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Differential Expression of Human Polycomb Group Proteins in Various Tissues and Cell Types

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Abstract Polycomb group proteins are involved in the maintenance of cellular identity. As multimeric complexes they repress cell type-specific sets of target genes. One model predicts that the composition of Polycomb group complexes determines the specificity for their target genes. To study this hypothesis, we analyzed the expression of Polycomb group genes in various human tissues using Northern blotting and immunohistochemistry. We found that Polycomb group expression varies greatly among tissues and even among specific cell types within a particular tissue. Variations in mRNA expression ranged from expression of all analyzed Polycomb group genes in the heart and testis to no detectable Polycomb group expression at all in bone marrow. Furthermore, each Polycomb group gene was expressed in a different number of tissues. *RING1* was expressed in practically all tissues, while *HPH1* was expressed in only a few tissues. Also within one tissue the level of Polycomb group expression varied greatly. Cell type-specific Polycomb group expression patterns were observed in thyroid, pancreas, and kidney. Finally, in various developmental stages of fetal kidney, different Polycomb group expression patterns were observed. We conclude that Polycomb group expression can vary depending on the tissue, cell type, and development stage. Polycomb group complexes can only be composed of the Polycomb group proteins that are expressed. This implies that with cell type-specific Polycomb group expression patterns, cell type-specific Polycomb group complexes exist. The fact that there are cell type-specific Polycomb group targets and cell type-specific Polycomb group complexes fits well with the hypothesis that the composition of Polycomb group complexes may determine their target specificity. J. Cell. Biochem. Suppl. 36:129–143, 2001.

Key words: Polycomb group; complex composition; immunohistochemistry; expression pattern; cell type-specificity

The Polycomb group (PcG) proteins have originally been identified in *Drosophila* as negative regulators of the homeobox genes [Simon, 1995]. These homeotic genes are important for defining the identity of the body parts. When PcG genes are mutated, homeotic genes are still correctly expressed during the earliest phases of embryogenesis. After germband extension, however, homeotic genes are expressed outside their normal expression pattern. This loss of correct homeobox gene expression leads to the change of the identity of certain body parts [Wedeen et al., 1986; Simon et al., 1992]. Thus PcG proteins are involved in maintaining the correct expression of the homeotic genes and consequently the maintenance of cellular identity [Pirrotta, 1997].

On *Drosophila* polytene chromosomes and the PcG proteins are associated with ~100 gene loci, indicating that there are many other PcG gene targets apart from the homeotic gene cluster. The association of multiple PcG proteins to the same loci suggested that PcG proteins might form multimeric protein complexes. This notion has been confirmed by the finding that PcG proteins Polycomb (Pc) and polyhomeotic (ph) can be coimmunoprecipitated [Franke et al., 1992]. The binding patterns of various PcG proteins to polytene chromosomes are not identical, but overlap considerably [Franke et al., 1992; Rastelli et al., 1993; Carrington and Jones, 1996; Sinclair et al., 1998]. As PcG proteins share some but not all

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chromosome binding sites, the composition of the PcG complex must inevitably vary at the different loci. Also, using in vivo formaldehyde crosslinking it has been shown that the PcG complex has different compositions at different target genes [Strutt and Paro, 1997]. This has led to the hypothesis that the composition of PcG complexes determines the specificity for their target genes.

Several human homologues of *Drosophila* PcG genes have been identified. As in *Drosophila*, the human PcG proteins coimmunoprecipitate [Gunster et al., 1997; Satijn et al., 1997a; Sewalt et al., 1998; Satijn and Otte, 1999a, 1999b], indicating that PcG proteins form multimeric complexes in humans as well. Several human PcG proteins interact directly with each other. Further support for human PcG complex formation is found in the observation that the different PcG proteins colocalize in specific nuclear domains within the nucleus of various cell lines, the so-called PcG domains [Gunster et al., 1997; Satijn et al., 1997a].

In humans two distinct PcG complexes have been described [Sewalt et al., 1998]. The HPC/HPH PcG complex contains HPC1, HPC2, RING1 [Satijn et al., 1997a; Satijn and Otte, 1999a] BMI1, HPH1, and HPH2 [Gunster et al., 1997]. The EED/EZH PcG complex contains EED, EZH2, and HDAC2 [Sewalt et al., 1998; van der Vlag and Otte, 1999]. The existence of two distinct PcG complexes has been confirmed in *Drosophila* [Kyba and Brock, 1998; Tie et al., 1998; Ng et al., 2000]. There are several observations that lead to this conclusion. First of all extensive protein–protein interactions have been observed between the components of the HPC/HPH complex [reviewed in Satijn and Otte, 1999a]. Also proteins that are part of the EED/EZH complex interact directly with each other. However, no proteins from the EED/EZH complex have been found to interact with proteins from the HPC/HPH complex [Sewalt et al., 1998]. Secondly, in immunoprecipitates of the HPC/HPH complex no proteins from the EED/EZH complex can be detected and vice versa, in immunoprecipitates of the EED/EZH complex no proteins from the HPC/HPH complex can be detected. This indicates that the two complexes are also biochemically distinct. Thirdly, at the level of nuclear localization the two complexes behave differently. Proteins from the HPC/HPH complex localize to pronounced PcG domains in the nuclei of U-2 OS cells [Gunster et al., 1997; Satijn et al., 1997a]. However, the proteins that are part of the EED/EZH complex are diffusely distributed throughout the nucleus [Sewalt et al., 1998]. Finally, the two PcG complexes display distinct patterns of expression at the issue level. The EED/EZH and the HPC/HPH complex have mutually exclusive expression patterns in tonsils [Raaphorst et al., 2000a]. This again indicates the existence of two distinct PcG complexes.

Although we known about PcG complex formation, very little is known about how PcG complexes are able to repress different target genes in various tissues. One hypothesis is that the composition of PcG complexes determines the specificity for their target genes. Support for this hypothesis mainly originates from *Drosophila* [Rastelli et al., 1993; Strutt and Paro, 1997; Sinclair et al., 1998]. To gain support for this hypothesis in mammals we wanted to find out if differentially composed PcG complexes exist in humans. To do this we analyzed PcG expression in various human tissues using Northern blotting and immunohistochemistry. The characteristic PcG expression pattern in tonsils already indicated that PcG expression is not ubiquitous, but can change dependent on the cell type. The varying expression of PcG genes is also evident from tissue to tissue. The analysis of PcG gene expression on Northern blot for various tissues reveals very distinct expression patterns for several PcG genes [Gunster et al., 1997; Satijn et al., 1997b; Sewalt et al., 1998], indicating that the composition of the PcG complex varies from tissue to tissue. However, a systematic comparison of the expression patterns and levels of several PcG genes in different tissues has not been done. Our study shows that PcG expression patterns are cell type-specific. A PcG complex can only be built up out of the PcG proteins that are expressed. This implies that the composition of PcG complexes must also be cell type-specific.

MATERIALS AND METHODS

Northern Blotting

Multiple Tissue Northern blots containing approximately 2 μg of poly(A)$^+$ RNA per lane from different human tissues were obtained commercially (CLONTECH). The blots were hybridized with [α-^{32}P] dATP-labeled DNA probes. X-ray film (Kodak X-OMAT AR) was preflashed and exposed to the blots with an

intensifying screen at −70°C for 1–4 days, depending on the probe. For *BMI1* and *RING1* full-length cDNA was used as probe [Alkema et al., 1993; Lovering et al., 1993]. The *HPH1* probe covers the coding cDNA sequence up to the homology regions it shares with *HPH2* [Gunster et al., 1997]. The probe for *HPC1* covers the coding sequence excluding the chromodomain and including 100 basepairs of 3′ untranslated region. The *HPC2* probe consists of the cDNA coding sequence between the chromodomain and the conserved C-terminus [Satijn et al., 1997b]. *EZH2* was detected with a cDNA probe encompassing 90 basepairs of 5′ untranslated region up unto amino acid 106 where it's homology with *EZH1* starts [Laible et al., 1997].

Immunohistochemistry

Human pancreas, thyroid, kidney, and fetal kidney were obtained freshly from the surgery room, fixed in 4% buffered formalin, and embedded in paraffin. Section (3 μm) were cut and deparaffinized, and endogenous peroxidase was inhibited with 0.3% H_2O_2 in methanol. Antigens were retrieved, by heating in the autoclave. After preincubation with swine or goat serum, primary antibodies were applied. The BMI1 protein was detected with 6C9 mouse monoclonal antibody. The following rabbit polyclonal antibodies were used for PcG protein detection: HPH1 (K344), RING1 (K320), HPC2 (K326), HPC1 (K350), EED (K365), and EZH2 (K358) [Gunster et al., 1997; Satijn et al., 1997a, 1997b; Sewalt et al., 1998]. Secondary antisera were biotinylated goat anti-mouse or biotinylated swine anti-rabbit antibody. Immunostaining was performed with 3-amino-9-ethylcarbazole as substrate using the streptavidin-biotin-avidin complex/horse radish peroxidase method and tyramine intensification. Sections were counterstained with hematoxylin and photographed with a Zeiss Axiophoto microscope (Zeiss Oberkochen, Germany).

RESULTS

Differential Transcription of PcG Genes in Various Tissues

To gain more insight into PcG function, we studied the expression of several PcG genes in various tissues using Northern blotting. We probed three Northern blots, containing poly $(A)^+$RNA from 23 different human tissues, for expression of *BMI1*, *RING1*, *HPH1*, *EZH2*, *HPC2*, and *HPC1* [Alkema et al., 1993; Lovering et al., 1993; Gunster et al., 1997; Laible et al., 1997; Satijn et al., 1997a].

The PcG gene transcription in the various tissues showed great diversity (Figs. 1–3). To be able to classify this variation, we grouped the data according to three criteria: (1) the number of distinct PcG genes that were expressed in one particular tissue, (2) the number of distinct tissues in which a certain PcG gene were expressed, (3) the expression level of individual PcG genes. According to these criteria we made an inventory of PcG expression in the various tissues (Table I). On the basis of criterion one we divided these tissues into three different classes: (i) it consists of tissues that express at least five out of the six PcG genes we analyzed, (ii) it consists of tissues that express two to four PcG genes, (iii) it consists of tissues that express one PcG gene or none at all. We detected PcG gene expression varying from high levels of expression to no detectable expression. To be able to give an indication about the expression level of the PcG genes we used the following semiquantitative determination. The term 'high' expression was used when the autoradiographic signal saturated the X-ray film. The term 'intermediate' expression was used when clear levels of expression were detected. The term 'low' expression was used when expression levels were low, but clearly above background levels. The term 'no detection' was used when no expression was detected or if expression levels were very hard to distinguish from the background.

Class I. It included six tissues. In the testis and heart all PcG genes we analyzed were expressed. In these two tissues PcG expression levels were mostly intermediate or high. In the pancreas, adrenal gland, thyroid, and liver the expression of five PcG genes could be detected. In these tissues expression of either *HPH1* or *EZH2* was below the detection level. The class I tissues all displayed intermediate to high level expression for one or more PcG genes.

Class II. It included eleven tissues. The kidney was the only tissue in class II in which four PcG genes could be detected. RING1 was expressed in all class II tissues, although at a low level. At the other end of the spectrum, in one of the class II tissues *EZH2* or *HPH1* expression could be detected. *HPC1* expression in class II was low and could only be detected in skeletal muscle and kidney. *HPC2* and *BMI1*

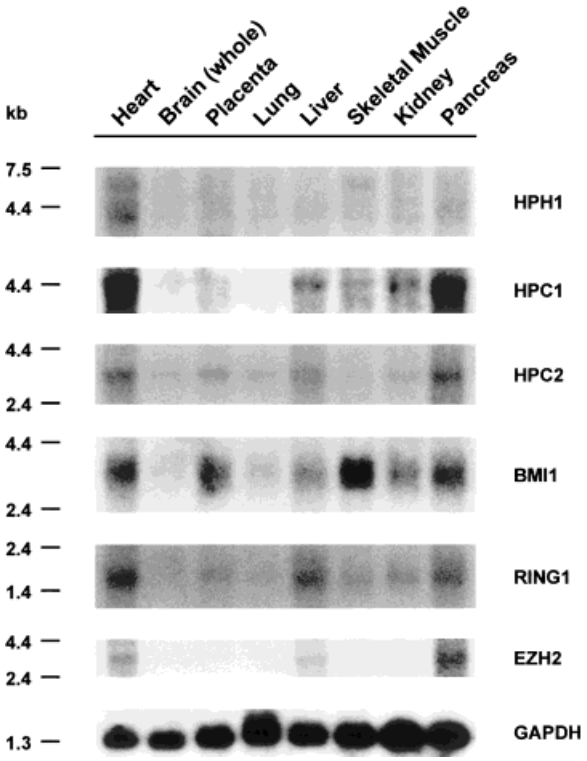

Fig. 1. PcG expression patterns in various human tissues I. Expression of *HPH1*, *HPC1*, *HPC2*, *BMI1*, *RING1*, and *EZH2* in human tissues. Shown are PcG expression levels in heart (**lane 1**), whole brain (**lane 2**), placenta (**lane 3**), lung (**lane 4**), liver (**lane 5**), skeletal muscle (**lane 6**), kidney (**lane 7**), and pancreas (**lane 8**). The filter was rehybridized with a probe for *GAPDH* to verify correct loading of the RNA. Molecular weight markers are indicated on the left in kilobases (kb). *HPH1* has a double transcript at 4.2 kb and at 5.0 kb. *HPC1* has a double transcript, the 4.2 kb and 4.4 kb transcripts are hard to discern. The *HPC2* transcript is 3.0 kb. The *BMI1* transcript is 3.3 kb. The *RING1* transcript is 1.6 kb. The *EZH2* transcript is 3.3 kb and the *GAPDH* transcript is 1.3 kb.

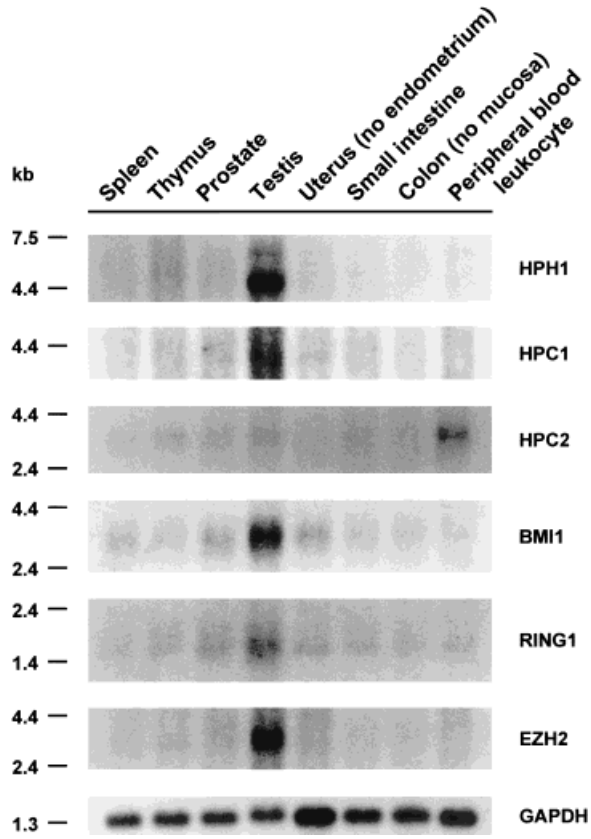

Fig. 2. PcG expression patterns in various human tissues II. Expression of *HPH1*, *HPC1*, *HPC2*, *BMI1*, *RING1*, and *EZH2* in human tissues. Shown are PcG expression levels in spleen (**lane 1**), thymus (**lane 2**), prostate (**lane 3**), testis (**lane 4**), uterus without endometrium (**lane 5**), colon without mucosa (**lane 6**), small intestine (**lane 7**), and peripheral blood leukocytes (**lane 8**). The filter was rehybridized with a probe for *GAPDH* to verify correct loading of the RNA. Molecular weight markers are indicated on the left in kilobases (kb).

were expressed in various combinations and mostly at low levels. High and intermediate *BMI1* expression could be detected in skeletal muscle and placenta respectively. Intermediate *HPC2* expression could be detected in peripheral blood leukocytes. Most of the class II tissues only showed low amounts of PcG expression. These tissues were the kidney, trachea, thymus, uterus, lymph node, spleen, prostate, and spinal cord.

Class III. It included six tissues, in which few PcG genes were expressed. Bone marrow was the only tissue in which no PcG expression could be detected. In the lung and the brain *HPC2* expression was detected. In the small intestine, colon, and stomach only *RING1* was expressed. BMI1, HPC1, EZH2, and HPH1 expression was not detected in class III tissues. When PcG expression could be detected in this class of tissues, it was always low.

In summary, the expression of the different PcG genes varied considerably according to all three criteria. Variation in criterion one ranged from no detected expression in the bone marrow to expression of all tested PcG genes in the heart and testis (Table I). Diversity in criterion two was also evident. *RING1* was expressed in most tissues, while *HPH1* and *EZH2* were expressed in only three and five tissues, respectively. As for criterion three, the more PcG genes expressed within a certain tissue, the higher the level of expression for those genes. Next we analyzed PcG expression for selected tissues at a cellular level.

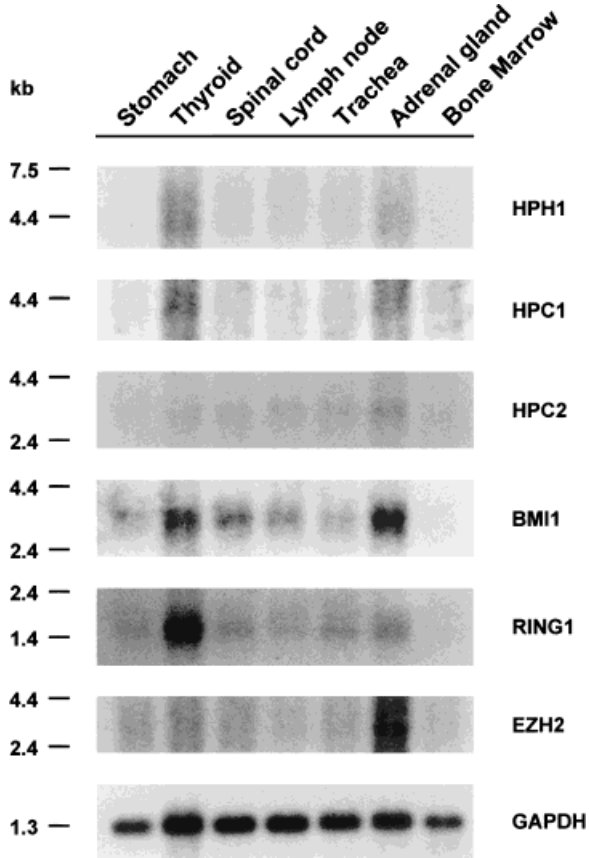

Fig. 3. PcG expression patterns in various human tissues III. Expression of *HPH1*, *HPC1*, *HPC2*, *BMI1*, *RING1*, and *EZH2* in human tissues. Shown are PcG expression levels in stomach (**lane 1**), thyroid (**lane 2**), spinal cord (**lane 3**), lymph node (**lane 4**), trachea (**lane 5**), adrenal gland (**lane 6**), and bone marrow (**lane 7**). The filter was rehybridized with a probe for *GAPDH* to verify correct loading of the RNA. Molecular weight markers are indicated on the left in kilobases (kb).

Differential Expression of PcG Proteins in Various Cell Types

Tissues are composed of several cell types. Co-expression of two PcG genes at the level of Northern analysis does, therefore, not necessarily mean that the PcG genes are co-expressed in the same cell. A clear example of this is PcG expression in the tonsil [Raaphorst et al., 2000a]. BMI1 and EZH2 are both expressed in the tonsil, but are not co-expressed in the same cell types. To gain more insight into PcG complex composition at the level of specific cell types, we performed immunohistochemistry. We choose the thyroid and pancreas, which expressed practically all PcG genes at high levels and the kidney, which expressed five PcG genes at a low level (Table I). To be able to compare the expression of particular PcG proteins in the different cell types, we established the following terminology. The term 'high expression' was used when expression could be detected in more than 90% of the cells of a specific cell type. The term 'varying expression' was used when expression could be detected in 10–90% of the cells of a specific cell type. The term 'no expression detected' was used when expression could only be detected in less than 10% of the cells of a specific cell type. As expected, in all the tissues that were analyzed an apparent nuclear staining for PcG proteins was evident [Gunster et al., 1997; Saurin et al., 1998; Sewalt et al., 1998]. However, in exocrine tissues sometimes some cytoplasmic background staining occurred (EED and EZH2 in pancreas; Fig. 5).

Thyroid. According to our Northern analysis the thyroid is classified as a class I tissue, expressing five out of the six PcG genes we analyzed. The thyroid is a hormone producing tissue. It predominantly comprises of one cell type, which is the endocrine epithelium (EE) that consists of thyrocytes, which secrete thyroxine (Fig. 4). In EE high expression was detected for BMI1, RING1, and EED (Fig. 4). Expression was varying for HPC2 and HPH1 (Fig. 4). HPC1 and EZH2 expression occurred in less than 10% of the cells and thus were considered as having no expression (Fig. 4). Importantly, in the EE of the thyroid the orthologues HPC1 and HPC2 are not expressed simultaneously. HPC2 is expressed while HPC1 is not. The composition of a PcG complex is limited to the PcG genes that are expressed in a certain cell type. Thus in EE the HPC/HPH complex could contain HPC2, HPH1, BMI1, and RING1. Interactions have been described for BMI1-RING1, BMI1-HPC2, BMI1-HPH1, and HPC2-RING1. Based on these interactions a model has been proposed for a possible composition of the HPC/HPH and EED/EZH complex [Satijn and Otte, 1999b]. We have incorporated the expression data of PcG proteins in the thyroid into this model and suggest a possible composition of the PcG complexes in the EE of the thyroid (Fig. 4). In EE proteins from both the HPC/HPH complex and the EED/EZH complex are expressed. As EZH2 is not expressed in the EE of the thyroid, EED might form a complex with other PcG proteins that were not analyzed.

Pancreas. The pancreas is, according to our Northern analysis, classified as a class I tissue.

TABLE I. Diversity of PcG Gene Transcription in Human Tissues[a]

	RING1	*HPC2*	*BMI1*	*HPC1*	*EZH2*	*HPH1*	
Testis	++	+	+++	++	+++	+++	Class I
Heart	++	++	++	+++	+	++	
Pancreas	++	++	++	+++	++	−	
Adrenal gland	+	+	++	+	++	−	
Thyroid	+++	+	++	+	−	+	
Liver	++	+	+	+	+	−	
Kidney	+	+	+	+	−	−	Class II
Skeletal muscle	+	−	+++	+	−	−	
Placenta	+	+	++	−	−	−	
Spinal cord	+	+	+	−	−	−	
Prostate	+	+	+	−	−	−	
Peripheral blood leukocytes	+	++	−	−	−	−	
Spleen	+	−	+	−	−	−	
Lymph node	+	+	+	−	−	−	
Uterus	+	−	+	−	−	−	
Thymus	+	+	−	−	−	−	
Trachea	+	+	−	−	−	−	
Lung	−	+	−	−	−	−	Class III
Brain	−	+	−	−	−	−	
Small intestine	+	−	−	−	−	−	
Colon	+	−	−	−	−	−	
Stomach	+	−	−	−	−	−	
Bone marrow	−	−	−	−	−	−	

[a]Tissues are arranged and divided into classes according to criterion one: the number of PcG genes they express; Class I: at least five out of six PcG genes are expressed; Class II: two to four PcG genes are expressed; Class III: one or none of the PcG genes are expressed. The PcG genes are arranged according to criterion two: the number of tissues in which they are expressed. Criterion three, the expression levels, are indicated: − no expression, + low expression, ++ intermediate expression, +++ high expression.

It primarily consists of two kinds of tissue, the exocrine cells (EC) and the endocrine islet cells (IC), (see Fig. 5). The EC mainly produce proteolytic digestive enzymes. There are several kinds of IC, however most of them are the so-called β-IC, which produce insulin. The IC showed high expression for BMI1 and RING1 and varying expression for HPH1 and HPC2 (Fig. 5). HPC1, EZH2, and EED expression could not be detected in the IC. In the EC expression of HPH1, EZH2, and EED was not detected. Expression of BMI1, RING1, HPC1, and HPC2 was detected with varying intensity and below the level of expression in IC (Fig. 5). In the pancreas no proteins from the EED/EZH complex are expressed. The possible composition of the HPC/HPH complex in IC and EC is presented in a model (Fig. 5). In the IC of the pancreas the possible composition of the HPC/HPH complex is different from that in EC (Fig. 5). In the IC HPC1 is lacking and HPH1 can be part of the complex. In EC however, HPH1 is lacking and HPC1 can be part of the complex (Fig. 5). HPH1 and HPC1, which seemed to be co-expressed in pancreas at the Northern level, apparently have a mutually exclusive expression pattern at a cellular level.

Kidney. The kidney is a class II tissue that expressed four out of the six PcG genes that were analyzed on Northern blot. In the kidney, waste products are separated from the blood in spherical structures called glomeruli. Each glomerulus (Glo) is encapsulated by Bowman's capsule. The blood flows through the glomerulus and the filtered fluid is secreted into the urinary space between the glomerulus and Bowman's capsule. The urinary space is drained to the proximal tubule, which further on becomes a distal tubule. For our study of PcG expression in the kidney we will observe three cell types: the cells comprising the proximal and distal tubules (Pt and Dt) and the cells comprising the Glo, (see Fig. 6).

In the cells comprising the Dt high expression was detected for BMI1, HPH1, HPC1, and EED, varying levels of expression were detected for RING1, while no expression was detected for HPC2 or EZH2 (Fig. 6). In the cells comprising the Pt BMI1, varying levels of expression for RING1 and EED were detected and no expression was detected for HPH1, HPC1, HPC2, and EZH2. Finally, in the Glo varying expression was detected for BMI1, RING1, HPH1, and EED, while no HPC1, HPC2, or EZH2 expression was detected (Fig. 6).

We made a model of the possible composition of PcG complexes in several cell types of the kidney (Fig. 6). In the Pt of the kidney RING1 and BMI1 make up the HPC/HPH complex. In the Glo also HPH1 can be part of the HPC/HPH

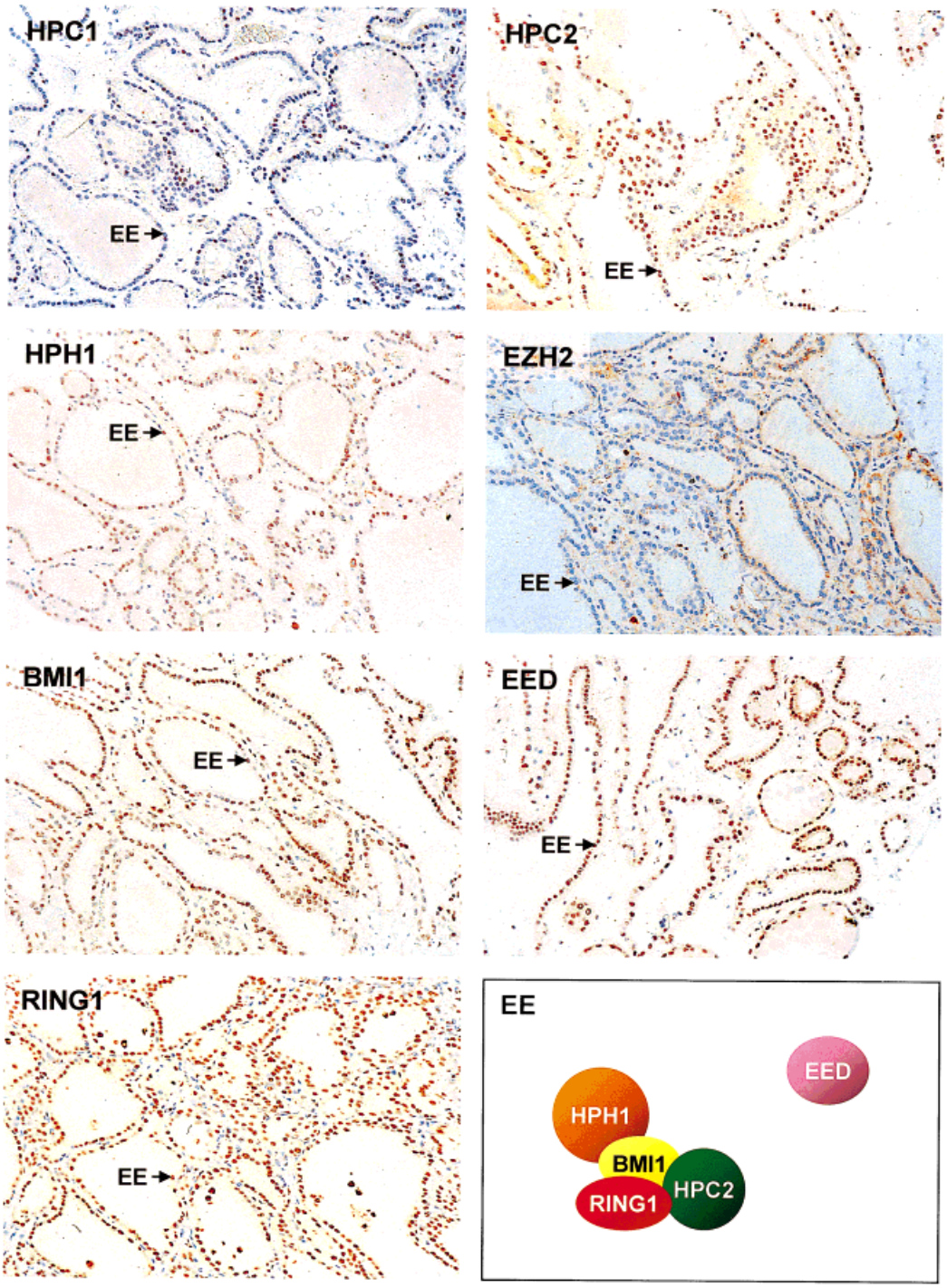

Fig. 4. Immunohistochemical detection of PcG proteins in the thyroid. PcG expression was detected with specific antibodies as indicated in the upper left corner. Positive nuclei are stained in red. Negative nuclei are blue, due to hematoxylin counter-staining. Endocrine epithelium is indicated (EE). Possible composition of PcG complexes in EE of the thyroid is depicted in a model. Overlapping spheres indicate the possibility of direct protein–protein interactions.

complex. Finally, in the Dt HPC1 can become part of the HPC/HPH complex (Fig. 6). EED is co-expressed with the HPC/HPH complex in Glo, the Pt, as well as the Dt (Fig. 6). In these three kidney cell types the possible composition of the HPC/HPH complex was different each time. On the other hand, no differences for the EED/EZH complex were found.

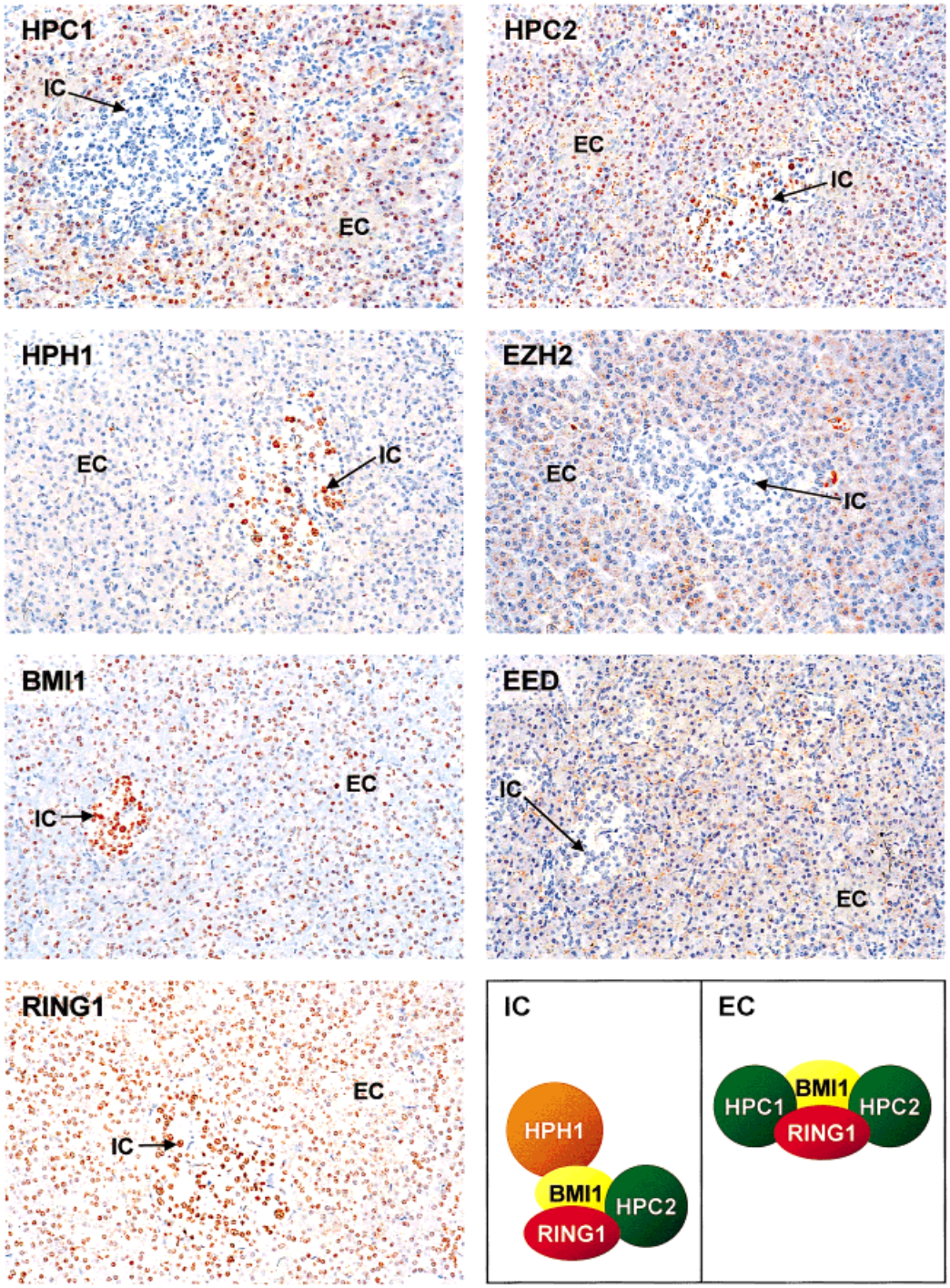

Fig. 5. Immunohistochemical detection of PcG proteins in the pancreas. PcG expression was detected with specific antibodies as indicated in the upper left corner. Positive nuclei are stained in red. Negative nuclei are blue, due to hematoxylin counter-staining. IC and exocrine epithelium cells (EC) are indicated. The possible compositions of PcG complexes in EC and IC are depicted in a model. Overlapping spheres indicate the possibility of direct protein-protein interactions.

Expression of PcG Proteins in Fetal Kidney

We observed characteristic combination of expressed PcG proteins in distinct cell types of various adult human tissues. We next asked if these characteristic PcG expression patterns change during embryogenesis or whether they are the same in the fetal and the adult stages of

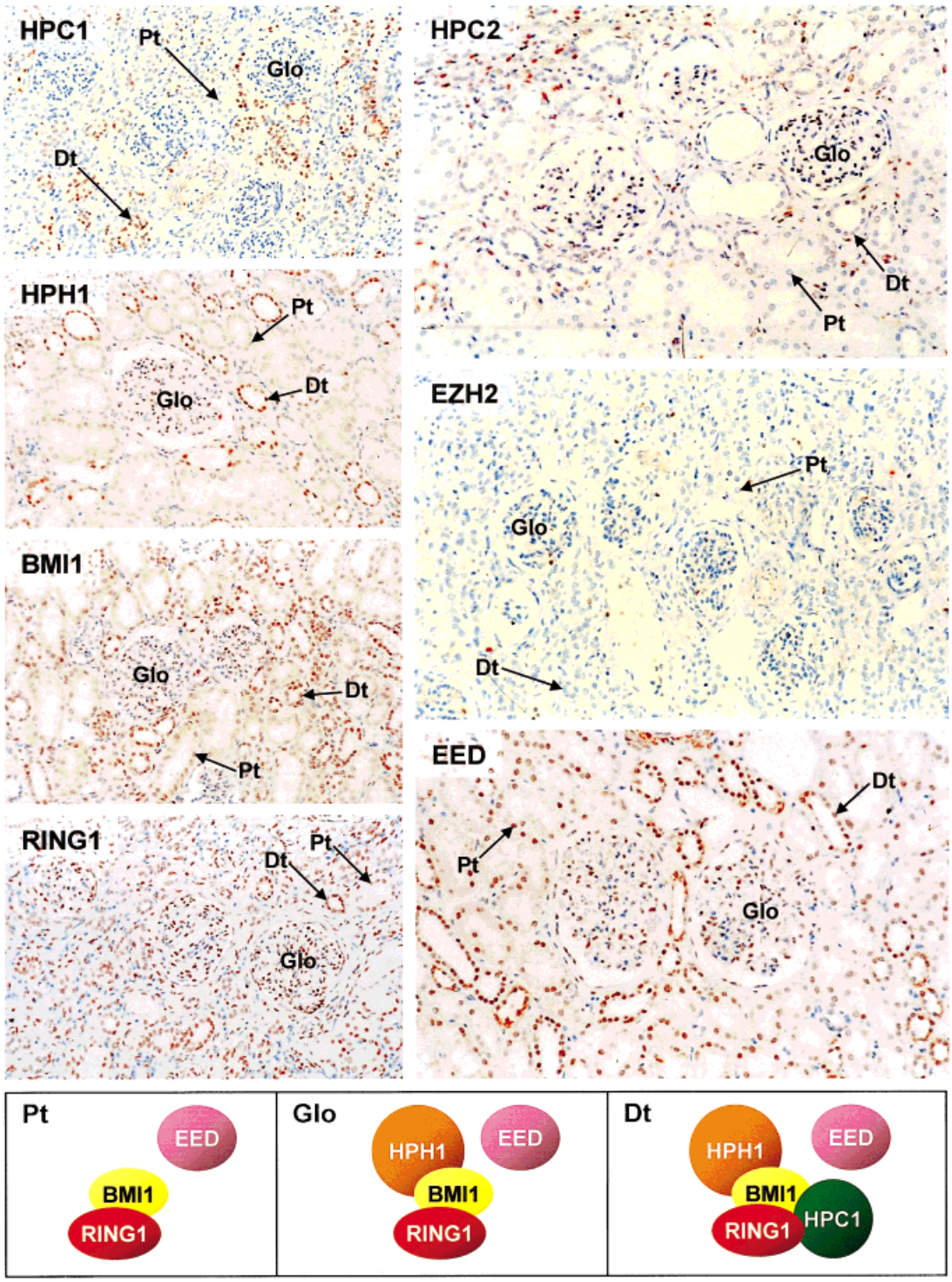

Fig. 6. Immunohistochemical detection of PcG proteins in the kidney. PcG expression was detected with specific antibodies as indicated in the upper left corner. Positive nuclei are stained in red. Negative nuclei are blue, due to hematoxylin counter-staining. Cells comprising the Dt, proximal tubules (Pt), and the Glo are indicated. In the tissue sections that were stained with HPC1 and EZH2 the Dt and Pt were hard to distinguish. The cells from the Dt have little cytoplasm and somewhat flattened nuclei (visible at higher magnification; data not shown). The cells from the Pt have abundant cytoplasm with an irregular apical border and a centrally placed nucleus. Possible compositions of PcG complexes in Pt, Dt and Glo cells of the kidney are depicted in a model. Overlapping spheres indicate the possibility of direct protein–protein interactions.

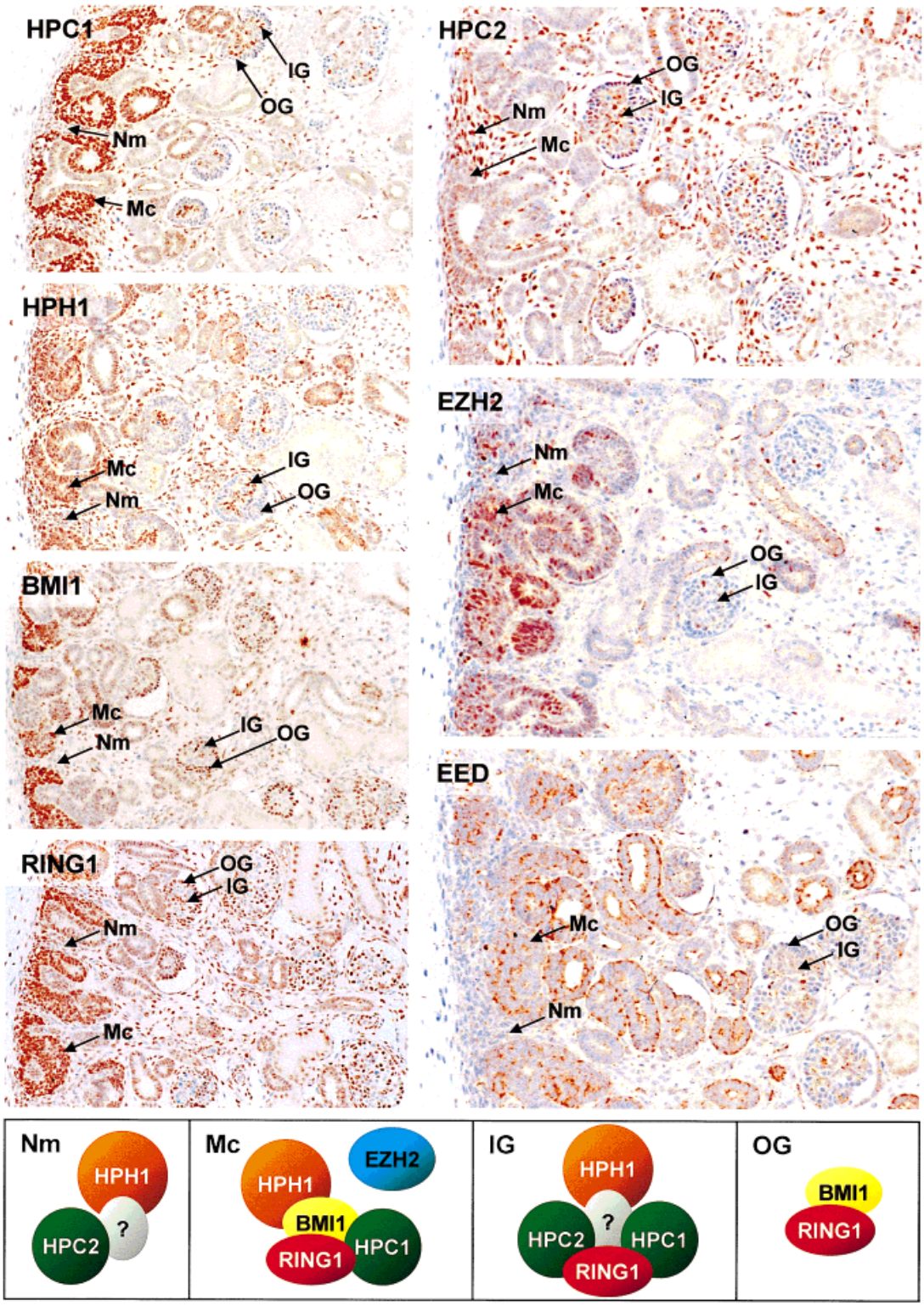

Fig. 7. Immunohistochemical detection of PcG proteins in fetal kidney. PcG expression was detected with specific antibodies as indicated in the upper left corner. Positive nuclei are stained in red. Negative nuclei are blue, due to hematoxylin counterstaining. Nm, Mc, inner glomerular precursor cells (IG) and OG are indicated. Possible compositions of for PcG complexes in various stages of glomerular development (Nm, Mc, IG and OG) in fetal kidney are depicted in a model. Overlapping spheres indicate the possibility of direct protein–protein interactions.

TABLE II. PcG Protein Expression at Various Stages of Glomerulogenesis[a]

	HPH1	HPC1	HPC2	BMI1	RING1	EZH2	EED
Nephrogenic mesenchyme	+	−	+	−	−	−	−
Mesenchymal condensates	+	+	−	+	+	+	−
Inner glomerular precursor cells	±	±	±	−	±	−	−
Outer glomerular precursor cells	−	−	−	+	+	−	−
Mature glomerular cells	±	−	−	±	±	−	−

[a]The expression of several PcG proteins in specific stages of glomerular development is displayed. The expression pattern of mature glomerular cells is shown again for comparison. Expression levels are indicated: − no expression, ± varying expression, + high expression.

the tissue. To compare the PcG expression patterns in fetal and adult tissue we stained tissue sections of fetal kidney. We chose the formation of Glo in fetal kidneys as a model. Glomerulogenesis takes place in the outer cortex of fetal kidneys. The first stage consists of proliferating cells, called nephrogenic mesenchyme (Nm), (see Fig. 7). In the next stage these cells have given rise to condensed structures, called mesenchymal condensates (Mc). In the following stage the Mc have developed into epithelial cells with comma- and S-shaped structures. In the subsequent stage, called the 'capillary loop' stage, the Glo is already preformed. During the final stage the fetal Glo matures towards an adult Glo, accordingly it is called the maturing stage [Nagata et al., 1998].

Human fetal kidney tissue of approximately 13 weeks gestational age was analyzed for Mc and the capillary loop stage. In Nm HPH1 and HPC2 expression was high, whereas no BMI1, RING1, HPC1, EED, or EZH2 expression was detected (Fig. 7; Table II). In Mc however, expression of BMI1, RING1, HPH1, HPC1, and EZH2 was high, while no HPC2 or EED expression was detected. At the capillary loop stage the inner and outer glomerular precursor cells (IG and OG) showed differential PcG expression. The IG expressed varying levels of RING1, HPH1, HPC1, and HPC2, while no BMI1, EED, or EZH2 was detected (Fig. 7; Table II). The OG expressed high levels of BMI1 and RING1, while HPH1, HPC1, HPC2, EED, and EZH2 could not be detected.

During glomerulogenesis striking changes in PcG expression were evident, giving rise to various possible PcG complexes (Fig. 7). Focusing on BMI1 and HPC2, a mutually exclusive expression pattern was evident in various stages of glomerular development. The other PcG proteins do not follow this pattern, but were expressed in their own distinctive way. A model of the possible compositions of PcG complexes in the various stages of glomerulogenesis is depicted in Figure 7.

We conclude that PcG expression changes dynamically during development. A comparison of the PcG expression pattern of several stages of glomerular development and mature glomeruli has been made (Table II). The PcG expression patterns in the several stages of glomerular development are all different from the adult situation. The expression pattern of the OG, one of the later stages in glomerular development, resembles most closely the adult situation (Table II).

DISCUSSION

Expression of PcG Genes at the Level of mRNA and Protein

In this paper we analyzed the expression levels of several human PcG genes in human tissues, both at the level of mRNA and protein. Northern blot analysis of the expression of six PcG genes in 23 human tissues revealed great diversity in human PcG gene expression. In the heart and testis all analyzed PcG genes were expressed, while in bone marrow no PcG expression could be detected. In the other tissues varying numbers of PcG genes could be detected (Table I). In some tissues identical expression patterns were detected (e.g. brain and lung, see Table I). Still there could be differences in PcG expression between these tissues. It is possible that Northern blotting does not detect very low levels of expression. In the bone marrow, for instance, we could not detect any expression of PcG genes. With RT-PCR, however, *BMI1* and *EZH2* expression could be detected in this tissue [Lessard et al., 1998]. Apart from sensitivity limitations there are also several PcG genes we didn't analyze, for example other PcG orthologues such as *EZH1* and *HPC3* or the human

homologue of sex combs on midleg, *SCML1* [Abel et al., 1996; van de Vosse et al., 1998; Bardos et al., 2000]. It is therefore possible that tissues that show identical expression patterns for PcG genes that were tested vary in expression pattern for other PcG genes.

In general, the PcG expression patterns as seen by RNA analysis coincided with immunohistochemistry. When expression of a PcG gene was detected for a certain tissue by Northern blotting, the corresponding PcG protein was also detected in one or more cell types of the same tissue. However, there were a few cases in which the expression as detected at the RNA level was different from the expression that was detected at the protein level. Expression of HPC1 was detected at the mRNA level, but not at the protein level in the thyroid. EZH2 expression was detected in the pancreas using Northern blotting, but not using immunohistochemistry, and vice versa, expression of HPC1 in the pancreas could not be detected at the mRNA level, but was detected at the protein level. Discrepancies in RNA and protein expression can be explained in several ways. It is possible that these genes are indeed both expressed at the mRNA and protein level, but that one or the other is just below the level of detection. In the case of HPC1 in the thyroid and EZH2 in the pancreas, the respective mRNAs could perhaps not be translated. Another possibility is that the mRNA is very unstable, while the corresponding protein is very stable. An example of this is the *Drosophila* PcG protein extra sex combs. This protein can be detected on polytene chromosomes long after RNA levels have already dropped below the detection level [Tie et al., 1998]. This could be an explanation for the lack of HPC1 mRNA in the pancreas where HPC1 protein was detected. However, overall the mRNA expression profile correlated with the tissues that were investigated for PcG protein expression.

Diversity in PcG Complex Composition

In our study we have found that there is a great diversity in PcG expression, both among tissues and among cell types within particular tissues. The differential expression of PcG proteins implies that also the composition of the PcG complex differs. PcG complexes are formed and held together by protein–protein interactions. However, PcG proteins do not directly interact with any other PcG protein. PcG proteins interact specifically with a limited number of so-called protein partners [see Satijn and Otte, 1999b for a review]. For example, RING1 interacts with itself, HPC1, HPC2, and BMI1, HPC1 and HPC2 in turn bind to BMI1, However, BMI1 is the only protein that has been shown to bind to HPH1. Since in Nm of the fatal kidney only HPC2 and HPC1 are expressed and BMI1 is not (Fig. 7; Table II), how then do HPH1 and HPC2 associate in a complex? Perhaps in these cells HPH1 and HPC2 are components of separate PcG complexes. It is also possible that there is another PcG protein that might interact with HPH1 as well as HPC2. It is possible that HPH1 is associated with other parts of the complex through its interaction with dinG, the orthologue of RING1. The dinG protein has been shown to directly interact with both Mph2 and M33 [Schoorlemmer et al., 1997; Hemenway et al., 1998]. Perhaps dinG also interacts with the human orthologues of Mph2 and M33, HPH1, and HPC2 respectively. If this is the case HPH1 can be associated with HPC2 without the presence of BMI1. Even though several direct protein–protein interactions between PcG proteins have been identified, our results may indicate that there are other interactions that facilitate the formation PcG complexes that have thus far not been identified.

Another way the composition of PcG complexes could be altered to obtain additional target specificity, is the stoichiometry of the proteins in the complex. A PcG complex could be composed of the same PcG proteins, but the ratio in which these proteins are present in the complex might differ. For example, in both heart and testis all six PcG genes we analyzed were expressed. Therefore the PcG complex in these two tissues could consist of the same PcG proteins. However, the expression levels of the different PcG genes were different in these tissues. In the heart more *HPC2* was expressed than in the testis. *HPH2* on the other hand, was expressed at a higher level in the testis than in the heart. This indicates that the PcG complex in the heart might contain more HPC2 than HPH1, while the PcG complex in the testis might contain more HPH1 than HPC2. Such stoichiometric diversity may be an additional level of variety in PcG complex composition.

Apart from variations in the composition of the PcG complex it has also been shown that there are two entirely separate PcG complexes, the HPC/HPH complex and the EED/EZH com-

plex. The first clue as to the existence of separate PcG complexes came from the study of interacting partners for the EED and EZH2 proteins. These proteins interact with each other, but not with BMI1, HPH1, HPC2, or RING1 [Sewalt et al., 1998]. These complexes also have been shown to have a mutually exclusive expression pattern in tonsils [Raaphorst et al., 2000a]. This mutual exclusivity in expression for the HPC/HPH and EED/EZH complexes is not universal. Co-expression of PcG proteins from the separate complexes in humans has been shown in fresh tumors [Raaphorst et al., 2000b] and cell lines [Sewalt et al., 1998]. We have found that proteins from both complexes can also be co-expressed in healthy tissue. In the EE of the thyroid (Fig. 4) and in several cell types of the kidney we found co-expression of the EED protein and proteins from the HPC/HPH PcG complex (Fig. 6). Co-expression of EZH2 and proteins from the HPC/HPH complex was seen in the Mc of fetal kidney (Fig. 7; Table II). Apparently co-expression of the two separate PcG complexes is not a specific trait of tumor cells, but can also appear in healthy cells. Unlike in tonsils, EED and EZH2 were never co-expressed in the tissues we analyzed. If EED or EZH2 are expressed on their own, how then is the EED/EZH complex built up? It is possible that there are other PcG proteins that have not yet been identified as being part of the EED/EZH complex. One such PcG protein could be a human homologue of *Drosophila* Polycomblike (Pcl) [Lonie et al., 1994]. Like E(z), Pcl did not co-purify with the complex containing the Pc and ph proteins [Shao et al., 1999]. Perhaps Pcl is part of the esc/E(z) complex. By analogy, a human Pcl homologue could be part of the EED/EZH complex. Identification of a Pcl homologue and the characterization of its protein interactions will bring more insight into this matter.

Implications for Varying Complex Composition

Apart from cell types, developmental stages have specific gene expression patterns. An example of developmentally changing patterns of expression is found in kidney development [Nagata et al., 1998]. We described the expression pattern of seven PcG proteins in the development of the Glo in fetal kidney. At each stage of glomerulogenesis a different PcG expression pattern was observed (Table II). This suggested that the composition of PcG complexes is not static throughout development but rather that PcG complex composition is dynamic, indicating that a specific PcG complex is established at each developmental stage. These data are in accordance with the idea that the composition of PcG complexes determines the specificity for their targets and that consequently different PcG complexes are needed to maintain the repression of different sets of genes. Apart from human fetal kidney, there are also other cases of changing PcG expression during development. In early mouse embryogenesis Ezh2 is expressed ubiquitously, while midway embryonic development Ezh2 is mainly expressed in the liver [Hobert et al., 1996]. By late mouse embryogenesis Ezh2 is mainly expressed in the thymus, showing Ezh2 is dynamically regulated during development. In human bone marrow, tissue with constantly differentiating cells, PcG gene expression has also been shown to be stage specific. The primitive $CD34^+$ population expresses BMI1 and no HPC1, Mel-18, or HPH1, while the mature $CD34^-$ population expresses HPC1, Mel-18, and HPH1, but no BMI1 [Lessard et al., 1998]. These data suggest that during development changes in PcG expression patterns occur and that different PcG complexes may exist at various developmental stages.

To be able to obtain specificity for cell-type and even developmental stage-specific sets of target genes, PcG complexes would need to be able to consist in many different compositions. The multiple copies of PcG genes in humans might be a way to achieve this specificity. Human PcG orthologues have considerable sequence homology in functionally conserved protein domains. The expression pattern of PcG orthologues differs considerably. The orthologues HPC1 and HPC2 have different expression patterns. Also, HPC2 was expressed in far more cell types than HPC1, which was only expressed in a subset of the cells that express HPC2 (Table I). The presence of HPC1 or HPC2 in a PcG complex could have significant consequences for its properties. HPC2 has a short motif through which it can bind to the co-repressor CtBP [Katsanis and Fischer, 1998; Sewalt et al., 1999]. HPC1 does not have this motif. It has been shown that the murine HPC1 homologue M33, which also lacks this motif, does not bind CtBP [Sewalt et al., 1999]. The *Drosophila* homologue of CtBP, dCtBP, binds to the DNA-binding gene repressors Krüppel, Snail, and Knirps [Nibu et al., 1998]. It could be that

through the interaction with these repressor proteins the PcG complex in *Drosophila* is targeted to some of the genes the PcG complex needs to repress. It is very well possible that a similar mechanism is used in humans. HPC2 might have an essential role in targeting the PcG complex to certain loci through its interaction with CtBP. While HPC1 might serve to modulate the PcG complex specificity. Thus the composition of the PcG complex could determine the specificity for its target genes.

We have shown that PcG expression in humans is extremely diverse. The expression pattern of human PcG genes varies greatly depending on the tissue, cell type, and developmental stage. The variety in PcG gene expression implies that there is also variety in PcG complex composition. In *Drosophila* differently composed PcG complexes have been shown to be associated with different target genes [Strutt and Paro, 1997]. Whether this is also the case in humans is as yet unknown. The identification of well defined human PcG target genes will open up more possibilities for the study of human PcG complex composition at various target gene loci.

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