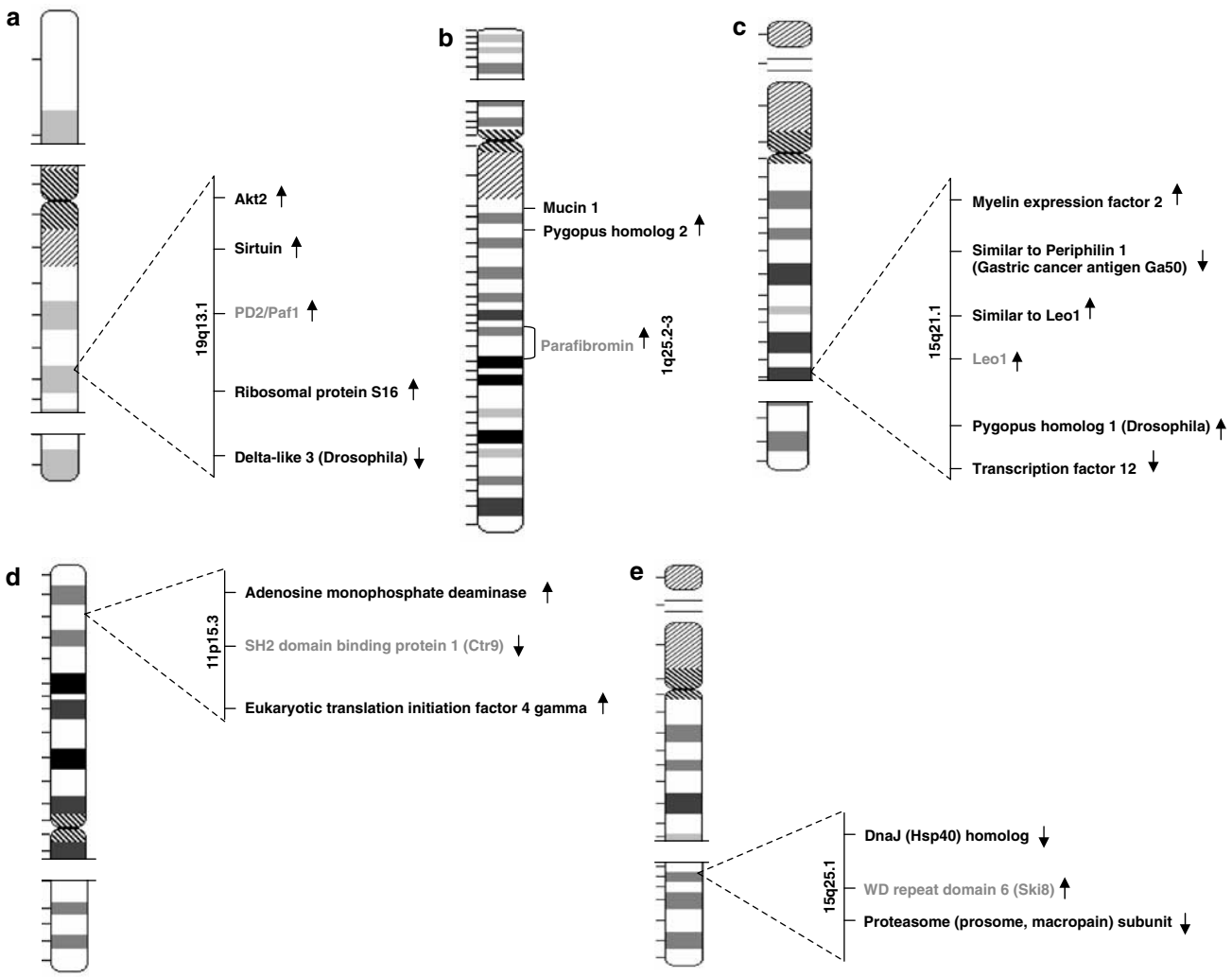

Figure 3 Chromosomal localization of various members of the hPAF complex. The direction of arrows indicates the orientation of transcription of the genes on the chromosome. (**a**) PD2/hPaf1, (**b**) Cdc73, (**c**) Leo1, (**d**) Ctr9 and (**e**) Ski8.

Table 1 Gain in locus 19q13 in various cancer cell lines and tumors

Cell line or tumor type	*Targeted gene*	*Method*	*Remarks*	*Reference*
AICPC-1 SU86.86	Mixed lineage leukemia (*MLL*) gene	FISH and slot blotting	Pancreatic cancer cell lines	Huntsman *et al.* (1999)
H4	Mixed lineage leukemia (*MLL*) gene	FISH and slot blotting	Glioblastoma cell line	Huntsman *et al.* (1999)
HPAC, PANC-1, BxPC-3, AICPC-1 AICPC-2	*OZF* and *AKT2*	FISH	Pancreatic cancer cell lines	Curtis *et al.* (1998)
UACC326, UACC1123, UACC2727, and OVCAR-3	*AKT2* and *SEI-1*	FISH	Ovarian cancer cell lines	Tang *et al.* (2002)
Pancreatic tumor cell lines	Hypothetical protein F231491 Mitochondrial ribosomal protein S12 p21 (CDKN1A)-activated kinase 4	CGH microarray		Mahlamaki *et al.* (2004)
	Ribosomal protein S16			Batra *et al.* (1991)
Pancreatic adenocarcinoma	—	CGH microarray		Schleger *et al.* (2000)
Panc-1	PD2/hPaf1	DNA microarray	Poorly differentiated pancreatic cell line	Heidenblad *et al.* (2005)
		Differential screening		Moniaux *et al.* (2006)

Table 2 Cancer cell lines and tumors exhibiting a gain of the locus 1q25

Cell line/tumor type	*Targeted gene/band*	*Method*	*Reference*
Primary liver carcinomas	1q25	G-banded with Wright's stain	Parada *et al.* (1998)
Invasive breast cancers	1q arm	Matrix-comparative genomic hybridization	Stange *et al.* (2006)
Childhood non-brainstem glioblastomas	1q25	FISH	Korshunov *et al.* (2005)
Pancreatic cancer	1q arm	CGH	Chang *et al.* (2005)

also possesses three helix–loop–helix (HLH) domains, a DEAD-box subfamily ATP-dependant helicase domain, one eukaryotic RNA recognition motif, RNP-1 region signature and a regulator of chromosome condensation signature (Figure 1a). In mammals, HLH proteins have critical roles in development, cell growth, differentiation and apoptosis. Many sequence-specific DNA-binding proteins that act as transcription factors share a conserved HLH domain, like Myc oncoprotein and human transcription factor AP-4. PD2/hPaf1 may thus have putative functions, which are in accordance to its domain structure and are similar to its yeast homolog.

Parafibromin, a tumor suppressor, participates in Wnt signaling

Parafibromin, a ~64 kDa protein, is a component of the human PAF complex and a homolog of *Saccharomyces cerevisiae* Cdc73 (Cell division cycle 73). It is encoded by the *HRPT2* gene and is associated with suppression of the hyperparathyroidism-jaw tumor (HPT-JT) syndrome. The *HRPT2* gene is mutated in the germ line of HPT-JT patients. Mutation of this component of the hPAF complex results in the loss of association between the remaining members of the complex and chromatin, and a significant reduction in binding of the complex to RNAP II (Rozenblatt-Rosen *et al.*, 2005). Recent studies on parafibromin have reported a nuclear localization of this protein (Rozenblatt-Rosen *et al.*, 2005), with another study reporting its localization as nucleocytoplasmic (Woodard *et al.*, 2005). A clear functional nuclear localization signal (NLS) was not identified until one study reported that parafibromin contains a functional bipartite (BP) NLS (Hahn and Marsh, 2005). However, a recent report reveals that it contains five putative NLSs, three BP and two monopartites (MP) (Figure 1b), and that only one of these is evolutionarily conserved and functions as MP NLS (Bradley *et al.*, 2007).

The behavior of parafibromin reported so far in cancers is its potential function as a tumor suppressor. Transient overexpression of wild-type parafibromin, but not that of its Leu64Pro missense mutant, inhibited cell proliferation and blocked the expression of cyclin D1, a key cell-cycle regulator previously implicated in parathyroid neoplasia (Woodard *et al.*, 2005). The Leu64Pro mutant form of parafibromin is associated with parathyroid cancer and familial isolated hyperparathyroidism.

Interestingly, the *HRPT2* locus, 1q25 (Figure 3b), is amplified in liver carcinoma (Parada *et al.*, 1998). Recently, it was also reported that the 1q arm (1q24.2–25.1 and 1q25.3–q31.3) is amplified in breast cancer (Stange *et al.*, 2006). Molecular analyses conducted on a set of 44 tumor samples obtained from pediatric patients with non-brainstem glioblastoma revealed a gain in the 1q25 band (Korshunov *et al.*, 2005) (Table 2). In another interesting report, Chang *et al.* (2005) found a gain in the locus of the parafibromin gene in pancreatic head cancers, suggesting a possible involvement of this subunit of the hPAF complex together with PD2/hPaf1 in the development of a subset of pancreatic cancers.

Recently, parafibromin and its *Drosophila* ortholog, Hyrax, were shown to interact with β-catenin/Armadillo

during Wnt/Wg signaling and were necessary for nuclear transduction of the Wnt/Wg signal (Mosimann *et al*., 2006). In humans, the Wnt pathway controls cell fate and homeostasis, and an altered Wnt signaling contributes to cancer (Figure 4). Mosimann *et al*. (2006) showed that the knockdown of Hyx in *Drosophila* and of parafibromin in cultured cells disturbed the β-catenin-mediated Wg/Wnt signal-transduction pathway. They further showed that overexpression of parafibromin led to a two- to threefold increase in Wnt target genes in human cells. These results suggest that parafibromin positively synergizes with components of the human Wnt cascade to enhance the transcription of Wnt target genes. Further confirmation of the role of parafibromin in Wnt signaling comes from the demonstration that parafibromin/Hyx directly binds β-catenin/arm via the N-terminal region. The basic difference between yeast Cdc73 and its metazoan homologs, parafibromin and Hyx, is the presence of an additional extended N-terminal region. The authors speculate that the metazoan homologs evolved in their N-terminal domain for specific signal-transduction pathways such as Wnt/Wg pathway, while conserving the C-terminal sequence for PAF complex and/or RNAP II association. Mosimann *et al*. (2006) also suggested the involvement of the whole PAF complex in Wnt signaling (Figure 4). Thus, parafibromin appears to be a key player along with PD2/hPaf1 in neoplastic transformation owing to the amplification of its locus and its involvement in the Wnt pathway.

Leo1: interacts with β-catenin during Wnt signaling cascade

Leo1 is a 105 kDa (Figure 1c) hPAF complex component and is of major interest due to its interaction with β-catenin in the Wnt cascade (Mosimann *et al*., 2006) (Figure 4). The 15q21 locus (Figure 3c) that contains the *Leo1* gene is amplified in colorectal cancer and malignant fibrous histiocytoma of bone (Camps *et al*., 2006; Tarkkanen *et al*., 2006). This region has a potential osteosarcoma suppressor gene identified by a

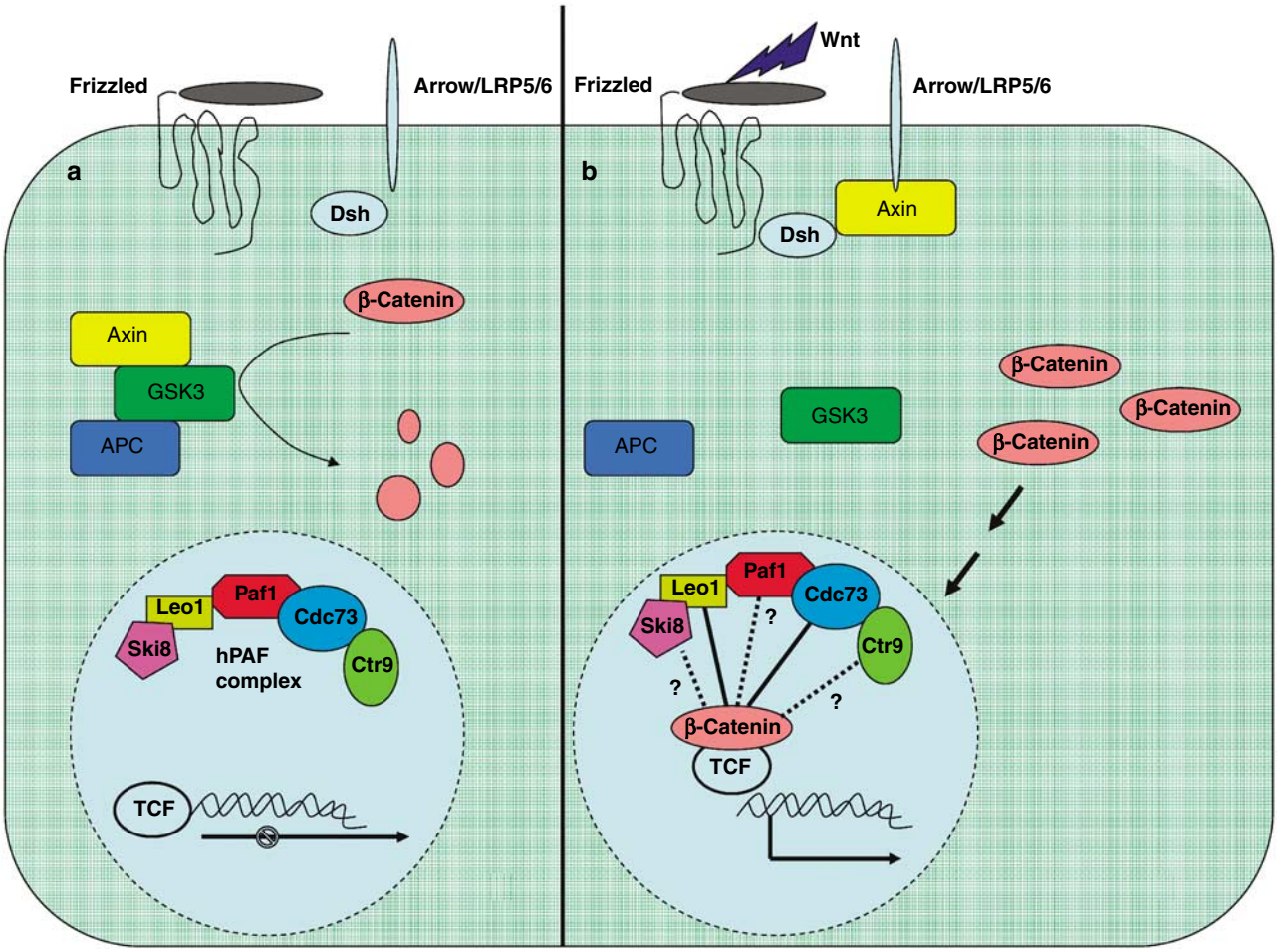

Figure 4 Mechanistic model of Wnt signaling and involvement of hPAF complex. (**a**) In the absence of a Wnt signal, axin, APC and protein kinase GSK-3 promote β-catenin degradation. (**b**) In the presence of a Wnt signal, the Frizzled/LRP receptor complex binds to Wnt proteins, which mediate a signal to stimulate the binding of Disheveled (Dsh) and Axin, leading to the inhibition of β-catenin degradation in cytoplasm. β-Catenin accumulates in the nucleus where subunits of PAF complex, parafibromin and Leo1, interact with it and regulate the transcription of certain genes.

combination of synteny and microsatellite mapping (Nathrath *et al*., 2002). Interestingly, the interaction of Leo1 with nuclear β-catenin suggests an alternate role of this protein in cancer development. Much remains to be known of Leo1 in terms of its interactions and functions, but its association with β-catenin and the imbalance of its locus, 15q21, are the primary areas for studying its potential role in tumor development.

Ctr9, together with Paf1, functions as a cell-cycle regulator

Koch *et al*. (1999) studied the transcriptional activation of G_1 cyclin genes, *CLN1* and *CLN2*, in late G_1 phase in yeast. They performed a genetic screen for novel regulators of Start-specific gene expression, where Start is the point at which cells commit to a new mitosis. They identified two novel genes involved in transcription in late G_1, *CTR9* and *PAF1*. They further showed that Ctr9 and Paf1 are components of a high molecular weight protein complex that also includes Cdc73. In their study, yeast mutants of *paf1* and *ctr9* showed nearly identical phenotypes, were morphologically similar and had reduced *CLN2* expression, suggesting that the two proteins might function as a complex.

The *CTR9* gene has been localized to chromosome 11p15 (Figure 3d). The 11p15.3 locus contains chromosomal aberrations associated with the pathogenesis of different tumor types including lung cancer and leukemia (Redeker *et al*., 1995). Interestingly, the Ctr9 gene locus is found to be deleted in pancreatic cancer (Bashyam *et al*., 2005) (Table 3). As mentioned previously, a gain of the 19q13 locus has been observed in pancreatic cancer cell lines, which suggests that hCtr9 may play an opposing role to PD2/hPaf1 in the pathogenesis of pancreatic cancer. Zhu *et al*. (2005), however, reported that siRNA-mediated silencing of hCtr9 leads to a reduced expression of other complex subunits. These observations suggest that Ctr9 plays multiple roles in the complex and further studies are required to elucidate its exact functions.

Ski8: a eukaryotic specific subunit of the hPAF complex

Proteins containing WD repeats (WDR) (also known as WD40 or β-transducin repeats) have been reported to play a crucial role in a wide range of physiological functions including signal transduction, RNA processing, remodeling of cytoskeleton, regulation of vesicular trafficking and cell division (reviewed by Rybakin and Clemen, 2005). The Ski8 subunit of the hPAF complex also contains a WDR domain (Figure 1e) and plays a role in RNA surveillance. hSki8 is also a component of the human SKI (hSKI) complex. In yeast, the SKI complex together with the exosome is essential for mediating 3′–5′ mRNA decay. The human SKI complex localizes to transcriptionally active genes in an hPAF-dependent manner. This novel link between hPAF and hSKI suggests that the hPAF complex coordinates events downstream of RNA synthesis, such as RNA surveillance (Zhu *et al*., 2005).

The human *Ski8* gene is present on locus 15q25.1 (Figure 3e). Armengol *et al*. (2000) performed comparative genomic hybridization (CGH) analyses of samples of xenografted human pancreatic tumors and of two metastases developed in mice. They observed a gain in chromosome 15 (15q25–q26) in six out of eight cases and in chromosome 19 (19q) in five out of eight cases. A loss in the 15q25 locus was seen in colorectal cancer (Birkenkamp-Demtroder *et al*., 2002), and both the 15q25 and 19q13 loci were deleted in primary cutaneous B-cell lymphoma (Mao *et al*., 2002). These studies suggest varying functions of hSki8 in different types of cancers.

Using the poorly differentiated Panc-1 cell line as a model, both hSki8 and PD2/hPaf1 were found to be overexpressed (unpublished lab data). This suggests that hSki8 may act as a mediator in RNA quality control during the transcription of genes responsible for the oncogenic phenotype in the Panc-1 cell line. This hypothesis is further supported by the observation that endonuclein, a protein that also possesses WDR sequences, is upregulated in pancreatic adenocarcinoma (Honore *et al*., 2002). However, detailed studies about this eukaryotic specific subunit of the hPAF complex are necessary to further understand its functions.

Conclusions and perspectives

Genetic imbalance is one among several mechanisms that contributes to the development of cancer. The *PD2/hPaf1* gene locus, 19q13, is well known owing to the presence of the oncogene *AKT2* and its amplification in pancreatic cancer. The stabilization of the 19q13 amplicon, detected in 10–20% of pancreatic adenocarcinomas, might involve a synergistic interaction of *PD2/hPaf1* with *AKT2*. The *Ctr9* gene locus, 11p15.3, is

Table 3 Gain/loss in locus 11p15 in various cancer cell lines and tumors

Cell line/tumor type	*Targeted gene/band*	*Method*	*Gain/loss*	*Reference*
Primary breast tumors and their matched sentinel lymph nodes	11p15	CGH	Loss	Cavalli *et al*. (2003)
Hepatocarcinoma samples	—	CGH	Gain	Schwienbacher *et al*. (2000)
Neuroblastomas	11p15	CGH	Loss	De *et al*. (2005)
Pancreatic cancer cell lines	11p15	CGH	Loss	Bashyam *et al*. (2005)

Abbreviations: CGH, comparative genomic hybridization; FISH, fluorescence *in situ* hybridization.

deleted in pancreatic cancer, suggesting a tumor suppressor function of this subunit. The *PD2/hPaf1* locus amplification with deletion of *Ctr9* gene locus may lead to initiation and progression of pancreatic cancer. The parafibromin gene locus, 1q25, is amplified in liver and breast cancers, while the Leo1 gene locus (15q21) shows an imbalance in many other types of cancers. The function of Leo1 and parafibromin in the pathogenesis of malignant change is still an open question, with suggestions that they can act as both pro- and anti-oncogenic factors. This dual role of the hPAF complex components is supported by the contrasting amplification and deletion of the *hSki8* locus, a member of both the hPAF and hSKI complexes, in pancreatic cancer and lymphoma, respectively.

In a cellular milieu, a biological complex is in a dynamic state and the stoichiometry of the complex components plays a crucial role in determining its varied functions. Therefore, studying the interactions and interdependence of the subunits, the variations in the expression of each subunit in relation to the different functions of the complex as well as in normal versus diseased states is important to understand fully the roles of the PAF complex and its relationship with disease.

This complex has an established role in transcription elongation and is also emerging as an important player in cancer biology. The Wnt cascade is dysregulated in cancer and, as discussed previously, two subunits of the hPAF complex, parafibromin and Leo1, interact with the Wnt cascade proteins and affect the target gene expression. Therefore, a detailed study elucidating the involvement of hPAF in the Wnt signaling pathway may provide clues to a potential link between hPAF and cancer pathogenesis. The hPAF complex is also associated with the cell cycle. In particular, parafibromin induces cell-cycle arrest in the G1 phase. Another link, although indirect, comes from the fact that the *PD2/hPaf1* gene is present on the same amplicon as the oncogene *AKT2*, and activation of *AKT2* triggers various cellular pathways involved in tumorigenesis, such as cell-cycle progression and cell survival. As cell-cycle regulation is a key phenomenon affected in cancer, it would be interesting to unravel the association between hPAF and cell cycle in relationship with tumorigenesis. Looking ahead, studies related to this novel complex may help deepen our understanding about its role in normal and cancer cell biology.

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