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REVIEW ARTICLE

PRH/Hex: an oligomeric transcription factor and multifunctional regulator of cell fate

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The PRH (proline-rich homeodomain) [also known as Hex (haematopoietically expressed homeobox)] protein is a critical regulator of vertebrate development. PRH is able to regulate cell proliferation and differentiation and is required for the formation of the vertebrate body axis, the haematopoietic and vascular systems and the formation of many vital organs. PRH is a DNA-binding protein that can repress and activate the transcription of its target genes using multiple mechanisms. In addition, PRH can regulate the nuclear transport of specific mRNAs making PRH a member of a select group of proteins that control gene expression at the transcriptional and translational levels. Recent biophysical analysis of the PRH protein has shown that it forms homo-oligomeric complexes *in vivo* and *in vitro* and that the proline-rich region of PRH forms a novel dimerization interface. Here we will review the current literature on PRH and discuss the complex web of interactions centred on this multifunctional protein.

Key words: development, gene regulation, haematopoiesis, haematopoietically expressed homeobox (Hex), homeodomain, oligomerization, proline-rich homeodomain (PRH), transcription.

INTRODUCTION

The PRH (proline-rich homeodomain) protein, also known as the Hex (haematopoietically expressed homeobox) protein (also termed Hhex or HHEX) is a transcription factor that contains the well-characterized DNA-binding domain termed the homeodomain. PRH is a relatively unusual homeodomain protein in that it appears to exist as a homo-oligomer *in vivo* and *in vitro*. Like most homeoproteins, PRH regulates cell development and differentiation. To regulate cellular development, PRH modulates gene expression using both transcriptional and post-transcriptional mechanisms. Some of the genes that PRH regulates encode proteins that are involved in control of the cell cycle or in growth factor signalling pathways. In addition PRH makes direct protein–protein interactions with many cellular proteins that influence the cell cycle. Thus PRH also functions as a potent regulator of cell proliferation.

PRH expression was first detected in avian and human haematopoietic cells [1,2]. Subsequently, PRH genes have been cloned from many species including, mouse (Hex) [3], *Xenopus* (Xhex), zebrafish (hhex) and rat [4–6] (Figure 1). Orthologues have also been identified in invertebrates such as *Caenorhabditis elegans* (pha-2) [7] but PRH is not present in yeast [2]. PRH is not confined to the haematopoietic compartment. PRH expression has been detected in cDNA libraries derived from unfertilized human oocytes and 2–8 cell stage embryos [8]. During mouse early embryogenesis, PRH mRNA is first detected in extra-embryonic tissues [9] that are involved in anterioposterior axis formation and formation of the primitive vasculature and blood system [9]. After gastrulation, PRH is expressed within the embryo itself: in mesodermal tissues that give rise to haematopoietic and vascular progenitors and the endocardium of the heart, and in endodermal tissues that are involved in the formation of organs such as the liver, thyroid, lung, thymus, gallbladder and pancreas [9–11]. Similar patterns of PRH expression, as determined by *in situ* hybridization, also occur during early development of the chick and frog [5,12]. In the adult, PRH is expressed in the thyroid, lung and liver, and in the haematopoietic compartment (bone marrow) [2,10,13,14]. PRH serves a variety of roles in the control of cell differentiation and cell proliferation of these tissues. As might be expected of a regulator of gene expression, misexpression of PRH and/or altered subcellular distribution is associated with a number of diseased states, including leukaemia and cancer.

Studies in avian, murine, *Xenopus*, zebrafish and human cells have shown that the functions of PRH are similar in all vertebrate species. In the present review we will focus on the structure of the PRH protein based upon studies of the human and avian proteins. In addition we will review the mechanisms that PRH proteins from all species use to regulate gene expression and cell proliferation. Finally we will review the role of PRH in development, with particular focus on the importance of PRH in haematopoietic and vascular differentiation.

Abbreviations used: ADE, anterior definitive endoderm; AML, acute myelogenous leukaemia; AP-1, activator protein-1; APL, acute promyelocytic leukaemia; AUC, analytical ultracentrifugation; AVE, anterior visceral endoderm; BMP, bone morphogenetic protein; BRE, BMP-responsive element; CML, chronic myelogenous leukaemia; CRE, cAMP-response-element; CREB, CRE-binding protein; E, embryonic day; eIF-4E, eukaryotic initiation factor 4E; EMSA, electrophoretic mobility-shift assay; ES, embryonic stem; ESM-1, endothelial cell-specific molecule-1; Fgf, fibroblast growth factor; Hex, haematopoietically expressed homeobox; HNF, hepatocyte nuclear factor; HOX, homeobox; HSC, haematopoietic stem cell; HUVEC, human umbilical-vein endothelial cell; NK, nuclear body-associated kinase; NMHC-B, non-muscle myosin heavy chain B; NTCP, sodium-dependent bile acid co-transporter; PML, promyelocytic leukaemic; PRH, proline-rich homeodomain; RARα, retinoic acid receptor α; SM, smooth muscle; SRF, serum-response factor; TBP, TATA-box-binding protein; Tg, thyroglobulin; TIE, TK with immunoglobulin-like and EGF (endothelial growth factor)-like domains; TK, thymidine kinase; TLE, transducin-like enhancer; TN, tinman; TSH, thyroid-stimulating hormone; TTF, thyroid transcription factor; VE, visceral endoderm; VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor; VSMC, vascular smooth muscle cell.

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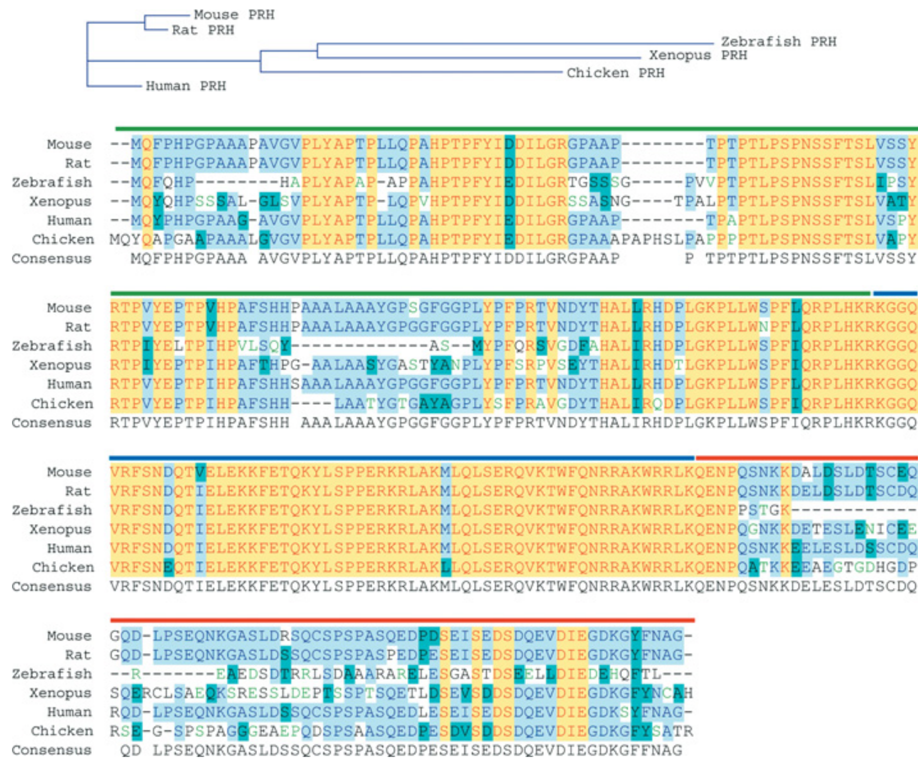

Figure 1 Sequence similarity of the PRH proteins from different vertebrate species

Upper part of the Figure: phylogenetic analysis of PRH. Lower part of the Figure: the amino acid sequence of mouse PRH (AAH57986), rat PRH (NP_077361), zebrafish PRH (NP_571009), *Xenopus* PRH (NP_989420), avian PRH (Q05502) and human PRH (Q03014) were aligned according to the ClustalW method. Identical residues are highlighted in yellow and similar residues in blue and green. The green line represents the N-terminal domain of PRH, the blue line the PRH homeodomain and the red line the C-terminal domain of PRH.

PRH PROTEIN

Human PRH is 270 amino acids in length and has a molecular mass of 30 kDa. The human and chicken PRH proteins share 97 % identity across the homeodomain region and 75 % identity over the whole protein [1]. In fact the human and murine PRH proteins differ by only a single amino acid across the homeodomain [13] and are 94 % identical over the whole PRH protein emphasizing the conservation of PRH (see Figure 1). A comparison of the human and avian proteins has revealed that they show very similar properties [15]. PRH appears to exhibit the modular nature found in most transcription factors and consists of three main domains: an N-terminal proline-rich domain (amino acids 1–136 in human PRH), the homeodomain (amino acids 137–196 in human PRH) and an acidic C-terminal region (amino acids 197–270 in human PRH) [1] (Figure 2).

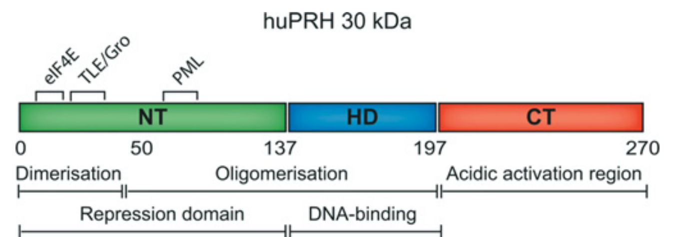

Figure 2 The domain organization of PRH

A schematic showing the PRH protein divided into three main domains: an N-terminal proline-rich domain (green; NT), a homeodomain (blue; HD) and an acidic C-terminal domain (red; CT). The correlated function of each region of the protein is also illustrated in the Figure. huPRH, human PRH.

The N-terminal domain

The PRH N-terminal domain (amino acids 1–136) is rich in proline (20 %), alanine (15 %) and glycine (10 %) residues [1]. Purification of the N-terminal domain and analytical ultracentrifugation and gel-filtration studies have shown that this domain forms dimeric structures in solution [15]. This dimeric form of the PRH N-terminal domain has a number of interesting properties. First, it is partially resistant to unfolding by SDS. Secondly, the CD spectrum of this domain does not show either typical α-helical or β-sheet secondary structures and, finally, fluorescence denaturation experiments indicate that the dimer is not a globular tightly folded domain but rather a more extended and mobile structure, similar to proteins such as elastin [15]. Two-hybrid analysis and pulldown assays demonstrate that although multiple regions of the N-terminal domain are involved in dimerization, the N-terminal 50 amino acids may play a critical role. A second region within the N-terminal domain downstream of these 50 residues is involved in interactions with the PRH homeodomain and hence in oligomerization [15] (see below).

The PRH N-terminal domain represses transcription independently of the PRH homeodomain when fused to a heterologous DNA-binding domain [6,16]. Deletion studies, together with mutagenesis and pulldown assays, show that the N-terminal domain contains several regions that contribute to transcriptional repression [16]. A strong repression region has been identified downstream of the N-terminal 45 amino acids of rat PRH or the N-terminal 48 amino acids of avian PRH, when fused to the GAL4 DNA-binding domain [6,16]. Interestingly, the N-terminal

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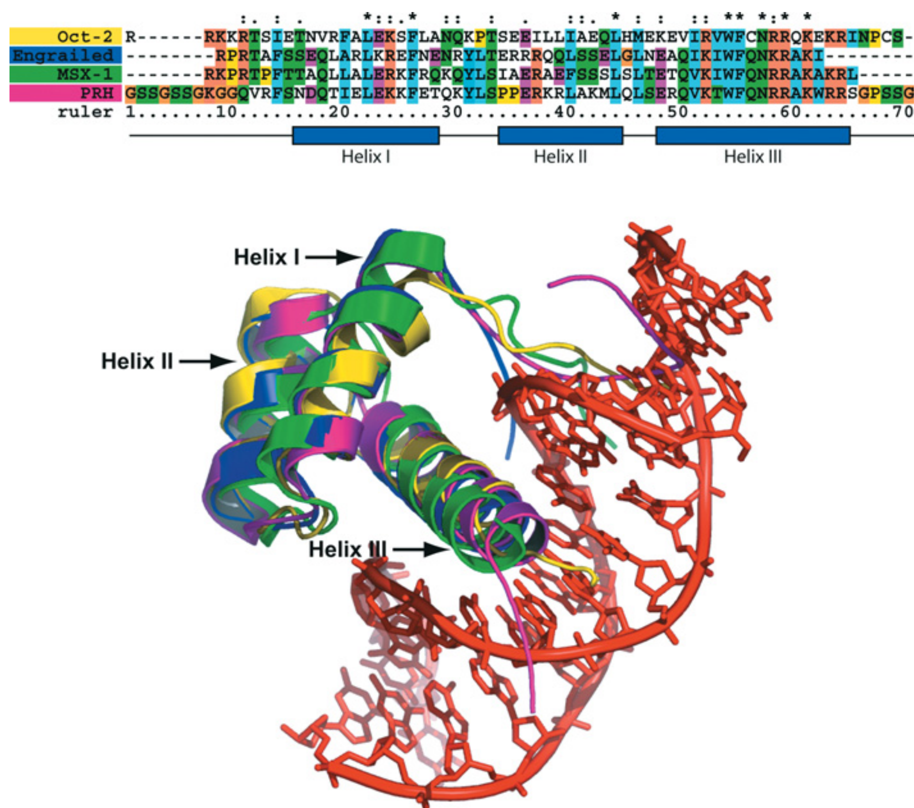

Figure 3 A molecular model of the PRH homeodomain bound to DNA

Upper part of the Figure: the amino acid sequences of the homeodomain proteins Engrailed, Oct-2, Msx-1 and PRH were aligned according to their three-dimensional structures. The alignment was carried out using the three-dimensional superimpose program CE. Lower part of the Figure: the structure of the three aligned homeodomain proteins Engrailed (blue), Oct-2 (yellow), Msx-1 (green) and PRH (magenta) bound to DNA (red). This molecular model shows that the PRH homeodomain contacts the major groove of DNA via helix III (the recognition helix) and the minor groove via the N-terminal arm. The loop between helix I and helix II makes extra contacts with the opposing phosphate backbone of DNA. This model shows that the PRH homeodomain binds to DNA following the general mode of specific DNA recognition employed by other homeodomain proteins. An interactive three-dimensional version of this structure can be found at http://www.BiochemJ.org/bj/412/0399/bj4120399add.htm.

domain of PRH serves as an interface for a variety of protein–protein interactions, including interactions with members of the Groucho/TLE (transducin-like enhancer) co-repressor family (amino acids 32–38; FxIxxIL, where x is any residue), the PML (promyelocytic leukaemic) protein (amino acids 50–115), translation factor eIF-4E (eukaryotic initiation factor 4E; amino acids 18–24; YxxxxLΦ, where x is any residue and Φ is a hydrophobic residue), and proteosome subunit C8 [17–20] (Figure 2). The PML interaction region lies downstream of amino acid 50 in the PRH N-terminus (amino acids 50–115) [19] and PML is known to be a co-repressor for some transcription factors, including the Mad tumour suppressor protein and SatB1 [21,22]. However, the function of the PML–PRH interaction in transcriptional repression has not been investigated. The interaction surface for the co-repressor protein Groucho/TLE (the Eh1 motif) in PRH is located within the N-terminal 50 amino acids (amino acids 32–38; FxIxxIL) in human PRH and the interaction between PRH and TLE results in co-repression [18].

The PRH homeodomain

The homeodomain comprises 60 amino acids and consists of an N-terminal arm and three α helices that are separated by a short loop and short turn respectively. The N-terminal arm and the third recognition helix are both involved in binding to specific DNA sequences. The recognition helix lies in the major groove and makes specific contacts with base pairs of DNA. The N-terminal arm wraps around DNA and makes specific contacts with base pairs in the adjacent minor groove. The loop also makes contacts with the phosphate backbone of DNA [23] (Figure 3). The PRH homeodomain is most similar to that of the haematopoietically expressed Hlx (56 % identity) protein and HOX11 (homeobox 11; 54 % identity), a protein that is misexpressed in some T-cell leukaemias [1,2,13]. The oncogenic HOX11 protein and PRH both contain a threonine residue at position 47 of the recognition helix. This amino acid contributes to the determination of sequence specificity by the homeodomain, suggesting that these proteins may recognize very similar DNA sequences [23]. PRH contains two consecutive proline residues at the beginning of the second helix in the homeodomain and this seems to be unique to PRH proteins (Figure 3).

PRH has been identified as a member of the NK (nuclear body-associated kinase) subclass of homeodomain proteins that carry a TN (tinman) motif [24,25]. The TN motif is a conserved decapeptide sequence (TPFSVKDILNLE) found N-terminally to the homeodomain in homeoproteins that are involved in heart development [26]. Although PRH is also involved in heart development [27] and contains an N-terminal sequence that loosely resembles the TN motif (amino acids 30–40, TPFYIEDILGRG), the PRH homeodomain lacks a conserved arginine residue at position 5 in the homeodomain found in all other members of the NK homeodomain family and lacks an NK-specific domain

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located downstream of the homeodomain in NK proteins. These differences suggest the possibility that PRH could equally be placed in a subclass of its own.

The consensus PRH-binding site is 5′-C/TA/TATTAAA/G-3′ as determined by SELEX (systematic evolution of ligands by exponential enrichment) experiments and DNase I footprinting assays [1]. A truncated protein consisting of the PRH homeodomain and C-terminal domain binds to degenerate A/T-rich sequences and can bind to some TATA boxes [16]. The isolated PRH homeodomain is able to bind DNA sequences containing: 5′-TAAT-3′, 5′-CAAG-3′ or 5′-ATTAA-3′ in EMSAs (electrophoretic mobility-shift assays) [28]. Thus the isolated PRH homeodomain appears to exhibit a more relaxed DNA-binding specificity than that shown by other homeodomains. This raises the interesting question, how does PRH find its target genes when its recognition site is so ill-defined and so short? This point will be addressed when the structure of the full-length protein is considered (see the section on oligomerization below).

A three-dimensional model of the PRH homeodomain bound to its consensus DNA sequence was built using sequence alignment and molecular modelling methods [29]. A recent global purification and NMR structural analysis project has shown that the structure of the PRH homeodomain corresponds closely with that of the model [PDB ID number 1WQI (http://www.rcsb.org)]. A three-dimensional alignment of this structure with the related structures of Oct-2, Engrailed and MSX-1 (msh homeobox 1) homeodomains bound to a DNA sequence containing the core motif, 5′-ATTA-3′, was used to build a molecular model of the PRH homeodomain bound to DNA (Figure 3).

The C-terminal domain

Many transcription factors contain acidic regions that are involved in transcriptional activation. The PRH C-terminal domain is composed of almost four times as many acidic residues as basic residues [1] and has been shown to be involved in transcriptional activation of the bile transporter gene NTCP (sodium-dependent bile acid co-transporter) [30]. PRH binds to the NTCP promoter and activates transcription using the homeodomain and the C-terminal domain. Although both the PRH homeodomain (amino acids 137–196) and the C-terminal region (amino acids 197–271) are required for this activating function of PRH, deletions of the PRH C-terminal domain show that amino acids 255–271 are critical for activation at this promoter [30,31]. The mechanism of activation has not been precisely determined but it is postulated that activation might arise through the interaction of the homeodomain and C-terminal domain with general transcription factors such as TBP (TATA-box-binding protein) [30] as these regions of PRH bind to TBP in pulldown assays [16].

Oligomerization

PRH is present in cells in discrete foci, and cross-linking studies have shown that the PRH protein forms oligomeric species within cells [15,18,19]. Recombinant PRH [32] is also oligomeric in cross-linking experiments and appears as discrete particles as well as larger self-associating aggregates under the electron microscope [15]. Gel filtration and AUC (analytical ultracentrifugation) sedimentation velocity experiments have provided estimates for the molecular mass of PRH that suggest that this protein forms octamers [15]. Pulldown experiments have revealed the presence of two regions within the N-terminal domain that allow self-association. The first is a dimerization region within the N-terminal 50 amino acids and the second is a homeodomain association region between amino acids 50 and 137. Together these data have resulted in a model for the formation of PRH octamers [15] (Figure 4, left-hand side).

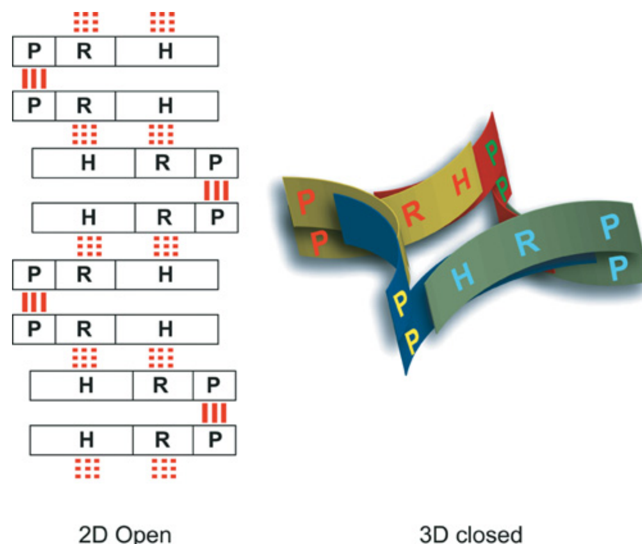

Figure 4 A model of PRH self-association

The representation on the left-hand side shows the two-dimensional (2D) open octamer. The representation on the right-hand side shows the three-dimensional (3D) model of the closed octamer. The P region represents amino acids 1–50, the R region amino acids 50–137 and the H region amino acids 137–270. In the octameric form of PRH, the R region interacts with the H region in a head-to-tail orientation via domain-swapping of two PRH molecules, whereas the P region interacts with another P region in a head-to-head orientation (dimerization).

AUC sedimentation velocity experiments with oligomeric PRH and DNA suggest that it is only one oligomeric form of PRH that binds to DNA [15]. Full-length recombinant PRH binds to a single DNA site with relatively low affinity compared with the PRH homeodomain and C-terminus, the isolated PRH homeodomain or a proteosomally digested PRH product [17,33]. This suggests that the PRH N-terminus has a regulatory role on DNA-binding activity. However, our current experiments suggest that the oligomeric PRH protein binds with very high affinity to DNA sequences with multiple PRH sites (H. Williams, K. Gaston and P.-S. Jayaraman, unpublished work). Interestingly, the promoter regions of the Goosecoid, Tle 4 and ESM-1 (endothelial cell-specific molecule-1) genes, which are all repressed by PRH, all contain multiple copies of putative PRH-binding sites [34–36]. This suggests that several clustered sites facilitate the binding of the oligomeric PRH protein (Figure 4, right-hand side) and provides an explanation for how PRH locates its target sites *in vivo*.

TRANSCRIPTIONAL REGULATION BY PRH

Like many other transcription factors, PRH exerts both positive and negative effects on transcription and can use both direct mechanisms (binding to the promoters of the regulated genes) or indirect mechanisms (modulating the activity of other transcription factors to regulate gene expression) [37].

Repression

PRH binds directly to sites located in the promoter or intron regions of several genes to bring about transcriptional repression (Table 1). For example, at the PRH-repressed ESM-1 promoter, a PRH-binding site has been identified using EMSA and ChIP (chromatin immunoprecipitation) and this site is critical for transcriptional repression [36]. The Tg (thyroglobulin) promoter

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Table 1 PRH target genes

Gene	Regulation	Model	Method	References
Goosecoid	Repressed	*Xenopus* embryos ES tissue cultured cells	Ectopic expression Transient transfection	[34]
Chordin	Repressed	*Xenopus* embryos	Ectopic expression	[34]
Xtle4, Nodal (Xnr1, Xnr2)	Repressed	*Xenopus* embryos ES tissue cultured cells	*In situ* hybridization Ectopic expression Conditional expression Affymetrix gene chips	[35]
Tg	Repressed	FRLT-5 tissue cultured cells	Transient transfection	[28]
VEGEF, VEGFR-1, VEGFR-2, TIE-1, TIE-2, neuropilin-1, integrin αV	Repressed	HUVEC tissue cultured cells	Adenoviral infection	[55–57]
		ES tissue cultured cells	cDNA microarray analysis, quantitative real-time RT (reverse transcription)–PCR	
ESM-1	Repressed	EOMA and HUVEC tissue cultured cells	Transient transfection Adenoviral infection Quantitative real-time RT (reverse transcription)–PCR OMM16K Genechip microarray	[36]
NTCP	Activated	PRH knockout in E10.5 embryos	Transient transfection	[85]
L-PK	Activated	Hep G2 tissue cultured cells HeLa cells Rat cultured hepatocytes	Transient transfection Adenoviral infection	[43]
NIS (sodium iodide symporter)	Activated	HeLa cultured cells	Transient transfection	[66]

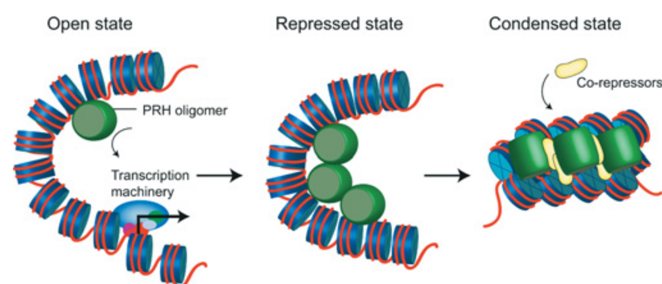

Figure 5 A representative model of transcription repression facilitated by PRH oligomerization

In the open state of chromatin, PRH octamers (green) bind specifically to multiple sites within a target promoter. Further self-association of PRH along the chromatin fibre will exclude the transcription machinery and bring about transcription repression (the repressed state). Recruitment of co-repressor proteins such as Groucho/TLE results in a condensed state of chromatin.

also contains PRH-binding sites that are bound by the isolated PRH homeodomain in EMSA, but these sites diverge considerably from the consensus PRH-binding site [28]. Although direct binding by the PRH homeodomain, or the PRH homeodomain and C-terminus, have been used in a number of studies to examine DNA binding [16,28], no studies have examined whether the full-length PRH protein binds to these promoter regions. However, purification of the full-length protein [32] and EMSA and DNAse I footprinting experiments have demonstrated that the full-length PRH protein binds with high affinity to multiple sites located in the human Goosecoid promoter (H. Williams., K. Gaston and P.-S. Jayaraman, unpublished work), a known target for regulation by PRH.

PRH interacts with members of the Groucho/TLE co-repressor protein family and may recruit TLE proteins to a subset of target promoters to bring about transcriptional repression [18]. The oligomeric Groucho/TLE proteins recruit histone deacetylases and also bind to hypoacetylated chromatin to form a repressive chromatin domain [38]. Thus a PRH–TLE complex can result in long-range transcriptional repression (Figure 5). In haematopoietic cells of myeloid lineage, the TLE co-repressor is co-expressed in the same cells as PRH [18], but it remains to be determined whether TLE is an obligate co-repressor for PRH or whether PRH can repress transcription with other co-repressor proteins. How the PRH–TLE interaction is regulated in cells and under what circumstances PRH is free to interact with other partners, including activators and co-activators, is yet to be explored and research in these areas will throw light on context-dependent functions.

PRH may also repress transcription by binding to AT-rich sequences such as those found in TATA boxes and competing with TBP for binding to these sites. In the context of a synthetic reporter construct, the PRH homeodomain can repress transcription of the TK (thymidine kinase) promoter in the absence of additional PRH-binding sites. EMSAs have shown that the PRH homeodomain and C-terminus can bind directly to the TK TATA box. Mutation of the homeodomain to block DNA binding inhibits transcriptional repression without altering the ability of the homeodomain to bind other factors such as TBP. Taken together, these results imply that DNA binding by PRH at the TK TATA box can repress transcription from this promoter [16]. There are precedents for DNA-binding proteins that utilize TATA box sequences to bring about gene-specific repression or activation. For example, the homeodomain repressor protein Engrailed can compete with TBP for TATA-binding sites and bring about transcriptional repression *in vitro* [39] and the transcriptional activator FoxM1c can bind to the TATA box and TBP and TAFs (TBP-associated transcription factors) and bring about transactivation of the c-myc promoter [40]. However, we do not yet know whether PRH uses its ability to bind to a single TATA box to repress any of its natural target genes *in vivo*. Given the oligomeric nature of PRH it is possible that only TATA sequences that are close to clusters of other PRH sites are bound by PRH *in vivo*. Clusters of PRH sites close to the TATA box sequence would allow highly co-operative binding of PRH oligomers, whereas isolated AT-rich sequences, such as a single TATA sequence, will represent a site that has a relatively low affinity for PRH. We predict that a preference for clustered sites in DNA will result in PRH recognizing structural features in DNA, that is, flexible regions of DNA similar to nuclear matrix attachment sequences in DNA. The requirement for clustered sites will also provide binding-site specificity and prevent PRH from regulating all promoters that

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contain a single TATA sequence. Promoters with a TATA sequence that lack additional PRH sites would only be regulated by PRH, if PRH was present at relatively high concentrations in cells. This of course means that many studies of promoter function using overexpressed PRH have to be considered with an understanding of the limitations of the experimental conditions in mind.

PRH has been shown to regulate some promoters independently of its ability to bind directly to DNA. For example, PRH interacts with GATA-2 in endothelial cells, resulting in reduced binding of GATA-2 to a 5′-UTR (untranslated region) GATA motif found in the VEGFR-2 [VEGF (vascular endothelial growth factor) receptor 2; also known as KDR (kinase insert domain-containing receptor)/flk-1] promoter. This brings about the repression of VEGFR-2 expression [41]. The PRH homeodomain is also reported to interact with members of the AP-1 (activator protein-1) family of transcription factors through direct binding to the Jun–Fos complex. This interaction results in the inhibition of Jun-mediated gene activation. However, in this case, repression occurs without interfering with Jun DNA-binding activity [42]. In both of these cases PRH modulates the activity of proteins that activate the transcription of genes that are required for cell proliferation.

Activation

PRH can activate a number of promoters (Table 1) and uses a number of different mechanisms to bring about transcriptional activation. PRH activates the NTCP promoter by directly binding to a site in the promoter [30,31]. A C-terminal region (amino acids 255–271) is critical for the activation function of rat PRH at this promoter [30]. PRH also activates transcription indirectly by interacting with other DNA-binding proteins. For example, PRH directly interacts with HNF (hepatocyte nuclear factor)-1α to stimulate the activity of HNF-1α at the L-PK (liver pyruvate kinase) gene promoter. The stimulation of HNF-1α requires the homeodomain and the acidic C-terminal region of PRH [43]. Similarly, PRH activates expression of the SM (smooth muscle) genes SM α-actin and SM22α in embryonic 10T1/2 fibroblasts by binding directly to SRF (serum-response factor) [30]. The PRH–SRF interaction requires the PRH homeodomain and results in increased binding of SRF to its binding site at this promoter. Thus PRH functions as a cofactor/co-activator for SRF [44]. PRH can also induce expression of the SMemb/NMHC-B (non-muscle myosin heavy chain B) gene in VSMCs (vascular smooth muscle cells) and in this case activation is also dependent on the PRH homeodomain. Over-expression of CREB [CRE (cAMP-response-element)-binding protein] increases PRH-induced SMemb promoter activity; however, EMSA assays have failed to demonstrate that PRH binds either to PRH-binding sites or to the CRE element in this promoter. Although a direct interaction between CREB and PRH seems likely, this has not been detected and therefore the precise mechanism for the activation of this promoter by PRH is not known [45].

Summary

In summary, PRH is able to repress or activate transcription in a context-dependent fashion. A central question is how are these functions of PRH controlled? We propose that the answer lies in the fact that the oligomeric/octameric PRH protein has two very fundamental capabilities that directly impinge upon each other and are very likely to be finely balanced, that is, DNA binding and protein–protein interactions. The proline-rich N-terminus contains a repression domain that participates in many protein–protein interactions. This domain also contains a region that is responsible for regulating the DNA-binding activity of the full-length protein. Removal of the proline-rich domain increases the DNA-binding activity of the homeodomain and C-terminus. Since the PRH N-terminus contains a region that interacts directly with the homeodomain, it is very likely that protein–protein interactions between the PRH N-terminus and other partner proteins will influence DNA binding by the homeodomain. Equally, direct interactions between the PRH homeodomain and other proteins may modulate both DNA binding and interactions of the PRH N-terminal domain or PRH C-terminal activation domain with other partner proteins. In this way the PRH octamer can be considered to be analogous with a nucleosome, in that it is capable of integrating signalling from the cell into a gene expression response (see below). Post-translational modifications of the PRH protein will undoubtedly also play a role in the regulation of PRH activities. Studies in our laboratory have shown that phosphorylation of PRH results in the regulation of DNA binding (A. Soufi, M. Buckle, K. Gaston and P.-S. Jayaraman, unpublished work). It is very likely that, just as with the nucleosome, post-translational modifications will strongly influence protein–protein interactions and thereby influence both transcriptional and non-transcriptional outcomes.

POST-TRANSCRIPTIONAL REGULATION BY PRH

PRH functions as a potent inhibitor of cell proliferation by acting as a repressor of mRNA transport, and ultimately as a repressor of translation, of genes that are required for proliferation. The eIF-4E is essential for cellular growth. It mediates the nucleocytoplasmic transport of specific transcripts (mRNA) that are important to growth control resulting in increased translation [46,47]. A number of homeodomain proteins interact with eIF-4E in a cell-type-specific manner and modulate its activity [47]. PRH is one such homeodomain protein and it interacts with eIF-4E through an N-terminal motif (amino acids 18–24; YxxxxLΦ in human PRH) that is shared by all proteins that bind to eIF-4E [20]. The PRH–eIF-4E interaction inhibits the mRNA transport activity of eIF-4E on transcripts such as that of the cell cycle protein cyclin D [20]. The PRH–eIF-4E interaction occurs within the nucleus and is thought to occur within PML bodies as both proteins are found within these structures [19,20]. Elevated PRH levels can lead to the disruption of PML nuclear bodies, the inhibition of the eIF-4E-mediated transport of cyclin D1 mRNA and a subsequent reduction of cyclin D1 protein levels [20]. HOXA9 can stimulate the mRNA transport activity of eIF-4E and PRH is able to antagonize this stimulatory activity because PRH and HOXA9 compete for a common binding site on the eIF-4E [48]. The ability of PRH to bind to growth control proteins such as PML and also factors that regulate mRNA transport and translation affords PRH the ability to integrate transcription, translation and control of proliferation.

PRH IN HAEMATOPOIETIC AND VASCULAR DEVELOPMENT

Haematopoiesis, or blood cell formation, is a continuous process throughout the life-time of the organism. In contrast with haematopoiesis, development of the vasculature occurs predominantly during the embryonic and fetal phase. In mice both vasculogenesis and haematopoiesis are initiated in an extra-embryonic tissue known as the blood islands of the yolk sac. The blood islands originate from a common mesodermal progenitor, the haemangioblast [49,50] (Figure 6). Later in development haematopoiesis occurs within the embryo, in the AGM (aorta gonad mesonephros) region and the fetal liver. Beginning at birth, the bone marrow is colonized by HSCs (haematopoietic stem cells) originating from the fetal liver. From birth and throughout

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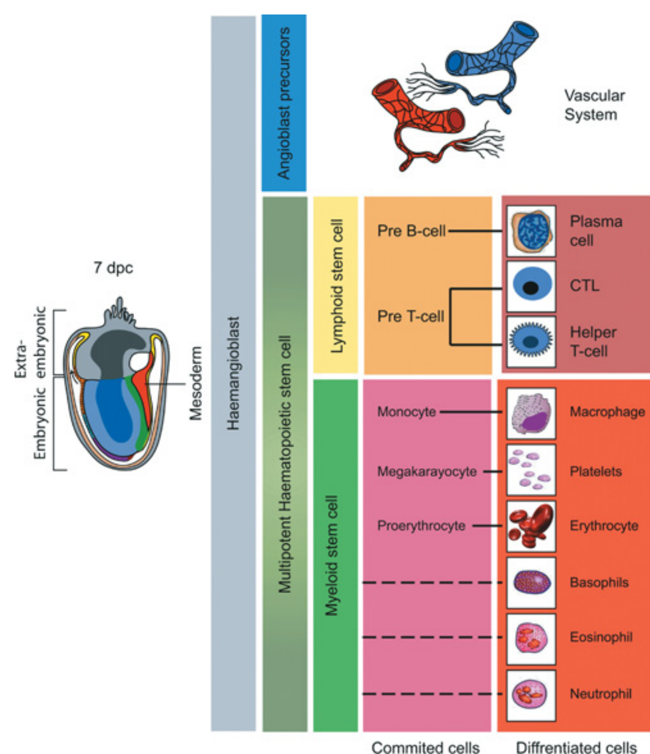

Figure 6 Diagram showing the different steps of haematopoiesis and vasculogenesis

Haematopoiesis and vasculogenesis begin in the blood islands. The inner part of these blood islands becomes HSCs, whereas the outer part gives rise to the vascular endothelial progenitors (angioblasts). The endothelial and haematopoietic precursors are thought to originate from the common mesodermal progenitor, the haemangioblast. HSCs differentiate to all cells found in the circulating blood, and the angioblasts develop to form the vascular endothelial system. PRH is expressed in the haemangioblast/haematopoietic stem cell but not in angioblasts or their derivatives. PRH is expressed in differentiating haematopoietic cells of B-cell lineages and myelomonocytic and erythroid lineages, but not in T-cell lineages. dpc, days *post coitum*.

adult life, all mature blood cells are produced in the bone marrow [49,50].

During early embryonic development, PRH is expressed in tissues that contribute to both haematopoietic and vascular systems. In mice, PRH is expressed in extra-embryonic mesoderm in blood islands of the yolk sac [9,51]. Later after gastrulation, PRH is expressed in the embryo proper in embryonic mesoderm at sites where (i) angioblasts are thought to arise and (ii) endocardial cells develop into the cardiac tubes of the heart [9]. Additionally PRH is expressed in regions where vessels are known to form, such as the dorsal aortae and the intersomitic vessels. However, once cells have differentiated to form committed endothelial precursors, PRH expression is lost in these cells [9]. The expression pattern of PRH during embryonic endothelial cell development is conserved between mouse and species such as *Xenopus* [5,9,12,52].

Still later in development, PRH is expressed in the murine fetal liver (see below) [9,11]. Consistent with the expression studies outlined above, mice that are homozygous for a disruption of the *Prh* gene show defects in haematopoiesis (monocyte development) and liver development and die at mid-gestation (E13.5–E15.5; E is embryonic day) [53,54]. A more recent gene-targeting experiment has shown that a null mutation of *Prh* also results in abnormalities of cardiac and vascular development [27].

Overexpression and knockout studies in different species have resulted in some apparent discrepancies with regard to the role of PRH in vasculogenesis. Overexpression of PRH in *Xenopus* embryos leads to disorganization of the vasculature and an increase in vascular progenitors [5] indicating that PRH may stimulate the proliferation of early endothelial progenitors. Mouse ES (embryonic stem) cell *in vitro* differentiation assays with $Prh^{-/-}$ cells, together with *in vivo* differentiation assays, and chimaeric mouse analyses, have revealed that PRH is required for differentiation of the haemangioblast into haematopoietic progenitors and, to a lesser extent, endothelial progenitors, but that formation of the haemangioblast is unaffected [55]. Moreover, $Prh^{-/-}$ endothelial progenitor cells generate lower numbers of vascular endothelial cadherin-positive cells and form fewer vascular sprouts compared with those from wild-type cells. This suggests that PRH also has a role as a positive regulator of endothelial cell maturation, organization and tube formation [55]. Kubo et al. [56] also showed that $Prh^{-/-}$ ES cells form fewer committed haematopoietic cells but, in contrast with Guo et al. [55], they show that $Prh^{-/-}$ cells form larger numbers of haemangioblasts and endothelial progenitors. In addition they demonstrate that overexpression of PRH leads to reduced numbers of haemangioblasts, reduced proliferation and decreased VEGFR-2 expression [56]. Consistent with this finding, $Prh^{-/-}$ mice and ES cells show up-regulation of VEGF-A, a growth factor that regulates endothelial proliferation [27,55]. Taken together, these findings support the idea that PRH negatively regulates proliferation of the haemangioblast and early endothelial progenitors and is required for the differentiation of haematopoietic progenitors. It has been suggested that the observed discrepancies between the various studies very probably reflect different functions of PRH at different stages in vascular development [56]. The initial role of PRH could be one of a negative regulator of haemangioblast and endothelial proliferation, and a positive regulator of haematopoietic differentiation. At later stages, PRH might positively regulate endothelial cell maturation, organization and tube formation [56].

The vasculature is made up of endothelial cells and VSMCs that are not proliferating. In the adult, PRH is not expressed in VSMCs or endothelial cells. Interestingly, overexpression of PRH in fully differentiated endothelial cells [HUVECs (human umbilical-vein endothelial cells)] results in repression of angiogenesis-related genes including VEGFR-1 (flt-1), VEGFR-2 (flk-1), TIE-1 [TK with immunoglobulin-like and EGF (endothelial growth factor)-like domains 1] and TIE-2, and inhibits the ability of these cells to form endothelial networks *in vitro* [57]. Thus PRH functions as a repressor of transcription in these cells and a negative regulator of angiogenesis. In contrast, elevated PRH expression activates expression of the embryonic SMemb/NMHC-B gene in adult VSMCs. The SMemb/NMHC-B gene is a marker of dedifferentiated VSMCs. PRH is itself up-regulated in VSMCs after a stent has been placed in the artery, that is during a period of proliferation and dedifferentiation after injury [45]. Moreover, overexpression of PRH in embryonic fibroblasts results in the expression of early, but not late, markers of differentiation into VSMCs [44]. Thus in adult VSMCs PRH functions as an activator of gene expression and the induction of PRH expression in VSMCs appears to be related to the dedifferentiation and proliferation of the VSMC [45].

The role of PRH in haematopoiesis is complex. $Prh^{-/-}$ ES cells do not contribute to the formation of lymphoid or myeloid tissues in chimaeric mouse analyses, indicating that PRH is required for the formation of the HSCs [55]. Retroviral expression of PRH in avian blastoderm showed that this protein influences the proliferation and differentiation of haematopoietic progenitors [58]. PRH is expressed in early haematopoietic progenitors of all lineages except that of T-cell lineages [13,14,58]. Although

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PRH expression is generally down-regulated during terminal differentiation of haematopoietic cells [14,58], PRH expression is maintained in terminally differentiated granulocytes [14]. Experiments with chimaeric mice, that are only homozygous null for *Prh* in the lymphoid lineages ($Rag^{-/-}Prh^{-/-}$) have shown that PRH is essential for B-cell development and function but does not affect T-cell development [59]. In the myeloid lineages, PRH functions as an inhibitor of cell proliferation, and PRH causes G_1 growth arrest when it is overexpressed in the myeloid cell line U937 [20,60].

At present, relatively few PRH-regulated target genes that are directly regulated by this protein have been well-characterized. Since PRH is expressed in so many tissues and at so many stages of development it is very difficult to make any conclusions about its function in cells. Its functions might also change in a single cell type during development. However, there appear to be some very general trends, that is PRH is highly expressed at early stages of differentiation in the haematopoietic and vascular compartments and is down-regulated during differentiation. Therefore the activity of PRH in haematopoietic and endothelial cell types appears to be related to blocking cell differentiation and the associated proliferation required for haematopoiesis and neoangiogenesis. Hence PRH down-regulates the expression of many genes required for proliferation and differentiation in these cell types. As mentioned earlier PRH inhibits the expression of genes such as VEGFR-1 (flt-1), VEGFR-2 (flk-1), TIE-1 and TIE-2 at the transcriptional level in HUVECs and inhibits the expression of the growth factor VEGF-A in the developing embryo and in the haemangioblast. Although at this point it is not clear whether these are direct target genes of PRH. In addition, cell-cycle genes regulated by AP-1, including many of the cyclins and p53, are potentially down-regulated by PRH at the transcriptional level through its action on AP-1. eIF-4E regulates the expression of cyclin D1, c-myc, VEGF-A and ornithine decarboxylase and many of these genes may also be subject to post-transcriptional regulation by PRH. It remains to be determined whether any of these genes can be regulated both transcriptionally and post-transcriptionally by PRH. It is possible that such a ‘belt and braces’ approach by PRH would not allow cells to respond with sufficient sensitivity to changes in the environment and a rapid requirement for increased proliferation.

PRH AND LEUKAEMIA

PRH is implicated in a number of different human leukaemias but the most direct evidence for PRH as a causative agent in leukaemia arises in one patient with acute myeloid leukaemia who has a sole cytogenetic abnormality [t(10;11)(q23;p15)] resulting in a nucleoporin–PRH fusion protein (Nup98–HHex). In this fusion protein the N-terminal domain of PRH is replaced with the N-terminal region of the Nup98 protein. The fusion protein results in a similar gene expression profile to that obtained by the Nup98–HoxA9 fusion protein which is highly leukaemogenic. In transgenic mice, this fusion protein results in a disease latency period of 8 months suggesting that this translocation, although necessary, is not sufficient for disease induction. The mechanism resulting in leukaemia is not known but one possibility is that the fusion protein may compete with endogenous PRH for PRH targets and block the repression of genes normally brought about by endogenous PRH [61].

PML is the growth control protein that forms PML nuclear bodies, sites of control of cell proliferation, apoptosis and gene regulation [62,63]. These bodies are disrupted in APL (acute promyelocytic leukaemia) by chromosomal rearrangements that result in the formation of fusion proteins between PML and the RARα (retinoic acid receptor α) [62,63]. Yeast two-hybrid experiments have shown that the N-terminal domain of PRH interacts directly with PML across a RING finger domain in PML that is required for the activity of PML in growth control [19]. Furthermore, PRH interacts with the PML–RARα fusion protein in APL [19]. It has been postulated that disruption of both PML and PRH functions by the PML–RARα fusion protein might well contribute to leukaemogenesis in APL [19], but this has yet to be demonstrated directly. There are a number of notable parallels between PML and PRH. Both proteins are oligomeric, both are involved in inhibition of cell proliferation, both are in nuclear bodies and both are potent inhibitors of neoangiogenesis. In addition, they both interact with the proteosome and are targeted by viral proteins to disrupt their activities. Furthermore, they partially co-localize in cells and they interact directly. Overexpression of PRH can lead to the disruption of PML nuclear bodies, and both proteins interact with eIF-4E to inhibit its activity although using subtly different mechanisms. However, there are two major differences between these proteins: first, PML is ubiquitously expressed whereas PRH shows tissue-specific expression; secondly, PML lacks DNA-binding activity and can only function as a co-repressor or co-activator of transcription, whereas PRH has strong DNA-binding activity and can use multiple mechanisms to regulate transcription. What is not known at present is how transcriptional regulation by PRH is modified through the interaction with PML and whether their effects on cell proliferation augment each other.

Loss of nuclear PRH

Down-regulation of PRH activity and aberrant PRH subcellular localization are associated with a subset of human leukaemias. Translation factor eIF-4E is up-regulated and PRH is down-regulated or mislocalized in the cytoplasm in a subset of AML (acute myelogenous leukaemia) and CML (chronic myelogenous leukaemia) cells [60]. The AML and CML cells show abnormally large nuclear bodies caused by the absence of PRH [60]. In a foci formation assay that measures transformation of NIH 3T3 cells by eIF-4E, overexpression of PRH was able to block transformation. Importantly, a PRH protein that does not bind to eIF-4E, or one that is not retained in the nucleus, does not block transformation, indicating that a PRH–eIF-4E interaction in the nucleus is responsible for this effect [60]. In addition to the loss of nuclear localization of PRH in patients with AML, the loss of nuclear localization of PRH is also implicated in the neoplastic transformation of thyroid and breast tissue [64–66]. In haematopoietic cells, PRH is clearly visible in PRH foci or dots in the nucleus and often these co-localize with PML or eIF-4E nuclear bodies [18,19]. Taken together these studies strongly suggest that a loss of nuclear PRH from nuclear bodies eliminates the growth inhibitory properties of PRH. Thus an additional layer of regulation of PRH activity may exist through regulation of the subcellular compartmentalization of PRH.

Overexpression of PRH

In direct contrast with the data demonstrating that loss or mislocalization of PRH results in human leukaemias or tumours, PRH overexpression in T-cell lineages has been linked to T-cell lymphomas. For example, mice that develop B-cell and T-cell leukaemias and lymphomas as a result of insertional mutagenesis have been shown to carry a retroviral insertion site (Lvis1) in close proximity to the *Prh* gene. This is a common retroviral insertion site that leads to the up-regulation of the *Prh* gene as well as

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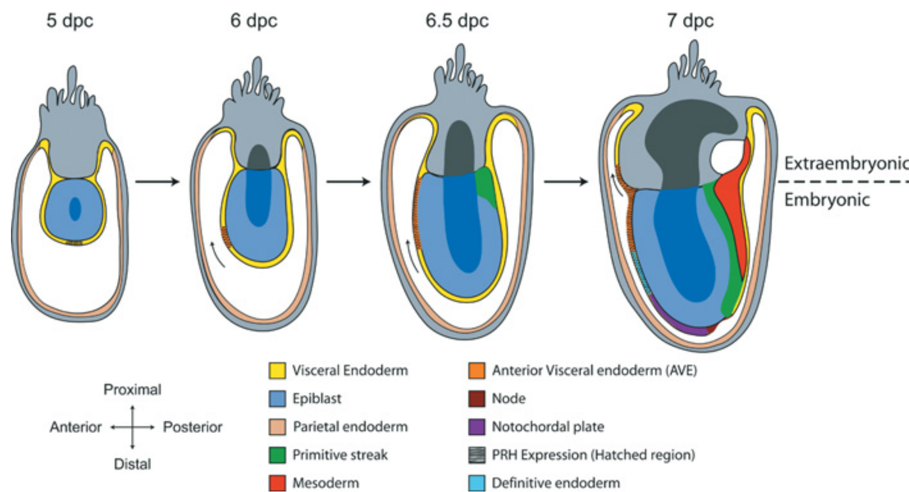

Figure 7 The expression of PRH during the early development of mouse embryos [from 5–7 dpc (days post coitum)]

At 5 dpc PRH is expressed in the AVE precursors, located at the distal tip of the embryo. The AVE moves towards the anterior–proximal region signalling to the epiblast in the posterior region where the primitive streak is formed at 6.5 dpc. During gastrulation the primitive streak elongates and cells at the tip form the node. The mesoderm and the definitive endoderm are also formed at this stage (7 dpc). The ADE where PRH is expressed moves anteriorly, displacing the AVE towards the extra-embryonic region.

other genes [67,68]. Moreover, elevated PRH levels influence T-cell proliferation in mouse transgenic experiments where the expression of *Prh* is under the control of T-cell-specific promoters in the bone marrow and the thymus [69]. High levels of PRH lead to increased cell cycling of haematopoietic progenitors and accumulation of T-cells that are arrested in differentiation in the thymus [69]. Retroviral expression of PRH in mouse bone marrow results in an aggressive T-cell lymphoma but death of all other haematopoietic cells in bone marrow indicating that PRH may function as a T-cell lineage oncogene [70].

It has been proposed that PRH might contribute to the deregulatory events that result in Hox11-dependent acute lymphoblastic T-cell leukaemias [69]. In these T-cell leukaemias there is a translocation event involving the Hox11 gene where the Hox11 locus (10q24) is translocated to the TCRδ locus leading to dysregulated Hox11 expression [71,72]. Since the human *PRH* gene (10q23) is located near the Hox11 gene it has been suggested that the translocation events might also involve the *PRH* gene which also becomes misexpressed.

How can high levels of expression of PRH cause PRH to function as an oncogene in a T-cell-specific fashion but result in cell death in other haematopoietic cells in bone marrow? Clearly PRH can function in many ways and the cell environment is liable to influence strongly the outcome of its activities. In cells that do not normally express PRH, such as T-cells, T-cell-specific proteins may block the binding of PRH to growth-control proteins such as eIF-4E or promote other unknown interactions that allow increased proliferation or decreased apoptosis, resulting in T-cell leukaemias. Equally, T-cell-specific interactions or post-translational modifications may block PRH transcriptional repression and instead allow increased activation by the C-terminal domain in PRH resulting in the activation of genes that lead to oncogenesis. In the other haematopoietic cells in the bone marrow, PRH activity presumably still inhibits cell growth and proliferation. Since PRH expression is uncontrolled in these cells the high levels of PRH expression simply lead to cell death [70]. Disruption of the normal activity of a transcription factor and/or gain of novel functions by that transcription factor is a common theme in oncogenesis. When PRH is misexpressed, the ability of PRH to form an oligomer and interact with multiple partners may result in the large-scale disruption of numerous regulatory networks and thereby magnify the dysregulation.

PRH EXPRESSION DURING EARLY DEVELOPMENT

In vertebrates, PRH plays a critical role in patterning of the body axis and in formation of forebrain and head structures. In mice, *Prh* is expressed in tissues prior to the formation of the primitive streak and gastrulation. *In situ* hybridization experiments have shown that PRH is first expressed in the primitive endoderm at E4.5 *post coitum* [9] and then in the VE (visceral endoderm). The VE differentiates to form the AVE (anterior VE), an early signalling centre identified in mouse, which appears to be involved in axis formation and neural patterning by signalling to the epiblast (embryo proper) before and during gastrulation [73,74] (Figure 7). Morphological and lineage analysis of VE cells shows that PRH-expressing cells have a tall, columnar epithelial morphology, which distinguishes them from other VE cells [75]. Real-time imaging of PRH-expressing VE shows that these cells actively migrate and form the AVE [76]. PRH expression is therefore used as a marker for the earliest molecular anterioposterior asymmetry in the mouse embryo before the formation of later signalling centres such as the primitive streak [9]. In chick and *Xenopus* development, PRH expression occurs in tissues analogous with the mouse AVE and similar movements of PRH-expressing cells have been observed [77–79].

After gastrulation (E7), *Prh* is expressed in the murine embryo proper in the ADE (anterior definitive endoderm) [9]. The ADE emerges from the primitive streak and the node signalling centres after gastrulation (E7) (Figure 8) [80]. The ADE then displaces the AVE, and is critical for correct head development [74]. *Prh*$^{-/-}$ embryos exhibit various degrees of anterior truncation ranging from none to severe lack of forebrain tissue as well as liver and thyroid dysplasia. Chimaeric embryos composed of *Prh*$^{-/-}$ cells developing within a wild-type VE show forebrain defects indicating that PRH is required in the ADE [54]. Thus for normal forebrain development PRH is needed in the ADE as well as the AVE.

The role of PRH in early patterning of the body axis has been addressed in *Xenopus* embryos by ectopic expression of PRH in two-cell and four-cell stage embryos. This results in embryos

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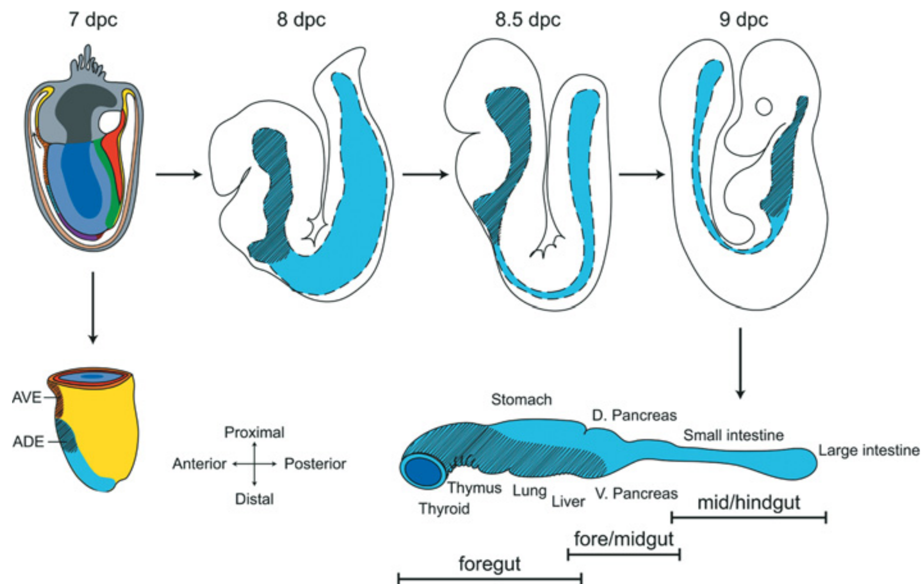

Figure 8 Schematic representation of PRH expression in the endoderm after gastrulation

At 7 dpc (days *post coitum*) PRH is expressed in the ADE (shaded). The ADE develops to give rise to the foregut (9 dpc). This region is fated to form the liver, pancreas, lungs, thyroid and thymus. PRH expression continues primarily in tissues that are derived from the foregut endoderm including liver, lung, thyroid, thymus and pancreas. The expression of PRH in the ADE is required for the normal development of the forebrain, which is derived from the overlying ectoderm. PRH expression in the ADE underlying the presumptive cardiac mesoderm is also required for cardiogenesis in vertebrate embryos.

with enlarged heads and small trunk structures [34]. Although embryonic developments in mouse and *Xenopus* are not identical, they have tissues that perform analogous functions. The anterior endoderm in *Xenopus* contains tissues analogous with the murine AVE and ADE [78]. PRH specifies the anterior mesendoderm (head formation) in *Xenopus* [34,54] by suppressing gene expression in the anterior endoderm that would promote trunk formation. It does this by repressing promoters associated with mesendodermal genes such as Goosecoid [34] and the Groucho-related co-repressor TLE4 [35]. TLE4 functions as a Wnt antagonist and repression of this gene allows increased Wnt signalling in the anterior mesendoderm and formation of the anterior endoderm. This mechanism is conserved between mice and *Xenopus* [35]. PRH can also induce the expression of proteins that block the action of signalling pathways in the posterior mesoderm including the expression of Cerberus, a Nodal and Wnt antagonist. [34,79,81].

In *Xenopus* embryos after head and axial patterning (at late gastrulation and early somite stages) Wnt/β-catenin is repressed in the anterior endoderm. Repression of Wnt signalling in the anterior endoderm allows PRH expression in the anterior endoderm because β-catenin represses PRH expression indirectly via the homeodomain repressor Vent2 [82]. Loss of PRH through the injection of PRH morpholino oligonucleotides resulted in the absence of liver- and pancreas-specific markers. Thus PRH expression is required for the maintenance of foregut identity and liver and pancreas development [82].

In mice the ADE self-differentiates to become the foregut endoderm (Figure 8) and PRH expression continues during embryogenesis in tissues that are derived from the foregut endoderm including liver, lung, thyroid, thymus and pancreas [5,6,9–12] (see Figure 8). In adults, PRH expression persists in several of these endoderm-derived cell types including lung, liver and thyroid [5,10].

Little is known about the role of PRH in the development of invertebrates. However, recent studies in *C. elegans* have shown that the PRH orthologue pha-2 is involved in the development of the pharyngeal isthmus and is required for expression of the acetylcholinesterase genes. Mutation in pha-2 results in grossly deformed pharyngeal muscle cells and neurons [7,83].

PRH in liver development

PRH is expressed in the developing murine embryonic liver [11] and $Prh^{-/-}$ mice show a pronounced defect in liver formation and loss of hepatic endoderm [5,11,27,53,54]. PRH promotes organogenesis by promoting the transition of hepatic endoderm to a pseudostratified epithelium, which in turn allows hepatoblasts to emerge into the stromal environment and continue differentiating [84]. The liver-specific transcription factors, HNF3 and GATA-4, are expressed in the developing liver and activate the expression of PRH [85]. PRH in turn activates the expression of several liver-specific genes [5,31,43,54]. PRH is nuclear in the endodermal cells of the developing liver and predominantly cytoplasmic in cells lateral to the liver-forming region. This suggests that nuclear localization of PRH is involved in early hepatic specification [51]. Conditional null mice for PRH have been developed to determine the role of PRH in the later stages of hepatic differentiation [86]. Deletion of PRH in the hepatic diverticulum leads to embryonic death, a small cystic liver and absence of the extrahepatic bile duct and gall bladder. Loss of PRH in the embryonic liver causes irregular development of the intrahepatic bile ducts and a progressive form of polycystic liver disease. In both cases there are disruptions of genetic networks regulating hepatic differentiation as evidenced by altered expression of HNF4α, HNF6 and HNF1α. Thus PRH also plays a role in later stages of hepatobiliary morphogenesis [86]. In the adult liver, PRH is thought to play a role in maintaining liver differentiation as PRH is expressed in adult liver, but not in undifferentiated hepatocytes or in undifferentiated liver cell lines [6]. During viral infection of the liver and haematopoietic cells with the virulent strain of lymphocytic choriomeningitis virus (termed LCMV-WE), PRH is down-regulated, resulting in

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the disappearance of PRH from the hepatic nuclei [87]. This is not observed when cells are infected with the related but non-virulent LCMV-ARM virus strain [87]. These observations are consistent with an important role for subnuclear compartmentalization of PRH in the regulation of PRH activity in liver cells as well as in haematopoietic cells.

PRH in thyroid development

PRH is expressed in the thyroid pimordia during development [9,12]. *Prh*$^{-/-}$ mice show arrested thyroid development at E9.5 [10,28,54]. PRH function is regulated in several complex loops together with other homeodomain proteins. The homeobox genes, TTF-1 (thyroid transcription factor 1), TTF-2 and Pax8, are expressed in the thyroid and are essential for its development. TTF-1 and Pax8 activate the expression of thyroid-specific genes, such as Tg, and PRH inhibits the activation of the Tg promoter by these proteins [28]. TSH (thyroid-stimulating hormone) increases the expression of Pax8 and TTF-1, which in turn up-regulates Tg gene expression. In contrast, TSH inhibits the expression of PRH allowing full activation of the Tg promoter by Pax8 and TTF-1 [28]. Pax8 is also thought to bind to the promoter of the PRH gene leading to its up-regulation [88] with the consequence that Tg expression will be negatively modulated. PRH is also expressed in adult thyroid [10] in both the nucleus and the cytoplasm. However, immunohistochemistry and immunocytochemistry have shown that the PRH protein is only present in the cytoplasm of thyroid adenomas, and differentiated and undifferentiated carcinomas [64] again underscoring the importance of subcellular compartmentalization in PRH function.

PRH in heart development

The inhibition of the canonical Wnt/β-catenin signalling by the antagonists Dkk-1 or Crescent initiates cardiogenesis in vertebrate embryos [89]. Loss of PRH expression or loss of PRH repression activity in the endoderm blocks endogenous heart development in *Xenopus* embryos and ectopic heart induction. Blocking Wnt signalling results in increased PRH expression in the endoderm, which is thought to result in the production of a diffusible heart-inducing factor, that acts on the cardiac mesoderm. Since the repression activity of PRH is involved in the induction of the heart-inducing factor, PRH probably induces expression of the heart-inducing factor indirectly [89]. The role of PRH in murine heart development may be slightly different as *Prh*$^{-/-}$ null mice do develop a heart. However, they have abnormal development of the heart ventricles, the AV (atrio-ventricular) valve, the myocardium and overabundant development of the endocardial cushions [27].

PRH in pancreatic development

In vertebrates, the pancreas originates from the ventral and dorsal domains of the gut endoderm. After organogenesis, PRH is expressed in ventral pancreas buds. Analysis of *Prh*$^{-/-}$ embryos and *Prh*$^{-/-}$ embryo tissue explant assays have shown that there is a failure in ventral pancreatic specification and morphogenesis, but not in the regulation of pancreatic genes [90]. PRH regulates pancreatic development by regulating the proliferation of endodermal cells and consequently the positioning of cells that later become pancreatic tissues rather than by regulating gene expression in differentiated pancreatic cells. Regulation of proliferation by PRH allows the presumptive pancreatic tissues to lie outside the cardiac mesodermal tissue, an inhibitor of pancreatic specification. Thus it has been suggested that in this case PRH regulates development 'morphogenetically' rather than through the regulation of tissue-specific gene expression [90]. However, it is important to point out that this morphogenetic effect occurs because PRH is able to regulate cell proliferation in the precursor endodermal cells and that this very probably occurs through its effects on transcription and translation. PRH expression is maintained in the adult pancreas suggesting that PRH may have additional functions in differentiated pancreatic cells [2,10].

PRH in skin development

PRH has recently been shown to be expressed in avian embryonic tarsometatarsal skin (which develops into feathers) and to up-regulate epidermal cell proliferation [91]. The pattern of PRH expression in the differentiating epidermis is similar to that of Wnt7a, and ectopic expression of PRH leads to increased expression of Wnt7a, β-catenin and Notch1 mRNA and increased cell proliferation [92,93]. The Prh gene is itself regulated by the Wnt signalling pathway (see below), suggesting that there are likely to be complex feedback loops in the regulation of PRH expression during development.

Summary

The wide-ranging studies on the role of PRH during development have highlighted the importance of studies in the whole embryo for understanding the role of a transcription factor in development. Studies on PRH in tissue culture, that is in more homogeneous populations of cells, only partially explain the functions of the protein in development. Three functions of PRH appear to be crucial to development. First, tissue-specific gene expression involving the direct regulation of target genes. For example, in the dorsal anterior mesendoderm in *Xenopus* embryos PRH expression results in the direct down-regulation of the Goosecoid gene. This gene would promote trunk formation at the expense of head formation if expressed and is therefore repressed in this tissue. Secondly, PRH also promotes the expression of diffusible proteins, such as Cerberus, that block the action of signalling pathways in the posterior region. This indirect effect of PRH on adjacent tissues is also seen in the patterning of the forebrain ectodermal tissue when PRH is expressed in the ADE, or the development of cardiac mesoderm through the effects of PRH on gene expression in the adjacent underlying endodermal tissues. In these cases, the effect of PRH is observed in tissues that it is not directly expressed in. Thirdly, PRH functions by regulating the proliferation of precursor endodermal cells so that the pancreatic endodermal progeny cells lie outside a zone of inhibition and are free to develop into differentiated cells. In this case the key point is that PRH does not perturb gene expression in the progeny cells but affects the growth of the precursor cells. It is worth noting however that all of the effects of PRH, whether in the cell type that PRH is expressed in or in adjacent tissues or at later time points, appear to be due to the gene regulatory activities of PRH.

REGULATION OF PRH GENE EXPRESSION

PRH regulates a plethora of developmental decisions and probably as a consequence of this the Prh gene is itself subject to multiple levels of control. The human Prh gene (HHEX) is located on chromosome 10 and the mouse gene is on chromosome 19 [2,94]. Single nucleotide polymorphism studies and linkage disequilibrium studies have highlighted the locus as a novel locus associated with Type 2 diabetes. The role of PRH in pancreatic development has been noted and allows the possibility that PRH could be involved in the development of diabetes [95].

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The Prh promoter region is GC-rich (69 %) and contains binding sites for the SP1 and SP3 proteins. These factors control PRH expression in liver and haematopoietic cell lines [96,97]. HNF3 and GATA-4 bind and further activate expression of the Prh gene in liver cells [85]. A blood-island-specific enhancer has been identified in the first intron of the Prh gene [98,99]. This enhancer (+775 to +1177) contains Myb and GATA-1- and GATA-2-binding sites and these factors are able to induce PRH expression in K562 haematopoietic cells [99]. In thyroid cells, the homeodomain proteins TTF and Pax8 are both able to up-regulate the Prh gene and binding sites for these proteins have been experimentally established [88,100]. In addition, PRH is able to up-regulate its own gene. However, PRH-binding sites have not been identified and it is not known whether PRH binds to the Prh promoter and activates expression of the Prh gene directly or regulates this promoter in a more indirect fashion by, for example, binding to the other homeodomain proteins [88,100].

Regulatory elements controlling PRH expression during early development have been mapped using mouse transgenic experiments. A 4.2-kb region upstream of the Prh gene is important for PRH expression in the endothelial cell precursors, liver and thyroid, and a 633-bp region in the second intron is both necessary and sufficient for PRH expression in the AVE and the embryonic ADE [98]. In zebrafish, PRH expression is regulated in the dorsal yolk syncytial layer, a tissue analogous with the mouse AVE, by Wnt and BMP (bone morphogenetic protein) signalling [4]. Experiments in *Xenopus* and zebrafish have also shown that PRH expression during early development is regulated by the maternal Wnt signalling pathway [81,82,101]. Repression of PRH in *Xenopus* foregut anterior endoderm occurs through repressor activity of the Vent homeodomain protein [82]. The PRH gene is also responsive to BMP signalling during development. In transgenic zebrafish that overexpress dominant-negative forms of Bmp or Fgf (fibroblast growth factor) receptors that block Bmp or Fgf signalling in the endodermal region that is fated to form the liver primordium, the expression of PRH is severely reduced in the liver region [102]. The mouse Prh promoter carries a 71-nucleotide BRE (BMP-responsive element) that is required for up-regulation of PRH by an activated Smad-dependent BMP signalling pathway. The PRH BRE contains Smad1–Smad4-binding sites and elements responsive to TGFβ (transforming growth factor β)/activin. These elements are required for PRH expression in tissues that give rise to the liver [103].

CONCLUSIONS AND FUTURE PROSPECTS

PRH regulates multiple processes in development and is an essential protein for the development of all vertebrates. PRH is a potent regulator of cell proliferation and a regulator of gene expression. It is likely that the ability of PRH to form unusual homo-oligomeric assemblies, to bind to DNA and to be involved in multiple protein–protein interactions, underpins many of the roles that PRH plays in the cell. The multi-subunit nature of the PRH octamer affords PRH the potential to integrate the multifarious developmental and growth regulatory cues from the cell into a gene expression response. For example, PRH can bind to AP-1 on DNA and block its transcription activity at genes that are required for cell proliferation. PRH can also bind to the growth control protein PML and translation factor eIF-4E to regulate cell proliferation. It is able to bind to co-repressors such as TLE and directly repress genes that need to be down-regulated for specific cell differentiation programmes (Figure 9). PRH octamers have the potential to bind to multiple DNA sites that potentially may be very distant from each other in the genome and this allows

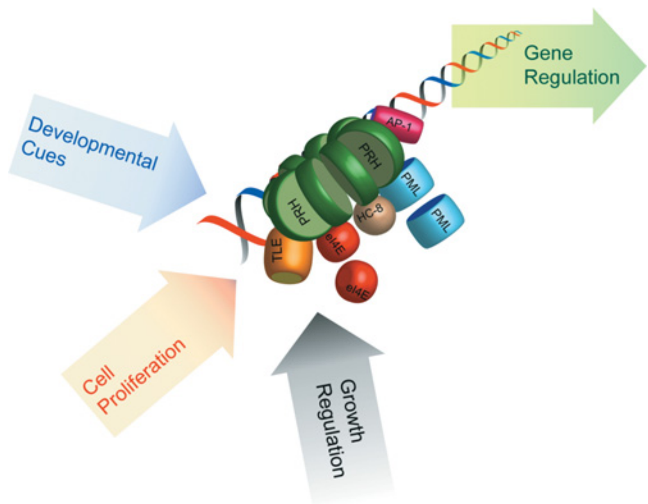

Figure 9 Model of PRH regulating gene expression in response to multiple developmental and cell-growth-related cues

A single octamer of PRH is shown bound to DNA and also to multiple PRH partners allowing PRH to respond to multiple signalling pathways simultaneously or temporally. For clarity, each subunit of PRH is not shown bound to DNA or bound to every interacting partner.

the possibility that many genes that are regulated by PRH may be clustered together at PRH foci. Regulation of PRH stability by interaction with the proteosome or post-translational modification will further add complexity to a model whereby PRH functions to integrate multiple signalling pathways into an output that results in a specific pattern of gene regulation.

Clearly some central questions that remain to be addressed surround the mechanisms regulating PRH DNA-binding activity. In addition, determining whether PRH has structural properties like that of nuclear PML will be of great importance. It is not clear at this point whether PRH binds to DNA and wraps the DNA around itself to form alternative nucleosomal-like particles which block transcription, or whether PRH spreads along the DNA and covers chromatin from a specific nucleation site, or indeed whether PRH forms a repressive domain by forming repressive loops through self–self protein–protein interactions. A rather important question given the oligomeric nature of PRH is why TLE is needed as a co-repressor for PRH. The precise role of oligomeric TLE proteins in the regulation of PRH-specific genes is yet to be established and will be important for understanding PRH function. Perhaps one role of the interacting partners such as TLE, PML and eIF-4E is to block unfettered self assembly by PRH. The mechanisms regulating the assembly or disassembly of PRH oligomers in the cell and the mechanisms regulating the interaction of PRH oligomers with other proteins are lacking at present but are key to understanding any role of PRH in chromatin organization.

The role of PRH in development has been well investigated. The cell-autonomous effects of PRH and the non-cell-autonomous effects coupled with the ability of signalling pathways to control PRH expression, post-translational modification, subcellular compartmentalization or activity through altering the levels of PRH-interacting partners, affords almost limitless possibilities in the outcomes in development. The challenge for the future will be to unravel further the effects of this transcription factor in response to multiple signalling pathways and developmental cues and to harness its activities in the treatment of disease.

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