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CUTTING EDGE

Cutting Edge: Polycomb Gene Expression Patterns Reflect Distinct B Cell Differentiation Stages in Human Germinal Centers

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Polycomb group (Pc-G) proteins regulate homeotic gene expression in *Drosophila*, mouse, and humans. Mouse Pc-G proteins are also essential for adult hematopoietic development and contribute to cell cycle regulation. We show that human Pc-G expression patterns correlate with different B cell differentiation stages and that they reflect germinal center (GC) architecture. The transition of resting mantle B cells to rapidly dividing Mib-1(Ki-67)⁺ follicular centroblasts coincides with loss of BMI-1 and RING1 Pc-G protein detection and appearance of ENX and EED Pc-G protein expression. By contrast, differentiation of centroblasts into centrocytes correlates with reappearance of BMI-1/RING1 and loss of ENX/EED and Mib-1 expression. The mutually exclusive expression of ENX/EED and BMI-1/RING1 reflects the differential composition of two distinct Pc-G complexes. The Pc-G expression profiles in various GC B cell differentiation stages suggest a role for Pc-G proteins in GC development. *The Journal of Immunology*, 2000, 164: 1–4.

Recent studies identified polycomb group (Pc-G)[2] genes as essential regulators of mouse lymphoid development (1–3). Pc-G proteins form an evolutionary conserved cellular memory system that ensures stable transmission of gene activity (4, 5). These gene-regulatory proteins were originally discovered in *Drosophila* as repressors of homeobox genes. Pc-G proteins and their trithorax group counterparts, which have an opposing, activating role, ensure correct embryonal development. Mutations in Pc-G genes can result in homeotic transformations, the change of one body part into another (4). Pc-G proteins form

heterogeneous multimeric protein complexes (6–11). In humans, two distinct Pc-G complexes were identified by yeast two-hybrid analyses. The BMI-1 Pc-G protein is part of the first complex and coimmunoprecipitates, cofractionates, and colocalizes with RING1, HPH1, HPH2, and HPC2 Pc-G proteins (7, 8, 12, 13). These Pc-G proteins have extensive interactions with each other. A second Pc-G complex contains the ENX and EED Pc-G proteins. ENX and EED interact with each other and coimmunoprecipitate. However, neither ENX nor EED interacts or coimmunoprecipitates with Pc-G proteins of the other complex, such as BMI-1 or HPC2 (9). The composition of these distinct Pc-G complexes probably contributes to target gene specificity and tissue-specific gene expression.

The role of Pc-G genes in developmental processes continues in adult life, and studies of Pc-G mutant mice demonstrated that Pc-G genes are essential for lymphoid development. Lymphocyte precursors in mice with a targeted deletion of MEL-18 (1) or BMI-1 (2) insufficiently respond to IL-7, which ultimately leads to severe lymphoid hypoplasia. Upon activation through their Ag receptor, B cells of MEL-18 transgenic mice enter cell cycle arrest through a c-*myc*/cdc25 cascade (14). By contrast, BMI-1 transgenic mice exhibit enhanced lymphoproliferation and induction of lymphomas, possibly by suppressing expression of p16/INKA4 and p19arf (15), and in collaboration with c-*myc* (3, 16). The latter observations demonstrate that Pc-G proteins also have an important role in regulation of the cell cycle.

The contribution of Pc-G genes to regulation of human hematopoiesis is relatively unexplored. PCR analysis of bone marrow subpopulations showed marked changes of human Pc-G transcription levels in developing lymphoid precursors (17). Pc-G transcription is detectable in tissues with high cell division rates, such as embryonic tissue or bone marrow, and appears to change at different stages of differentiation. Since germinal centers (GCs) are characterized by high cell proliferation rates, with maintenance of spatial maturity patterns of lymphoid cells, we questioned whether differentiation of B cells in GCs is reflected in altered expression of human Pc-G genes. We demonstrate that the GC mantle zone, dark zone (DZ), and light zone (LZ) are associated with distinct expression profiles of the BMI-1/RING1 and ENX/EED Pc-G proteins. These patterns reflect the differential composition of the two previously identified distinct Pc-G complexes and suggest that human Pc-G proteins play a role in GC development and Ag-specific B cell differentiation.

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[2] Abbreviations used in this paper: Pc-G, polycomb group; DZ, dark zone; GC, germinal center; LZ, light zone.
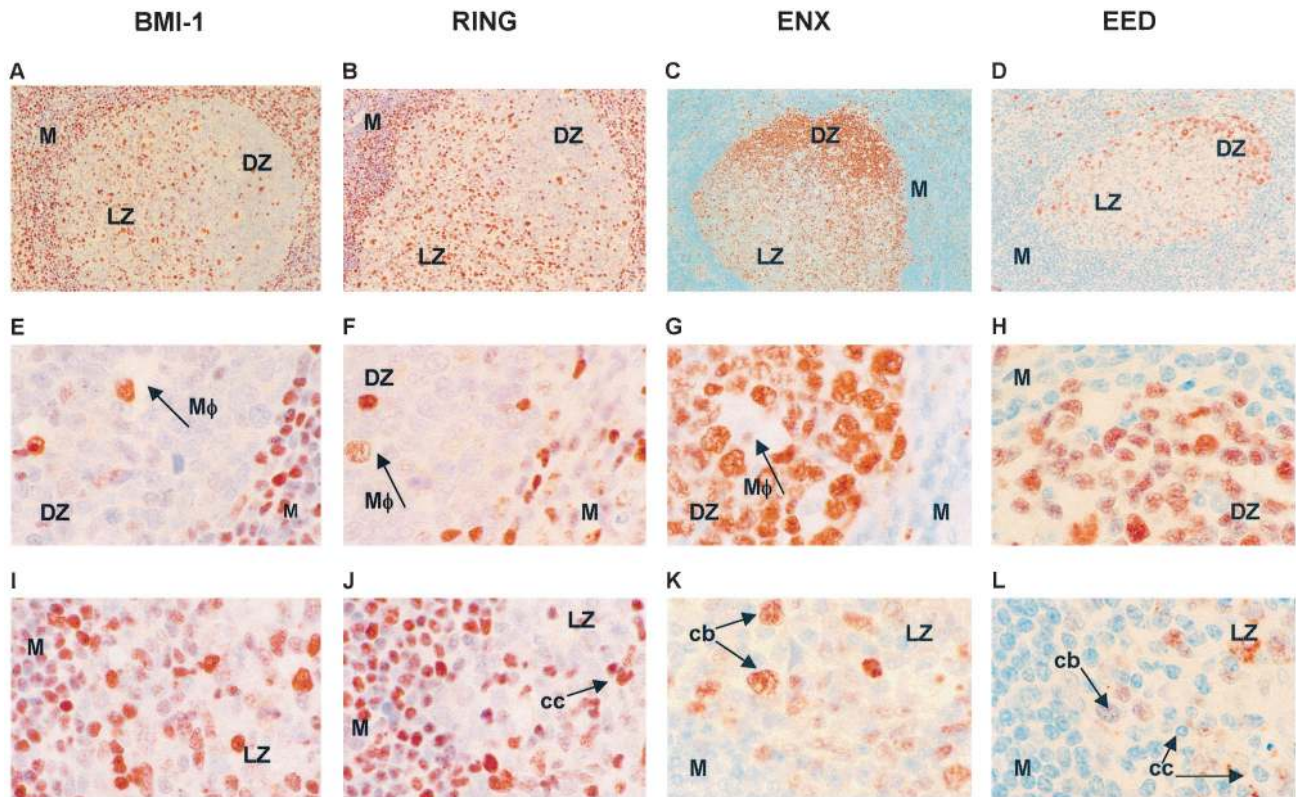
FIGURE 1. Immunohistochemical detection of human Pc-G BMI-1, RING-1, ENX, and EED proteins in secondary GCs of the tonsil. *A–D*, GC overview. *E–H*, Detail of the DZ and mantle zone (M). *I–L*, Detail of the LZ and M. *A*, *E*, and *I*, Detection of BMI-1; *B*, *F*, and *J*, detection of RING-1; *C*, *G*, and *K*, detection of ENX; *D*, *H*, and *L*, detection of EED. Centroblasts (cb) contain large, plump nuclei and primarily reside in the DZ. Centrocytes (cc) contain angular or cleaved nuclei and primarily reside in the LZ. Mϕ, tingible body macrophage.

Materials and Methods

Tonsils and lymph nodes were obtained freshly from the surgery room, fixed in 4% buffered Formalin, and embedded in paraffin. Sections (3 μm) were cut and deparaffinized, and endogenous peroxidase was inhibited with 0.3% H_2O_2 in methanol. For immunohistochemistry, primary Abs were applied following microwave Ag retrieval and preincubation with swine or goat serum. BMI-1, RING-1, ENX, and EED Pc-G proteins were detected with the 6C9 mouse monoclonal and K320, K358 and K365 rabbit polyclonal antisera, respectively (9). Secondary antisera were biotinylated goat anti-mouse or biotinylated swine anti-rabbit. Immunostaining was performed with 3-amino-9-ethylcarbazole using the streptavidin-biotin-avidin complex/HRP method and tyramine intensification. Sections were counterstained with hematoxylin and photographed with a Zeiss Axiophoto microscope (Zeiss, Oberkochen, Germany).

For double immunofluorescence, tissue sections were fixed in 2% formaldehyde and endogenous peroxidase was inhibited as above. Following preincubation with 5% BSA, primary Abs against BMI-1 (6C9) or Mib-1/Ki-67 were applied in combination with K358 (anti-ENX) or A452 (anti-CD3). BMI-1 and Mib-1 were detected by biotinylated goat anti-mouse antiserum followed by streptavidin^{CY3}. ENX and CD3 were detected by swine anti-rabbit Ig-peroxidase-antiperoxidase), followed FITC. Cross-reactivity of the antisera was excluded by appropriate controls, and sections were analyzed and photographed with a Leica DMRB immunofluorescence microscope (Leica, Deerfield, IL).

Results and Discussion

### B cell subpopulations in the GC have distinct BMI-1 and ENX expression patterns

GCs are sites of multiplication and selection of Ag-specific B cells and consist of a follicle surrounded by a mantle zone (18, 19). The follicle contains a DZ and a LZ. During the GC reaction, rapidly dividing centroblasts give rise to centrocytes that are subject to antigenic selection. The DZ is primarily composed of centroblasts and centrocytes, whereas the LZ mainly contains centrocytes, a

follicular network of Ag-presenting dendritic cells and helper T cells. B cells in the mantle zone are regarded as the precursors of centroblasts (the primary follicle).

We performed an immunohistochemical analysis of Pc-G expression in GCs of the human tonsil. BMI-1 and RING1, and ENX and EED, were investigated as representative of two different human Pc-G complexes. Expression of these proteins was detected in nuclear staining patterns, as shown previously (7–9), and depended on the stage of lymphocyte development in the GC reaction (Fig. 1). Similar results were obtained with GCs in lymph nodes (data not shown). Resting B cells in the mantle zone stained for BMI-1/RING1 with varying intensity, but did not express ENX or EED (Fig. 1). By contrast, centroblasts in the DZ area were BMI-1$^-$ and RING1$^-$ (Fig. 1, *E* and *F*), whereas the vast majority of such cells expressed ENX and EED (Fig. 1, *G* and *H*). A large portion of LZ centrocytes expressed BMI-1 and RING1 (Fig. 1, *I* and *J*), whereas ENX and EED expression in the LZ was notably reduced (Fig. 1, *K* and *L*). Most ENX$^+$ or EED$^+$ cells in the LZ had a centroblast appearance. Note that occasional BMI-1$^+$ or RING$^+$ cells in the DZ were ENX$^-$- and EED$^-$-tingible body macrophages (Fig. 1, *E–H*). In addition, a minority of BMI-1$^+$ follicular lymphocytes were T cells (Fig. 2*A*).

The BMI-1/RING1 and ENX/EED staining patterns suggested that expression of these proteins is mutually exclusive during the GC reaction. We therefore detected BMI-1 and ENX by double immunofluorescence to further explore follicular Pc-G expression patterns. As predicted by immunohistochemistry, BMI-1 and ENX expression did not overlap. Mantle zone B cells were single positive for BMI-1 and did not stain for ENX, whereas follicular cells expressed either BMI-1 or ENX (Fig. 2*B*). Since transition from

FIGURE 2. Immunofluorescent detection of the human BMI-1 and ENX Pc-G genes in secondary GCs of the tonsil. *A*, Double immunofluorescence analysis of BMI-1 (red fluorescence) and CD3 (green fluorescence, identifying T cells). *B*, double immunofluorescence analysis of BMI-1 and ENX expression (red and green fluorescence, respectively). Coexpression is visualized as yellow fluorescence. *C*, Analysis of Mib-1 (Ki-67, red fluorescence) and BMI-1 (green fluorescence) coexpression demonstrating that BMI-1$^+$ cells are Mib-1$^-$. *D*, Analysis of Mib-1/Ki-67 and ENX coexpression (green and red fluorescence, respectively) signifying the relationship between ENX expression and proliferation. Note that nuclei coexpressing ENX and Mib-1/Ki-67 do not stain evenly yellow because ENX and Mib-1 appear to be present in different nuclear regions. For legend, see Fig. 1. T: T cell area.
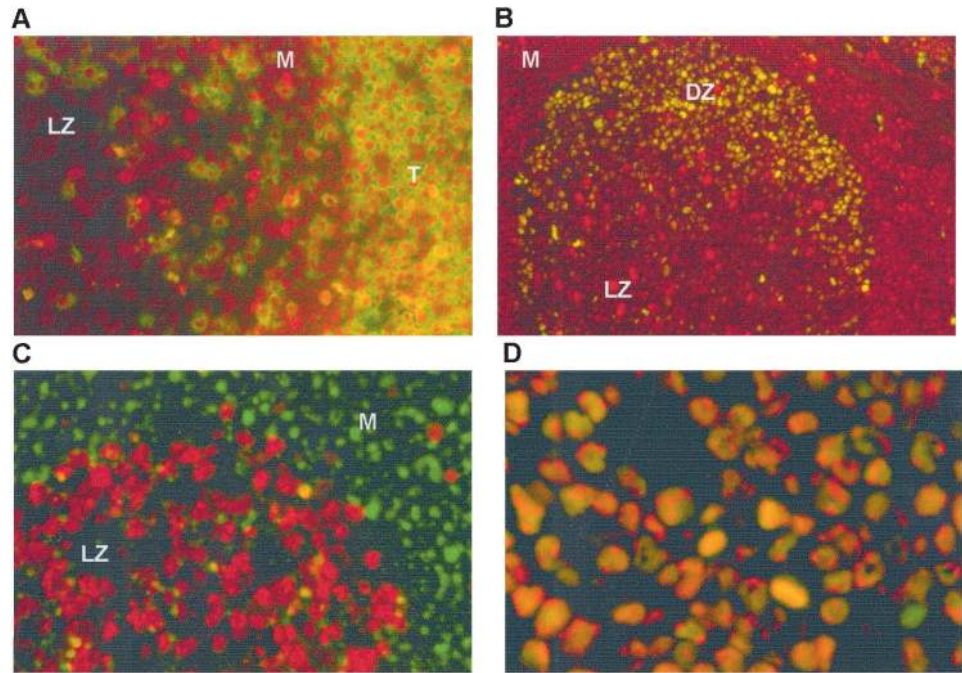
resting mantle B cell to follicular centroblasts is associated with a high rate of cell division, we questioned whether ENX$^+$ follicular B cells expressed Mib-1 (Ki-67), a marker for proliferating cells (20). Double immunofluorescence showed that mantle cells were negative for Mib-1 (Fig. 2*C*), whereas follicular ENX$^+$ B cells were double positive for Mib-1 (Fig. 2*D*). By contrast, BMI-1$^+$ cells, which are ENX$^-$ (Fig. 2*B*), did not express Mib-1 (Fig. 2*C*).

GC architecture is reflected in differential Pc-G expression and reflects the composition of two different Pc-G complexes

The known kinetics of GC formation (18, 19) and differential Pc-G expression patterns observed in the current study suggest that human Pc-G genes play a role in the GC reaction. Resting naive B cells in the mantle zone are remnants of the primary follicle that was present before initiation of the GC reaction (19). These cells can be viewed as precursors of DZ centroblasts, which in turn give rise to DZ and LZ centrocytes. These mantle B cells expressed BMI-1 and RING1 with varying intensity, but did not express ENX or EED. Because DZ centroblasts were BMI-1$^-$/RING1$^-$ and ENX$^+$/EED$^+$, we conclude that initiation of the GC reaction coincides with the appearance of ENX and EED expression, whereas BMI-1 and RING1 expression is lost. This process is linked to initiation of increased cell division, because ENX$^+$ centroblasts also stain for Mib-1. The next step, differentiation of centroblasts into centrocytes (18, 19), appears to be associated with loss of ENX, EED, and Mib-1 expression while BMI-1 and RING1 reappear. Sporadically observed BMI-1/ENX and BMI-1/Mib-1 double-positive cells probably represent the transitional stage between centroblasts and centrocytes.

A very interesting aspect of the BMI-1/RING1 and ENX/EED staining patterns in secondary GCs is that they reflect the composition of two different human Pc-G complexes containing either BMI-1 and RING1 or ENX and EED (7–9, 12, 13). We speculate that the GC reaction is associated with formation of different Pc-G complexes, with different functions and different target genes. In this regard, the classification of BMI-1 as a classic Pc-G gene and ENX as a gene that shares similarities to both Pc-G and trithorax group proteins (21) may be relevant. Earlier studies demonstrated

that interaction of CD40L/gp39 on T cells with CD40 on the B cell during Ag-specific responses move the B cell into the cell cycle (22). CD40L-CD40 interaction and antigenic triggering are therefore likely contributing factors to the switch in Pc-G expression profiles.

Concluding remarks

Pc-G genes are important regulators of cellular differentiation during embryonic development and also appear to contribute to regulation of hematopoiesis. Our analysis of human Pc-G expression demonstrated that BMI-1/RING1 and ENX/EED expression patterns were mutually exclusive in the GC and dependent on the developmental stage of the B cell. Follicular precursor B cells in the mantle, and follicular centroblasts and centrocytes, exhibited characteristic Pc-G staining patterns that can be interpreted as generation of different Pc-G complexes and consecutive on/off switching of Pc-G gene expression during GC formation. We propose that these gene-regulatory proteins play a role in GC development and Ag-specific differentiation of B cells.

Acknowledgments

References

1. Akasaka, T., K. Tsuji, H. Kawahira, M. Kanno, K. Harigaya, L. Hu, Y. Ebihara, T. Nakahata, O. Tetsu, M. Taniguchi, and H. Koseki. 1997. The role of mel-18, a mammalian polycomb group gene, during IL-7-dependent proliferation of lymphocyte precursors. *Immunity 7:135.*
2. Van der Lugt, N. M. T., J. Domen, K. Linders, M. van Roon, E. Robanus-Maandag, H. te Riele, M. van der Valk, J. Deschamps, M. Sofroniew, M. van Lohuizen, and A. Berns. 1994. Posterior transformation, neurological abnormalities, and severe hematopoietic defects in mice with targeted deletion of the *BMI-1* proto-oncogene. *Genes Dev. 8:757.*
3. Alkema, M. J., H. Jacobs, M. van Lohuizen, and A. Berns. 1997. Perturbation of B and T cell development and predisposition to lymphomagenesis in EμBMI1 transgenic mice require the BMI-1 RING finger. *Oncogene 15:899.*
4. Simon, J. 1995. Locking in stable states of gene expression: transcriptional control during *Drosophila* development. *Curr. Opin. Cell Biol. 7:376.*
5. Pirrotta, V. 1998. Polycombing the genome: PcG, trxG and chromatin silencing. *Cell 93:333.*

6. Alkema, M. J., M. Bronk, E. Verhoeven, A. P. Otte, L. J. van't Veer, A. Berns, and M. van Lohuizen. 1997. Identification of Bmi-1-interacting proteins as constituents of a multimeric mammalian polycomb complex. *Genes Dev.* 11:226.

7. Gunster, M. J., D. P. E. Satijn, K. M. Hamer, J. L. den Blaauwen, D. de Bruijn, M. J. Alkema, M. van Lohuizen, R. van Driel, and A. P. Otte. 1997. Identification and characterization of interactions between the vertebrate polycomb-group protein BMI-1 and human homologues of polyhomeotic. *Mol. Cell. Biol.* 17:2326.

8. Satijn, D. P. E., M. J. Gunster, J. van der Vlag, K. M. Hamer, W. Schul, M. J. Alkema, A. J. Saurin, P. S. Freemont, R. van Driel, and A. P. Otte. 1997. RING1 is associated with the polycomb group protein complex and acts as a transcriptional repressor. *Mol. Cell. Biol.* 17:4105.

9. Sewalt, R. G. A. B., J. van der Vlag, M. J. Gunster, K. M. Hamer, J. L. den Blaauwen, D. P. E. Satijn, T. Hendrix, R. van Driel, and A. P. Otte. 1998. Characterization of interactions between the mammalian polycomb-group proteins Enx1/EZH2 end EED suggests the existence of different mammalian polycomb-group protein complexes. *Mol. Cell. Biol.* 18:3586.

10. Van Lohuizen, M., M. Tijms, J. W. Voncken, A. Schumacher, T. Magnuson, and E. Wientjens. 1998. Interaction of the mouse polycomb-group (Pc-G) proteins Enx1 and Enx2 with Eed: indication for separate Pc-G complexes. *Mol. Cell. Biol.* 18:3572.

11. Satijn, D. P. E., and A. P. Otte. 1999. RING1 interacts with multiple polycomb-group proteins and displays tumorigenic activity. *Mol. Cell. Biol.* 19:57.

12. Satijn, D. P. E., D. J. Olson, J. van der Vlag, K. M. Hamer, C. Lambrechts, H. Masselink, M. J. Gunster, R. G. A. B. Sewalt, R. van Driel, and A. P. Otte. 1997. Interference with the expression of a novel human polycomb protein, hPc2, results in cellular transformation and apoptosis. *Mol. Cell. Biol.* 17:6076.

13. Schoorlemmer, J., C. Marcos-Gutierrez, F. Were, R. Martinez, E. Garcia, D. P. E. Satijn, A. P. Otte, and M. Vidal. 1997. RING1A is a transcriptional repressor that interacts with the polycomb-M33 protein and is expressed at rhombomere boundaries in the mouse hindbrain. *EMBO J.* 16:5930.

14. Tetsu, O., H. Ishihara, R. Kanno, M. Kamiyasu, H. Inoue, T. Tokuhisha, M. Taniguchi, and M. Kanno. 1998. Mel-18 negatively regulates cell cycle progression upon B cell antigen receptor stimulation through a cascade leading to c-*myc*/cdc25. *Immunity* 9:439.

15. Jacobs, J. J. L., K. Kieboom, S. Marino, R. A. DePinho, and M. van Lohuizen. 1999. The oncogene and polycomb-group gene Bmi-1 regulates cell proliferation and senescence through the *ink4a* locus. *Nature* 397:164.

16. van Lohuizen, M., S. Verbeek, B. Scheijen, E. Wientjens, H. van der Gulden, and A. Berns. 1991. Identification of cooperating oncogenes in Eμ-myc transgenic mice by provirus tagging. *Cell* 65:737.

17. Lessard, J., S. Baban, and G. Sauvageau. 1998. Stage-specific expression of polycomb group genes in human bone marrow cells. *Blood* 91:1216.

18. Liu, Y. J., G. D. Johnson, J. Gordon, and I. C. MacLennan. 1992. Germinal centers in T-cell-dependent antibody responses. *Immunol. Today* 13:17.

19. Camacho, S. A., M. H. Kosco-Vilbois, and C. Berek. 1998. The dynamic structure of the germinal center. *Immunol. Today* 19:511.

20. Cattoretti, G., M. H. Becker, G. Key, M. Duchrow, C. Schluter, J. Galle, and J. Gerdes. 1992. Monoclonal antibodies against recombinant parts of the Ki-67 antigen (MIB 1 and MIB 3) detect proliferating cells in microwave-processed Formalin-fixed paraffin sections. *J. Pathol.* 168:357.

21. Lajeunesse, D., and A. Shearn. 1996. *E(z)*: a polycomb group gene or a trithorax group gene? *Development* 122:2189.

22. Liu, Y. J., and J. Banchereau. 1997. Regulation of B-cell commitment to plasma cells or to memory B cells. *Semin. Immunol.* 9:235.