Video Article

In vitro Differentiation Model of Human Normal Memory B Cells to Long-lived Plasma Cells

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Abstract

Plasma cells (PCs) secrete large amounts of antibodies and develop from B cells that have been activated.PCs are rare cells located in the bone marrow or mucosa and ensure humoral immunity.Due to their low frequency and location, the study of PCs is difficult in human. We reported a B to PC *in vitro* differentiation model using selected combinations of cytokines and activation molecules that allow to reproduce the sequential cell differentiation occurring *in vivo*. In this *in vitro* model, memory B cells (MBCs) will differentiate into pre-plasmablasts (prePBs), plasmablasts (PBs), early PCs and finally, into long-lived PCs, with a phenotype close to their counterparts in healthy individuals.We also built an open access bioinformatics tools to analyze the most prominent information from GEP data related to PC differentiation. These resources can be used to study human B to PC differentiation and in the current study, we investigated the gene expression regulation of epigenetic factors during human B to PC differentiation.

Introduction

The differentiation of B cells to plasma cells (PCs) is essential for humoral immunity and protect the host against infections[1]. B to PC differentiation is associated with major changes in transcription capacity and metabolism to accommodate to antibody secretion.The transcription factors that control B to PC differentiation have been extensively studied and revealed exclusive networks including B- and PC-specific transcription factors (TFs)[2]. In B cells, PAX5, BCL6 and BACH2 TFs are the guardians of B cell identity[2,3]. Induction of *IRF4*, *PRDM1* encoding BLIMP1 and *XBP1* PC TF will extinguish B cell genes and induce a coordinated antibody-secreting cell transcriptional program[3,4,5]. These coordinated transcriptional changes are associated with Ig genes transcription activation together with a switch from the membrane-bound form to the secreted form of the immunoglobulin heavy chain[2,3,4]. B to PC differentiation is linked with induction of genes involved in endoplasmic reticulum and Golgi apparatus functions concomitant with unfolded protein response (UPR) activation known to play a key role in PC by accommodating the synthesis of secreted immunoglobulins[6,7]. The TF XBP1 plays a major role in this cellular adaptation[8,9,10].

B cells and PCs are key players of humoral immunity. Understanding the biological processes that control the production and the survival of normal plasma cells is critical in therapeutic interventions that need to ensure efficient immune responses and prevent autoimmunity or immune deficiency. PC are rare cells with early differentiation stages taking place in anatomic locations that hamper full biological characterization, particularly in human. Using multi-step culture systems, we have reported an *in vitro* B to PC differentiation model. This model reproduces the sequential cell differentiation and maturation occurring in the different organs *in vivo*[11,12,13].In a first step, memory B cells are first activated for four days by CD40 ligand, oligodeoxynucleotides and cytokine combination and differentiate into preplasmablasts (PrePBs). In a second step, preplasmablasts are induced to differentiate into plasmablasts (PBs) by removing CD40L and oligodeoxynucleotides stimulation and changing the cytokine combination. In a third step, plasmablasts are induced to differentiate into early PCs by changing the cytokine combination[11,12]. A fourth step was introduced to get fully mature PCs by culturing these early PCs with bone marrow stromal cells or selected growth factors[13]. These mature PCs could survive several months *in vitro* and secrete high amounts of immunoglobulin (**Figure 1**).Interestingly, our *in vitro* model recapitulates the coordinated transcriptional changes and the phenotype of the different B to PC stages that can be detected *in vivo*[11,12,13,14,15]. PCs are rare cells and our *in vitro* differentiation model allows to study human B to PC differentiation.

Protocol

The protocol follows the guidelines in accordance with the Declaration of Helsinki and agreement of the Montpellier University Hospital Centre for Biological Resources.

1. *In Vitro* Normal Plasma Cell Differentiation Model

NOTE: PCs are generated through a four-step culture[11,12,13].

1. **B cell amplification and differentiation**
    1. Use peripheral blood cells from healthy volunteers for memory B cell purification.
    2. Dilute 7.5 mL of blood with 24.5 mL of RPMI 1640 medium.
    3. Add 12.5 mL of room temperature density gradient (*e.g.*, Ficoll) to a sterile 50 mL conical tube. Gently overlay the density gradient with the 32 mL of diluted blood. Minimize the mixing of the two phases.
    4. Centrifuge 20 min at 500 x g with the brake off. Collect the PBMCs from the diluted plasma/density gradient interface and place the cells in a sterile 50 mL tube and complete to 45 mL with Hank's balanced salt solution.
    5. Centrifuge the cells for 5 min at 500 x g. Carefully remove the supernatant and keep the pellet.
    6. Add 45 mL of RPMI1640/10% fetal calf serum (FCS) and centrifuge 5 min at 500 x g. Carefully remove the supernatant and keep the pellet. Add 30 mL of PBS/2% FCS.
    7. Remove CD2+ cells using anti-CD2 magnetic beads. Add 4 beads for one target cell. Incubate 30 minutes at 4 °C.
        1. Separate bead-bound cells from unbound cells using a magnet for cell separation application. Collect unbound cells and incubate with anti CD19 APC and anti CD27 PE antibodies for 15 minutes at 4 °C (2 μL of antibody for 10^6 cells).
        2. Wash twice in PBS/10% goat serum.
        3. Remove contaminating events on both FCS and SSC plots. Plot singlets on FSC-A vs FSC-H and SSC-A vs SSC-H plots to remove debris and to select the total leukocyte population. Select memory B cells on CD19/CD27 and $CD19^+CD27^+$.
        4. Purify MBCs using a cell sorter with a 95% purity.
    8. Perform all the culture steps using IMDM and 10% FCS, supplemented with 50 mg/mL human transferrin and 5 mg/mL human insulin.
    9. Plate purified MBCs in six-well culture plates (1.5×10^5 MBCs/mL in 5 mL/well), for 4 days, with IL-2 (20 U/mL), IL-10 (50 ng/mL), and IL-15 (10 ng/mL).
        1. Add Phosphorothioate CpG ODN 2006, histidine-tagged recombinant human soluble CD40L (sCD40L; 50 ng/mL) and anti-polyhistidine mAb (5 mg/mL) for MBCs activation (**Figure 1**). Activation by sCD40L or ODN only with the same cytokine cocktail yields to lower amplification[12]. The combination of activation signals described here leaded to the maximum number of activated B cells and PrePBs[11,12] (**Figure 1**).
        2. For gene expression profiling, purify $CD38^-/CD20^-$ PrePBs, at day 4, using a cell sorter (**Figure 2**).

2. **Plamablastic cell generation**
    1. At day 4, count the cells.
    2. Plate cells in 12-well culture plates at 2.5×10^5/mL in 2 mL/well.
    3. Induce differentiation by removal of CpG oligonucleotides and sCD40L and addition of a new culture medium with a new cytokine cocktail including IL-2 (20 U/mL), IL-6 (50 ng/mL), IL-10 (50 ng/mL) and IL-15 (10 ng/mL) (**Figure 1**). Culture cells for 3 days (from day 4 to day 7) at 37 °C.
    4. For gene expression profiling, purify $CD38^+/CD20^-$ PBs, at day 7, using a cell sorter (**Figure 2**).

3. **Early plasma cell generation**
    1. At day 7, count the cells.
    2. Plate cells in 12-well culture plates at 5×10^5/mL in 2 mL/well.
    3. Differentiate PB in early PCs using fresh culture medium with IL-6 (50 ng/mL), IL-15 (10 ng/mL) and IFN-α (500 U/mL) for 3 days at 37 °C. For gene expression profiling, purify $CD20^-/CD38^+/CD138^+$ early PCs, at day 10, using a cell sorter (**Figure 2**).

4. **Long-lived plasma cell generation**
    1. Differentiate Early PCs into long lived PCs by changing the culture conditions.
    2. Culture the cells in 12-well culture plates at 5×10^5/mL in 2 mL/well or in 24-well culture plates in 1 mL/well at 37 °C, using IL-6 and stromal cell conditioned medium and APRIL (200 ng/mL).
    3. Obtain stromal cell-conditioned medium by culturing stromal cells for 5 days with culture medium. Filter the stromal cell culture supernatant (0.2 μm) and freeze.
    4. Add 50% of stromal cell-conditioned media to PC cultures with renewal every week. Generated long-lived PCs could be maintained for months[13]. Long term survival of PCs could be obtained with IL-6 and APRIL only as reported[13].
    5. Measure Ig secretion from flow cytometry sorted PCs.
    6. Culture PCs at 10^6 cells/mL for 24 h and harvest culture supernatant. Measure IgG and IgA using human enzyme-linked immunosorbent assay (ELISA) kits[11,12,13]. The median IgG secretion ranged from 10 pg/cell/day at day 10 to 17 pg/cell/day at day 60[13].

2. Molecular Atlas of B to PC Differentiation

NOTE: We built a convenient and open access bioinformatics tools to extract and visualize the most prominent information from Affymetrix GEP data related to PC differentiation (GenomicScape)[15]. GEP are publicly available from ArrayExpress database (http://www.ebi.ac.uk/arrayexpress/) including purified MBCs, PrePBs, PBs and EPCs: E-MTAB-1771, E-MEXP-2360 and E-MEXP-3034 and BMPC E-MEXP-2360[14,15]. Genomicscape is a freely available webtool.

1. **Selection of B to PC dataset**
    1. Select B to PC dataset in the **Browse Data** menu in GenomicScape open access bioinformatic tool (http://www.genomicscape.com) called **Human B cells to plasma cells**. Visualization of gene expression profile of unique gene or a list of genes is available using the **Expression-Coexpression Report** tool in the **Analysis Tools** available in the home page.

2. **Supervised analysis and principal component analysis (PCA)**
    1. Select **Analysis Tools | webSAM** to load the SAM tool.
    2. Choose the **Human B cells to plasma cells** dataset and select the groups of samples to compare.

3. Use the different filters available to apply the filtering options of interest.
4. To compare gene expression profiles with SAM tool, modify the filtering options including Fold change, number of permutations, False discovery rate and the type of comparison. GenomicScape will compute the analysis and provide genes differentially expressed between the selected groups.
5. Run principal component analysis by selecting **Principal Component Analysis** in **Analysis Tools** menu.
6. Choose the **Human B cells to plasma cells** dataset and select the groups of interest.
7. Paste a list of genes of interest or select genes according to the variance. To paste a list of genes, select **To analyse a list of genes/ncRNAs, click here….** Genomicscape will compute PCA that could be visualized and downloaded.
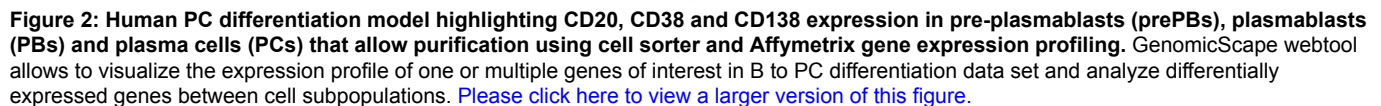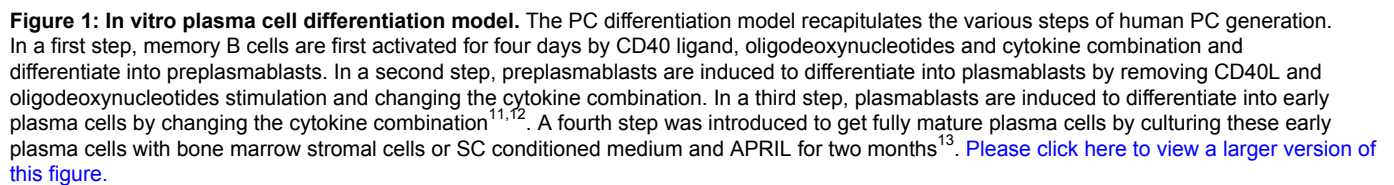
Representative Results

The overall procedure of *in vitro* normal PC differentiation is represented in **Figure 1**. Using the protocol presented here, we could generate adequate quantity of cells that could not be obtained with *ex vivo* human samples. Although the role of the complex network of transcription factors involved in PC differentiation has been investigated, the mechanisms regulating key PC differentiation transcription networks remain poorly known. Cellular differentiation is mostly driven by epigenetic and transcriptional changes. Using our *in vitro* model and the B to PC GEP atlas, we investigated the expression changes of the epigenetic factors in normal plasma cell differentiation.
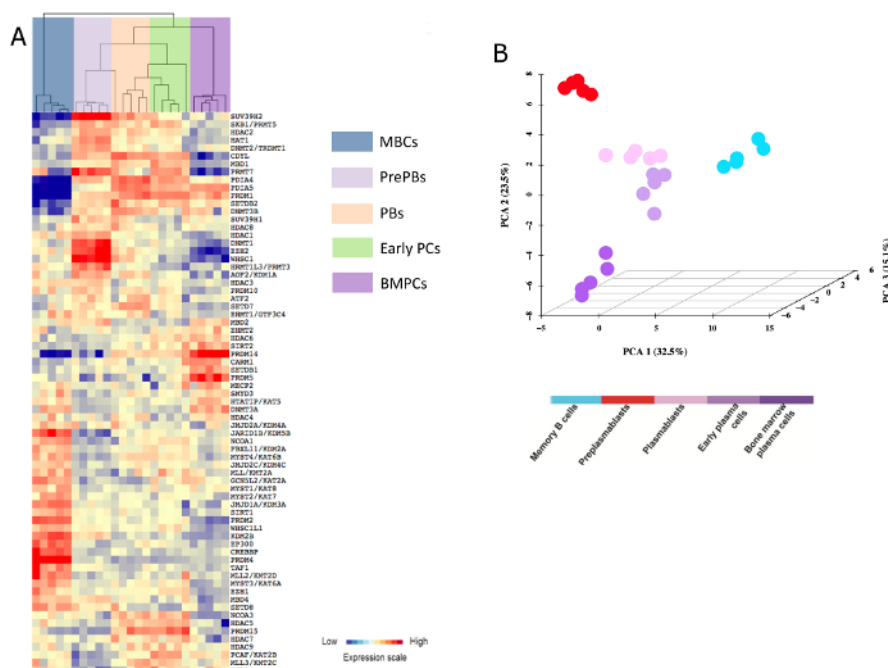
Epigenetic factor expression in normal plasma cell differentiation
We defined as epigenetic factors those belonging to the following families: DNA methyltransferases (DNMT), methyl-CpG-binding domain (MBD) proteins, histone acetyltransferases (HAT), histone deacetylases (HDAC), histone methyltransferases (HMT) and histone demethylases (HDM) as previously described for cancer cells[16] (**SupplementaryTable 1**). We investigated the epigenetic factors differentially expressed during PC differentiation using our *in vitro* model, purified mature bone marrow PCs (BMPCs) and "B to PC atlas" available using the GenomicScape webtool (**Figure 2**). Using multi-class SAM analysis, we found 71 genes significantly differentially between the MBCs, PrePBs, PBs, early PCs and BMPCs with a false discovery rate < 5% (**Figure 3A**&**3B** and **SupplementaryTable 2**). MBC stage is characterized by the overexpression of 23 epigenetic player genes including 39.13% of histone acetyltransferases (HATs), 30.43% of histone methyltransferases (HMTs), 21.74% of histone demethylases (HDMTs), 4.35% of methyl binding proteins and 4.35% of HDACs (**Figure 4**). 14 epigenetic players were overexpressed specifically at the PrePBs stage including 50% of HMTs, 14.28% of DNMTs, 21.43% of HDACs, 7.14% of HDMTs and 7.14% of HATs (**Figure 3**). PBs stage is distinguished by the overexpression of 5 epigenetic players with 2 HDMTS, 2 HMTs and 1 DNMT. 4 epigenetic players are overexpressed in early PCs (1 HDAC, 1 HAT, 1 HMT and 1 methyl binding protein). In BMPCs, 10 epigenetic factors were specifically overexpressed including 50% of HMT (**SupplementaryTable 2**).

The most important changes in epigenetic factor expression occur during the MBCs to PrePBs transition with a downregulation of 23 MBCs genes and upregulation of 14 PrePBs epigenetic genes (**Figure 4**). MBCs significantly overexpressed HATs involved in posttranslational modifications of histones. Histone acetylation affects chromatin structure resulting in decondensation of chromatin and increasing transcription of genes that are epigenetically silenced by chromatin compaction. The MBCs to PrePBs transition is also associated with a significant downregulation of HATs, a major shift in HMTs expression and enrichment in HDACs and DNMTs gene expression. Interestingly, PrePB stage is also characterized by the overexpression of *MMSET* HMT. MMSET/WHSC1/NSD2 is a SET domain containing histone lysine methyltransferase that can di- and trimethylate histone H3 at lysine 36. MMSET is involved in the reciprocal t(4;14)(p16;q32) translocation in a subgroup of multiple myeloma that is associated with poor prognosis. It was reported that MMSET is involved in DNA repair regulating the induction of H4K20 methylation on histones around DNA double strand breaks, which, in turn, facilitates 53BP1 recruitment[17]. PrePBs are highly proliferating compared to MBCs with 50% of cells in the S phase[11] suggesting that the PrePBs stage could be associated with a high replicative stress. Overexpression of MMSET could participate in the prevention of replication stress-induced DNA damage in PrePBs.

PB and early PCs presented similar epigenetic factor expression profiles (**Figure 2**). BMPCs are characterized by a specific overexpression of HMT and HDAC that are related to Ig secretion stress adaptation including *EHMT2* and *HDAC6*. Mature BMPCs have the characteristic to synthetize and secrete large amounts of antibodies. This high synthesis of immunoglobulins is associated with a misfolded protein-induced stress and the development of endoplasmic reticulum (ER) response to accommodate this production. Our results demonstrate major gene expression changes of epigenetic factors that may play an important role during B to PC differentiation through epigenetic-mediated reprogramming events.

Figure 1: In vitro plasma cell differentiation model. The PC differentiation model recapitulates the various steps of human PC generation. In a first step, memory B cells are first activated for four days by CD40 ligand, oligodeoxynucleotides and cytokine combination and differentiate into preplasmablasts. In a second step, preplasmablasts are induced to differentiate into plasmablasts by removing CD40L and oligodeoxynucleotides stimulation and changing the cytokine combination. In a third step, plasmablasts are induced to differentiate into early plasma cells by changing the cytokine combination[11,12]. A fourth step was introduced to get fully mature plasma cells by culturing these early plasma cells with bone marrow stromal cells or SC conditioned medium and APRIL for two months[13]. Please click here to view a larger version of this figure.

Figure 2: Human PC differentiation model highlighting CD20, CD38 and CD138 expression in pre-plasmablasts (prePBs), plasmablasts (PBs) and plasma cells (PCs) that allow purification using cell sorter and Affymetrix gene expression profiling. GenomicScape webtool allows to visualize the expression profile of one or multiple genes of interest in B to PC differentiation data set and analyze differentially expressed genes between cell subpopulations. Please click here to view a larger version of this figure.

Figure 3: **Multi-Class SAM Analysis.** The signals of the 71 epigenetic factors significantly differentially expressed during B to PC differentiation (SAM analysis; FDR < 0.05) in memory B cells (MBCs, n=5), preplasmablasts (PrePBs, n=5), plasmablasts (PB, n = 5), early plasma cells (Early PCs, n =5) and normal bone marrow plasma cells (BMPCs, n = 5) are displayed from low (deep blue) to high (deep red) expression. Please click here to view a larger version of this figure.
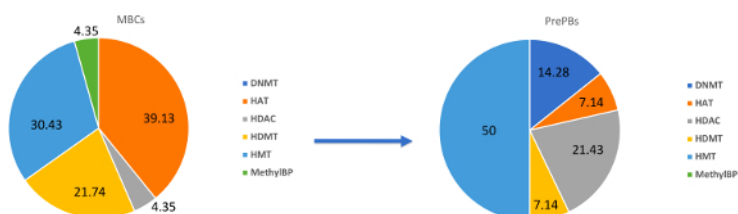
Figure 4. **Percentage of epigenetic genes belonging to DNMTs, methyl-CpG-binding domain (MethylBP) proteins, histone acetyltransferases (HAT), histone deacetylases (HDAC), histone methyltransferases (HMT) and histone demethylases categories significantly overexpressed in MBCs or PrePBBs.** Please click here to view a larger version of this figure.

Supplementary Table 1. Please click here to download this file.

Supplementary Table 2. Please click here to download this file.

Discussion

In human, PC are rare cells with differentiation stages taking place in anatomic places that hamper full biological characterization. We have developed an *in vitro* B to PC differentiation model using multi-step culture systems where various combinations of activation molecules and cytokines are subsequently applied in order to reproduce the sequential cell differentiation occurring in the different organs/tissues *in vivo*[11,12,13].

Other efficient *in vitro* B to PCs differentiation models were reported[18,19]. The model developed by Le Gallou *et al.* explored T-cell dependent B to PC differentiation with a two-step culture methodology starting from CD19^{+}/CD27^{-} naïve B cells. Cocco *et al.* reported an *in vitro* model to generate long-lived PCs starting from total B cells[19]. Our strategy mimics the activation and differentiation occurring in germinal center with CD40 and Toll-like receptor activation mimicking T cell help and antigen activation used in combination with cytokines produced by dendritic cells, macrophages and T helper. Activation with sCD40L and CpG ODN yields to the generation of PrePBs that have been identified in lymph nodes, tonsil and bone marrow in human[11,20]. This transitional stage is characterized by the absence of CD20, CD38, and CD138 markers and the coexpression of B and PC TFs, but at a reduced level compared with B cells, PBs, or PC[11]. This transitional stage is of particular interest since it is associated to proteasome inhibitor resistance in multiple myeloma patients[20,21]. Even if PCs could be generated without IL-15[18,19], IL-15 addition provided, in our hands, optimized results in the generation of CD20^{-}CD38^{++} cells at day 4[11,12]. Addition of IL-21 would be of particular interest since IL-21 promotes PC differentiation through BLIMP induction mediated by STAT3 activation as previously reported[19,22]. As reported by Cocco *et al.* complex culture conditions containing IL-21, IFN-α, IL6 and stromal cell-conditioned medium support the generation of long-lived PCs. We reported that long-term survival of PCs could be supported, *in vitro*, using IL-6, APRIL and stromal cell-conditioned medium or the two growth factors only. Stromal cells produce major PC growth factors, particularly IL-6 and galectin[13,19,23,24,25] and sustain the interactions between hematopoietic cells and PCs[26]. Furthermore, APRIL is one of the major growth factors involved in PCs survival, produced by hematopoietic

cells[27,28]. To avoid heterogeneity related to different sources of stromal cells, Resto-6 stromal cells, developed and provided by Karin Tarte's lab, were used between passages 8 and 15[13,29]. Resto-6 cells express CD90, CD73 and CD105 stromal cell markers and support efficiently the growth of normal and malignant B cells[29].

However, a moderate heterogeneity could be observed in the percentage of activated B cells, PrePBs, PBs and PCs depending on healthy donor's blood used for memory B cell purification[11,12,13]. The generated long-lived PCs are non-cycling PCs, surviving and producing immunoglobulins for more than three months *in vitro*, as their *in vivo* counterpart[13]. Long-lived PCs express highly CD138 and gene expression profiles related to *in vivo* PCs[13]. Furthermore, a higher ratio of spliced to unspliced *XBP1* together with higher expression of *IRF4* and *PRDM1* PCs transcription factors characterized the long-lived PCs obtained *in vitro* compared to early PCs obtained at Day 10[13].

It is thought epigenetic and transcriptional changes control cellular transitions during development. However, the terminal differentiation of B lymphocytes into plasma cells is a unique process whose epigenetic modifications remain largely unknown. According to that, we recently analyzed the miRnome of normal PC differentiation and identified novel key miRNAs regulating networks of significance for normal PC differentiation[14]. We showed that several miRNAs could also participate into the regulation of expression of key transcription factors during PCD, including IRF4, PRDM1, ELL2 and ARID3A[14]. According to that, we extended here the characterization of epigenetic related gene profiles during B to PC differentiation using GenomicScape open access platform. This approach allowed us to identify chromatin modifying enzyme genes specifically expressed in the different stages of PC differentiation. MBCs significantly overexpressed HATs known to cause nuleosomal remodeling that exposes transcription binding sites. Histone post-transcriptional modifications, including histone acetylation have been reported in promoter region of major B cell TFs including *PAX5, CIITA, SPIB* and *BCL6*[30,31]. Furthermore, proteomic analysis demonstrated that CREBBP and EP300 (overexpressed in MBCs) interact with BCL6 and could play a role in the transcriptional regulation of BCL6[32]. PAX5, was also shown to regulate target gene expression by recruiting chromatin remodeling and histone modifying proteins[33]. Pax5 was shown to induce H3K4 methylation and H3K9 acetylation at promoters of their activated target genes[33].

Major epigenetic factor GEP changes were identified during the MBCs to PrePBs transition with a major downregulation of HATs together with an increase in HMTs expression and enrichment of HDACs and DNMTs gene expression. Transitional PrePB stage is a highly proliferating cell population[11]. According to that, several epigenetic factors known to be involved in cell proliferation control including DNMT1[34], EZH2[35,36,37,38] and MMSET[39,40] have been identified and could be involved in cell activation and proliferation induction during the PrePB stage.

At the end stage of B to PC differentiation, BMPCs overexpressed epigenetic factors, including *EHMT2* and *HDAC6*, known to be involved in ER stress response. ER and Golgi apparatus play a major role in PC to accommodate the synthesis of secreted Ig. In bladder cancer cells, EHMT2 inhibition stimulates ER stress and induces apoptosis[41]. HDAC6 belongs to the class 2b family of HDACs. It is known to play a role in protein homeostasis and the UPR[42,43] and HDAC6 inhibitors are tested in clinical trials to target malignant PCs in multiple myeloma. Our data underline that epigenetic factors may play a role in homeostasis of long-lived Ig secreting PCs.

Using lentiviral vectors and/or specific inhibitors, it could be interesting to investigate the role of these biological pathways and chromatin modifying enzymes in chromatin remodeling, PC differentiation and functions as previously described by our group [44].

The knowledge derived from this research could inform and instruct on diagnostic and therapeutic strategies for PC disorders, such as in the case of multiple myeloma, a cancer without a definitive cure for which better prognosis and improved therapeutic strategies are critically needed.

Disclosures

The authors have nothing to disclose

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