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TITLE:
In vitro differentiation model of human normal memory B cells to long-lived plasma cells

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SUMMARY:
Using multi-step culture systems, we report an *in vitro* B cell to plasma cell differentiation model.

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[3-5]. 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[8-10].

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*[11-13]. 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* [11-15]. 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[11-13].

1.1. B cell amplification and differentiation

1.1.1. Use peripheral blood cells from healthy volunteers for memory B cell purification.

1.1.2. Dilute 7.5 mL of blood with 24.5 mL of RPMI 1640 medium.

1.1.3. 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.

1.1.4. 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.

1.1.5. Centrifuge the cells for 5 min at 500 x g. Carefully remove the supernatant and keep the pellet.

1.1.6. 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.

1.1.7. Remove $CD2^+$ cells using anti-CD2 magnetic beads. Add 4 beads for one target cell. Incubate 30 minutes at 4 °C.

1.1.7.1. 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).

1.1.7.2. Wash twice in PBS/10% goat serum.

1.1.7.3. 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 $CD19^+CD27^+$.

1.1.7.4. Purify MBCs using a cell sorter with a 95% purity.

1.1.8. Perform all the culture steps using IMDM and 10% FCS, supplemented with 50 mg/mL human transferrin and 5 mg/mL human insulin.

1.1.9. Plate purified MBCs in six-well culture plates (1.5 x 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).

1.1.9.1. 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**).

1.1.9.2. For gene expression profiling, purify $CD38^-/CD20^-$ PrePBs, at day 4, using a cell sorter (**Figure 2**).

1.2. Plamablastic cell generation

1.2.1. At day 4, count the cells.

1.2.2. Plate cells in 12-well culture plates at 2.5 x 10^5/mL in 2 mL/well.

1.2.3. 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.

1.2.4. For gene expression profiling, purify $CD38^+/CD20^-$ PBs, at day 7, using a cell sorter (**Figure 2**).

1.3. Early plasma cell generation

1.3.1. At day 7, count the cells.

1.3.2. Plate cells in 12-well culture plates at 5 x 10^5/mL in 2 mL/well.

1.3.3. 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**).

1.4. Long-lived plasma cell generation

1.4.1. Differentiate Early PCs into long lived PCs by changing the culture conditions.

1.4.2. Culture the cells in 12-well culture plates at 5 x 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).

1.4.3. 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.

1.4.4. 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].

1.4.5. Measure Ig secretion from flow cytometry sorted PCs.

1.4.6. 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[11-13]. 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.

2.1. Selection of B to PC dataset

2.1.1. 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.

2.2. Supervised analysis and principal component analysis (PCA)

2.2.1. Select **Analysis Tools** | **webSAM** to load the SAM tool.

2.2.2. Choose the **Human B cells to plasma cells** dataset and select the groups of samples to compare.

2.2.3. Use the different filters available to apply the filtering options of interest.

2.2.4. 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.

2.2.5. Run principal component analysis by selecting **Principal Component Analysis** in **Analysis Tools** menu.

2.2.6. Choose the **Human B cells to plasma cells** dataset and select the groups of interest.

2.2.7. 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] (**Supplementary Table 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 **Supplementary Table 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 (**Supplementary Table 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 Legend

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].

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.

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.

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.

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*[11-13].

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[13,19,23-25] 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[11-13]. 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[35-38] 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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Figure 1

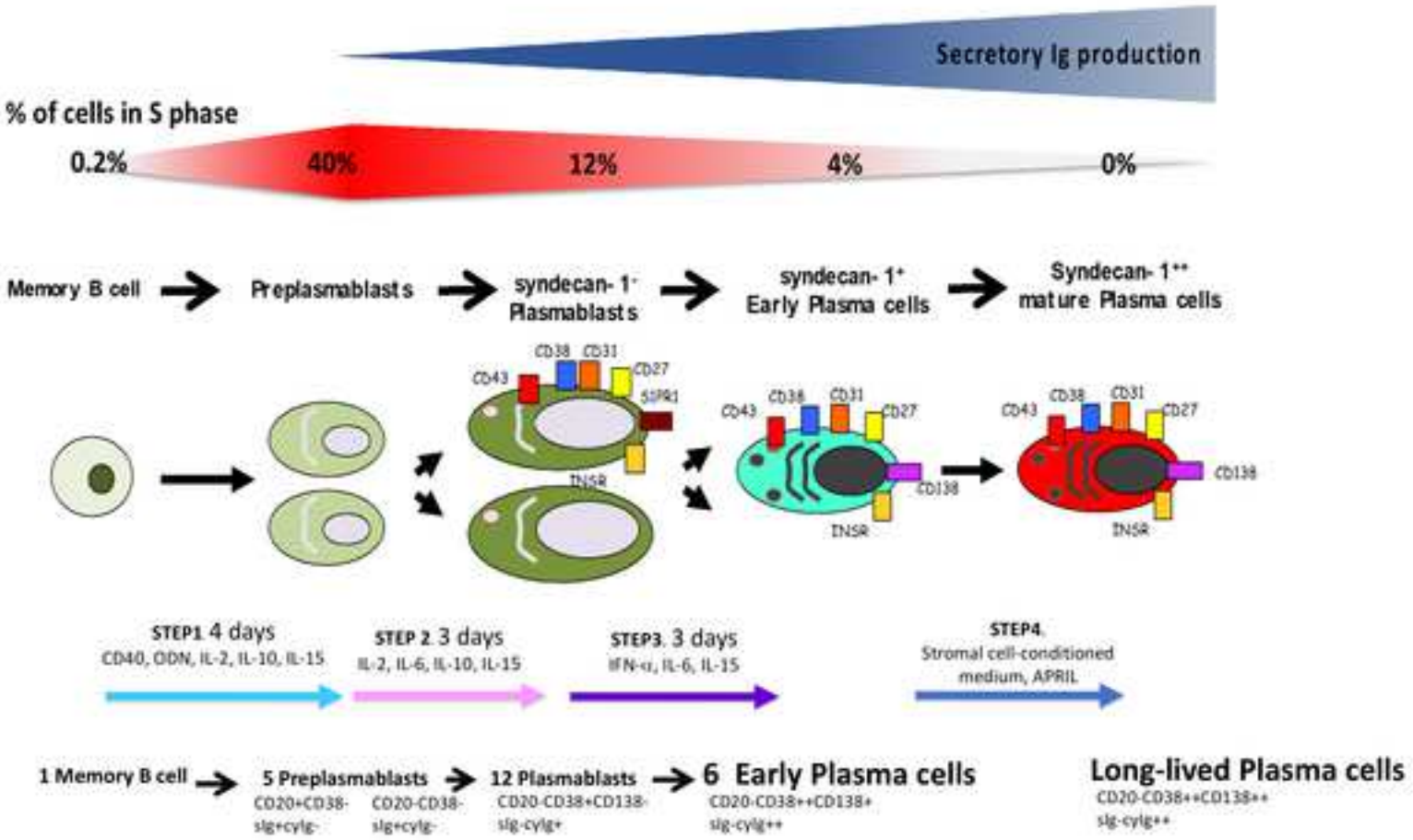

Figure 2

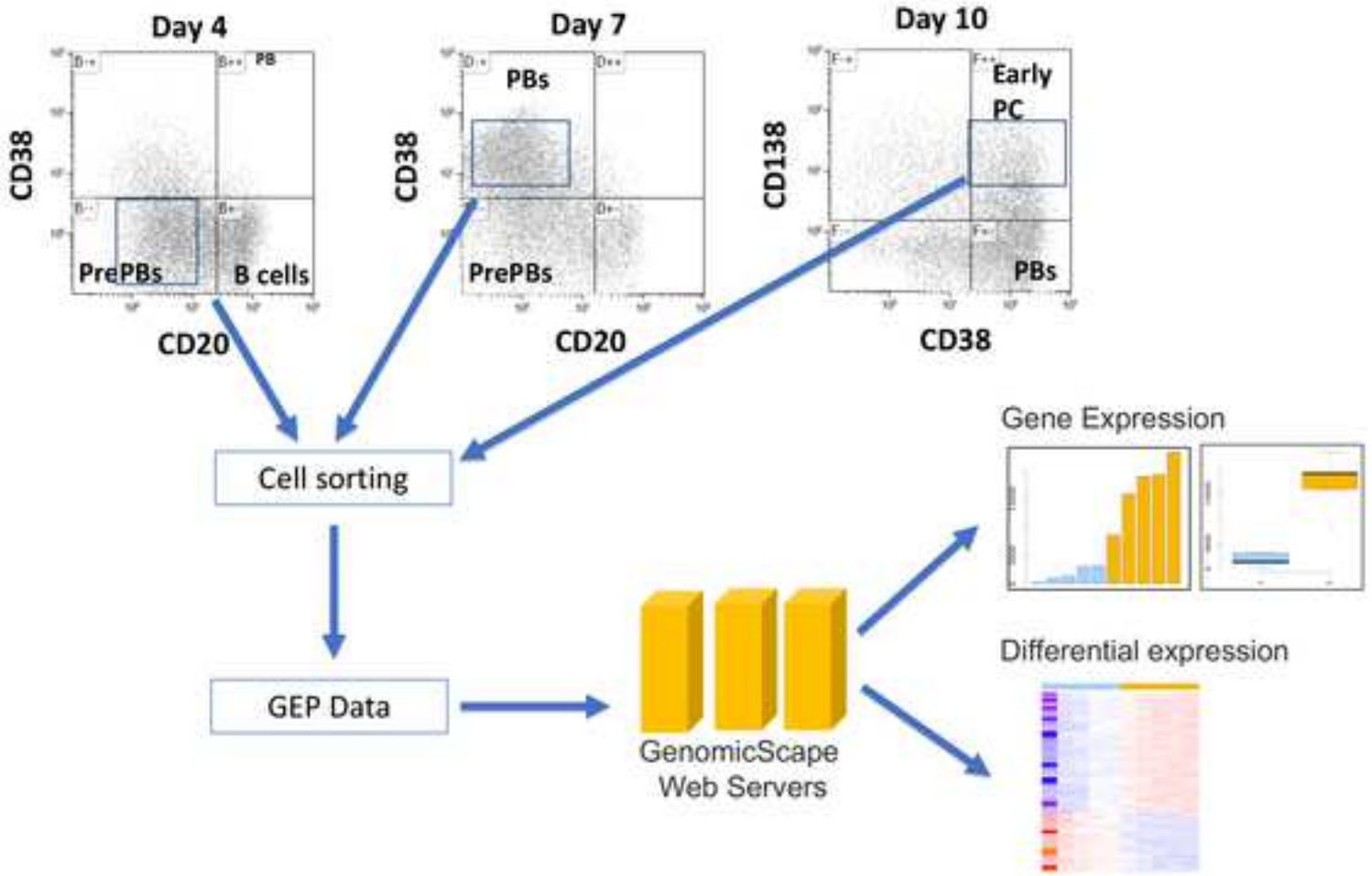

Figure 3A

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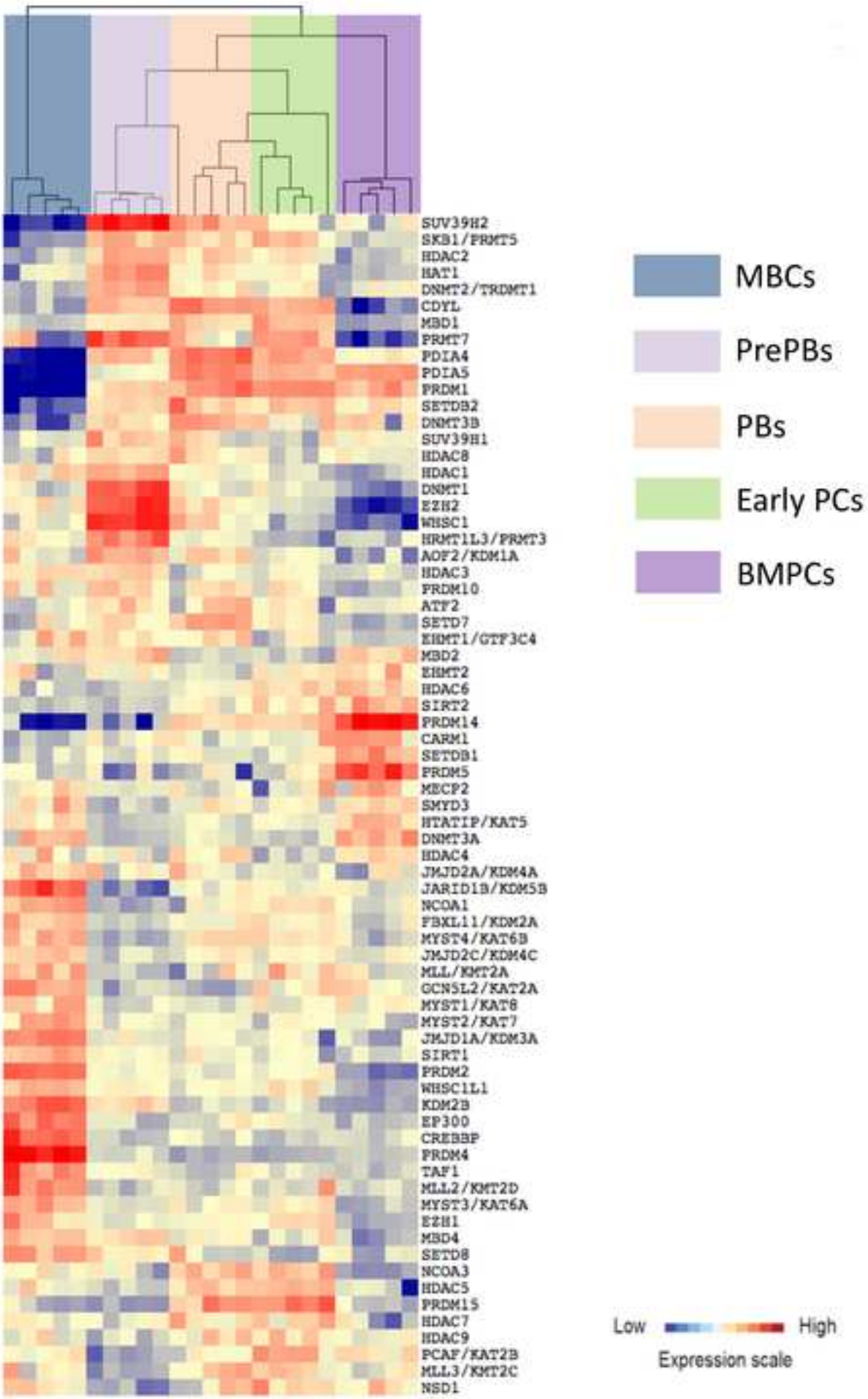

Figure 3B

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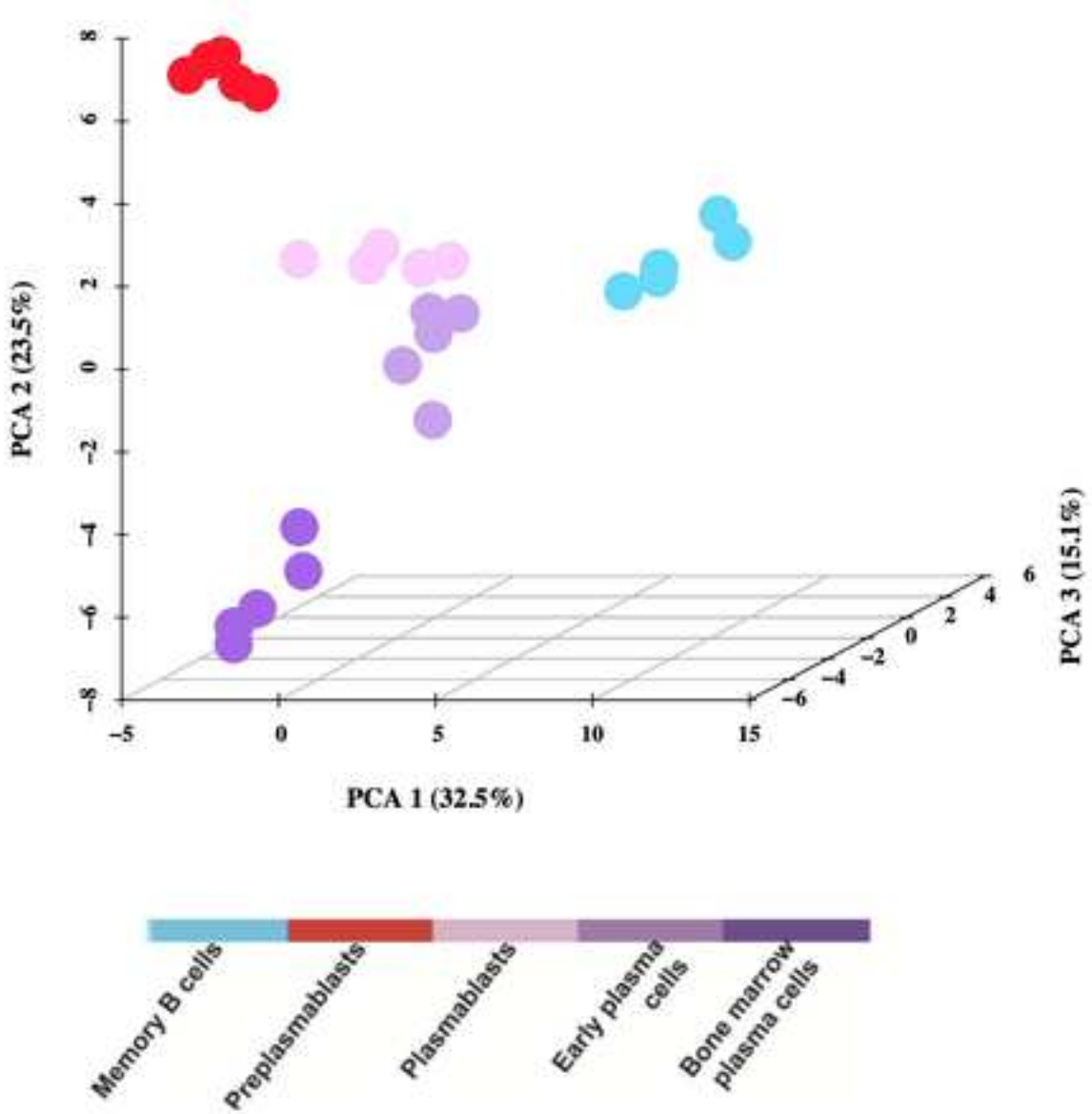
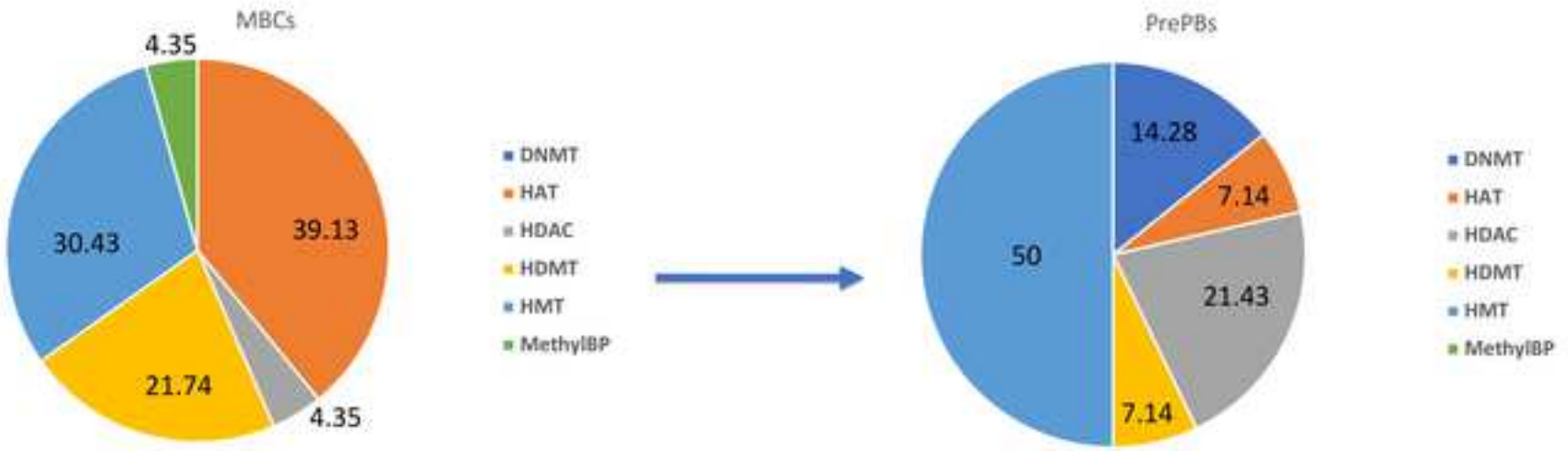


Figure 4



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Name of Material/ Equipment	Company	Catalog Number	Comments/Description
anti-CD2 magnetic beads	Invitrogen	11159D	
Anti-CD138-APC	Beckman-Coulter	B49219	
Anti-CD19-APC	BD	555415	
Anti-CD20-PB	Beckman-Coulter	B49208	
Anti-CD27-PE	BD	555441	
Anti-CD38-PE	Beckman-Coulter	A07779	
Anti-histidine	R&D Systems	MAB050	
CpG ODN(PT)	Sigma		T*C*G*T*C*G*T*T*T*T*G*T*C*G*T*T*T*T*G*T*C*G*T*T
human Transferin	Sigma-Aldrich	T3309	
IFN-α	Merck	Intron A	
IMDM	Gibco	31980-022	
Recombinan Human CD40L-hi	R&D Systems	2706-CL	
Recombinant Human APRIL	R&D Systems	5860-AP-010	
Recombinant Human IL-10	R&D Systems	217-IL-	
Recombinant Human IL-15	Peprotech	200-15-10ug	
Recombinant Human IL-2 Protein	R&D Systems	202-IL-	
Recombinant Human IL-6	Peprotech	200-06	

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Author(s): Michel Jourdan, Hugues de Boussac, Elena Viziteu, Alboukadel Kassambara, Jérôme Moreaux

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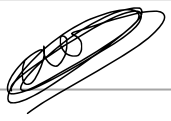
Signature:	
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October 6th, 2018

Dear Editor,

Please, find enclosed our revised manuscript, entitled "**In vitro differentiation model of human normal memory B cells to long-lived plasma cells**" to be considered for publication in Jove.

We thank the reviewers for their comments that help us to improve our manuscript. We have answered all comments and we hope that our manuscript could be published in Jove.

The manuscript has been seen and approved by all listed authors.
The authors have no conflict of interest to declare.

With my best regards,

Dr Jerome MOREAUX

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Editorial Comments:

• *Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.*

• ***Protocol Language:*** *Please ensure that all text in the protocol section is written in the imperative voice/tense as if you are telling someone how to do the technique (i.e. "Do this",*

"Measure that" etc.) Any text that cannot be written in the imperative tense may be added as a "Note", however, notes should be used sparingly and actions should be described in the imperative tense wherever possible.

We checked the manuscript.

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1) Please include an ethics statement before your numbered protocol steps indicating that the protocol follows the guidelines of your institutions human research ethics committee.

An ethics statement was included in the manuscript.

2) Line 87: Unclear how peripheral blood cells are obtained. Please describe the stepd from the time of blood draw to when the PBC are obtained (e.g. centrifugation etc). Mention all centrifugation speeds in g and report durations and temperatures.

The manuscript was modified with more details included (p 3, lines 89-97)

3) Line 88: Unclear exactly is done. What is the bead concentration? How is the purification done? Describe the gating strategy.

We described these points with more details.

4) Lines 91-99: Mention culture duration and environmental conditions.

We modified the manuscript including these informations.

5) Line 103: Unclear how these steps are performed as most details are absent.

We included more details within the manuscript.

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The numbering was modified.

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We highlighted parts of the manuscript accordingly.

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The discussion was extended.

• ***Figures:***
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We modified the figure.

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This was removed.

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Comments from Peer-Reviewers:

Reviewer #1:

In this manuscript by Jourdan et al. authors propose a strategy that enables in vitro MBC to plasma cell differentiation in humans. This offers a convenient alternative to obtaining differentiated cells from subjects which are both rare and remotely located. While the idea behind the manuscript is useful, article falls short in multiple aspects.

Major comments:
1-In vitro Plasma cell generation has already been very well documented (Le Gallou et al. Journal of Immunology 2012; Cocco et al. Journal of Immunology 2012) Unfortunately authors, despite using a similar procedure to those described in these articles, do not refer to these publications and discuss why we need another method on top of theirs.

As indicated in the manuscript, our protocol was defined in 2009 (Jourdan et al. Blood 2009) and improved in 2011 and 2014 (Jourdan et al. Journal of Immunology 2011 and Jourdan et

al. Leukemia 2014). We discussed the plasma cell generation studies published in 2012 and the different methodologies p 6 to 7, lines 245 to 266.

2-The differences in the protocol described in this manuscript compared to the previously published protocols is the use of IL15. The authors should justify the need to use these cytokines while others can generate PCs without them. Also authors should discuss the requirement for each reagent with relevant citations so that readers can have a better understanding of the protocol.

We modified the manuscript p 6 to 7, lines 257 to 260.

3-The functionality of the plasma cells generated through this strategy has not been shown. Is there a life expectancy limit for these cells? Also, how are their antibody production rates compare with equal amounts of in vivo generated plasma cells? There is no single demonstration of antibody production which should be included with representative ELISAs for each stage of differentiation.

The goal of the manuscript is to present an illustrate the in vitro model of B to PC differentiation and GEP analyses using tools developed by the group. Antibody production rates were previously fully described by the team for the different differentiation steps (Jourdan et al, Blood, 2009; Jourdan et al. JI, 2011 and Jourdan et al. Leukemia, 2014). We modified the manuscript p 4, lines 144 to 147 and p 7 lines 273 to 277.

Other comments:
1-The term 'Epigenetic genes' should be replaced. This is not an existing term and authors should avoid generating new terminology.

We modified the manuscript.

2-Why are only certain genes compared but not the entire transcriptome?

Entire transcriptional analyses were already done and published. Here, we focused on

3-The methodological details of PCA analysis are irrelevant and can be excluded. This is a method that describes plasma cell generation.

The method reported also a molecular atlas of B to PC differentiation using publicly available tools developed by our group. This molecular atlas includes publicly available GEP data related to B to PC differentiation (murine and human GEP data) (kassamabara et al. Plos Comp Biol. 2015).

4-Authors should check the manuscript for grammatical errors and typos.

The manuscript was reviewed.

Reviewer #2:

Manuscript Summary:

The authors described the cell culture method that would recapture in vivo differentiation process from human memory B cells to plasma cells. They also represent the bioinformatics tool for analyzing the gene expression data during memory B cell to plasma cell differentiation.

Major Concerns: None

Minor Concerns:
The description of 'I - In vitro normal plasma cell differentiation model' would better be more precise. For example, the plating cell number was not described for the culture stage 2, 3 and 4. Also the explanation on 'Resto6 stromal cell' would be necessary. If the culture efficiency depends on the donor conditions etc, it should also be noted. This referee is interested whether the system is applicable to single cell culture.

We defined the part 1 of the protocol more precisely (p 3, lines 89-116). We also extended the description of Resto 6 stromal cells p 7, lines 266 to 270. We also add more details within the discussion p 7, lines 271-277.

We never tried to transpose the system to single cell culture. This is an interesting question that we will investigate in the future.

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Supplementary Table 1

Gene	Aliases
DNA methyltransferases (n = 4)	
DNMT1	CXXC9; DNMT; FLJ16293; MCMT; MGC104992
TRDMT1	RP11-406H21.1; DNMT2; M.HsaIIP; PuMet; RNMT1
DNMT3A	DNMT3A2; M.HsaIIIA
DNMT3B	ICF; M.HsaIIIB
Methyl-binding proteins (n = 5)	
MBD1	RFT; PCM1; CXXC3
MBD2	DMTase; NY-CO-41; DKFZp586O0821
MBD3	
MBD4	MED1
MECP2	RTS; RTT; PPMX; MRX16; MRX79; AUTSX3; DKFZp686A24160
HATs (n = 19)	
P300/CBP	
CREBBP	CBP; RTS; RSTS
EP300	p300
HAT1	
HAT1	
MYST	
MYST1	MOF; hMOF; FLJ14040
MYST2	HBO1, HBOA
MYST3	MOZ, RUNXBP2, ZNF220
MYST4	DKFZp313G1618, FLJ90335, KIAA0383, MORF, MOZ2, qkf, querkopf
HTATIP	TIP; ESA1; PLIP; TIP60; cPLA2; HTATIP1
GCN5	
PCAF	CAF; P/CAF; p300/CBP-associated factor
GCN5L2	GCN5; hGCN5; PCAF-b; MGC102791
p160	
NCOA1	SRC1; NCoA-1; RIP160; F-SRC-1; MGC129719; MGC129720
NCOA3	ACTR; AIB1; RAC3; SRC3; pCIP; AIB-1; CTG26; SRC-1; CAGH16; TNRC14; TNRC16; TRAM-1; MGC141848
Other	
SRCAP	KIAA0309
TAF1	BA2R, CCG1, CCGS, NSCL2, OF, P250, TAF2A, TAFII250
TAF1L	MGC134910, TAF2A2
GTF3C4	TFIII90; FLJ21002; TFIIIC90; MGC138450; TFiiiC2–90; TFIIICdelta
ATF2	HB16; CREB2; TREB7; CRE-BP1; MGC111558
CDY1	CDY; CDY1A
CDYL	CDYL1; MGC131936; DKFZP586C1622
HDACs (n = 13)	
Class I	
HDAC1	HD1; RPD3; GON-10; RPD3L1; DKFZp686H12203
HDAC2	RPD3; YAF1
HDAC3	HD3; RPD3; RPD3–2
HDAC8	RPD3; HDACL1
Class II	
HDAC4	HD4; HDACA; HA6116; HDAC-A; KIAA0288
HDAC5	HD5; NY-CO-9; FLJ90614
HDAC6	HD6; JM21; FLJ16239
HDAC7A	HDAC7; DKFZP586J0917
HDAC9	HD7; HDAC; HDRP; MITR; HDAC7; HDAC7B; HDAC9B; HDAC9FL; KIAA0744; DKFZp779K1053
HDAC10	MGC149722; DKFZP761B039
Class III	
SIRT1	SIR2L1
SIRT2	SIR2L; SIR2L2
Class IV	
HDAC11	FLJ22237

Gene	Aliases
HMTs (n = 41)	
Arginine HMTs	
PRMT1	ANM1; HCP1; IR1B4; HRMT1L2
PRMT5	JBP1; SKB1; IBP72; SKB1Hs; HRMT1L5

PRMT7	FLJ10640; KIAA1933
PRMT8	HRMT1L3; HRMT1L4
CARM1	PRMT4
Lysine HMTs	
SUV39	
EHMT1	GLP; FP13812; FLJ12879; KIAA1876; EUHMTASE1; Eu-HMTase1; bA188C12.1; DKFZp667M072; RP11-188C12.1
EHMT2	G9A; BAT8; NG36; C6orf30; FLJ35547; NG36/G9a
SUV39H1	MG44; SUV39H
SUV39H2	FLJ23414
SUV420H2	MGC2705
SETDB1	ESET; KG1T; KIAA0067
SETDB2	CLLL8
SET1	
SETD7	SET7; SET9; SET7/9; FLJ21193; KIAA1717
SETD8	SET8; SET07; PR-Set7
MLL	HRX; TRX1; ALL-1; CXXC7; HTRX1; MLL1A
MLL2	ALR
MLL3	HALR; FLJ12625; FLJ38309; KIAA1506; MGC119851; MGC119852; MGC119853; DKFZp686C08112
MLL4	HRX2; MLL2; TRX2; KIAA0304
EZH1	KIAA0388
EZH2	ENX-1; MGC9169
SET2	
NSD1	STO; SOTOS; ARA267; FLJ22263; FLJ44628; DKFZp666C163
WHSC1	WHS; NSD2; TRX5; MMSET; REIIBP; FLJ23286; KIAA1090
WHSC1L1	NSD3; pp14328; FLJ20353; MGC126766; MGC142029; DKFZp667H044
ASH1L	ASH1; ASH1L1; FLJ10504; KIAA1420
RIZ	
PRDM1	BLIMP1
PRDM2	RIZ; RIZ1; RIZ2; MTB-ZF; HUMHOXY1
PRDM3	EVI1, MDS1-EVI1
PRDM4	PFM1
PRDM5	PFM2
PRDM6	
PRDM7	PFM4
PRDM8	PFM5
PRDM9	PFM6
PRDM10	PFM7, KIAA1231
PRDM11	PFM8
PRDM12	PFM9
PRDM13	PFM10
PRDM14	PFM11
PRDM15	ZNF298
PRDM16	MEL1, PFM13
Other	
SMYD3	ZMYND1; ZNFN3A1; FLJ21080; MGC104324; bA74P14.1

Histone demethylases (n = 8)	
PADI4	PAD; PDI4; PDI5; PADI5
AOF2	LSD1; BHC110; KIAA0601
JMJD2A	JMJD2; JHDM3A; KIAA0677
JMJD2C	GASC1; JHDM3C; FLJ25949; KIAA0780; bA146B14.1
JARID1B	PUT1; PLU-1; FLJ10538; FLJ12459; FLJ12491; FLJ16281; FLJ23670; RBBP2H1A
JMJD1A	TSGA; JMJD1; JHMD2A; KIAA0742; DKFZp686A24246; DKFZp686P07111
FBXL10	CXXC2; Fbl10; PCCX2; JHDM1B
FBXL11	FBL7; CXXC8; FBL11; JHDM1A; LILINA; FLJ00115; FLJ46431; KIAA1004; DKFZP434M1735
KDM2B	JHDM1B, NDY1

Supplemental Table S2

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Supplementary Table S2: Genes significantly differentially expressed during B to PC differentiation using SAM multiclass analysis.

Gene ID	Gene Name	Score(d)	Numerator(r)	Denominator	contrast-MBC	contrast-PreP	contrast-PBs	contrast-Early	contrast-BMP	q-value(%)	Overexpressed in
212079_s_at	MLL/KMT2A	0.300294137	89.31042492	297.4098184	1.586064376	-1.141419095	-0.571686790	0.614685454	-0.487639455	0	MBC
32259_at	EZH1	0.459360478	352.1382428	766.5836731	1.893292669	-0.671443951	-0.250270325	0.576642785	-1.548221173	0	MBC
209106_at	NCOA1	0.644489948	215.9239940	335.0308170	3.536425800	-2.324597351	-0.772664460	0.572068879	-1.011232867	0	MBC
212462_at	MYST4/KAT6I	0.673914341	379.9434947	563.7860351	2.773898983	-2.325098856	0.572358155	0.565564586	-1.586722868	0	MBC
224076_s_at	WHSC1L1	0.803807761	382.7198185	476.1335198	3.732890200	-0.114976941	-0.724292128	0.321664164	-3.215285295	0	MBC
208988_at	FBXL11/KDM	0.579816761	562.0646493	969.3832367	2.556560469	-1.562162526	-0.234808048	0.312058345	-1.071648238	0	MBC
202182_at	GCN5L2/KAT2	0.568579195	178.6556912	314.2142602	3.214999081	-1.500031874	-1.904616028	0.232094034	-0.042445212	0	MBC
226547_at	MYST3/KAT6A	0.773910011	460.9201558	595.5733214	3.739779997	-1.322856386	-0.265223596	0.125372642	-2.277072656	0	MBC
49485_at	PRDM4	1.701540504	1549.370061	910.5690150	8.468645687	-1.915345585	-2.256251266	-2.250137427	-2.046913407	0	MBC
203057_s_at	PRDM2	2.667459711	2904.268483	1088.776887	12.73370170	-2.156693746	-2.817320007	-1.915936637	-5.843751318	0	MBC
226215_s_at	KDM2B	1.204117068	1200.503809	996.9992459	5.704724821	0.075510346	-1.628670139	-1.725767508	-2.425797520	0	MBC
212689_s_at	JMJD1A/KDM	1.165333937	842.9591982	723.3627816	5.945223092	-1.184693279	-1.149806450	-1.603335226	-2.007388136	0	MBC
228443_s_at	SETD8	0.557984603	524.2796029	939.5951067	2.562033923	0.165839514	-0.346652291	-1.323456951	-1.057764194	0	MBC
218878_s_at	SIRT1	0.639471487	350.3815949	547.9237178	3.326570912	-0.158270043	-0.568225485	-1.200514746	-1.399560636	0	MBC
202160_at	CREBBP	0.907888884	2331.367731	2567.899850	4.205551299	-1.196389281	-0.854106656	-1.149119372	-1.005935989	0	MBC
213579_s_at	EP300	0.980542186	529.4548006	539.9612664	5.252309712	-1.529307439	-0.946134875	-0.961340294	-1.815527103	0	MBC
209579_s_at	MBD4	0.652362381	1211.956614	1857.796599	2.200398030	1.143880638	-0.411434432	-0.702645253	-2.230198981	0	MBC
201549_x_at	JARID1B/KDM	0.745963159	350.3497160	469.6608827	4.087675550	-1.733895244	-0.726223843	-0.684303864	-0.943252597	0	MBC
227205_at	TAF1	0.477425548	396.2379769	829.9471585	2.385800685	-0.341836350	-0.690997765	-0.574339328	-0.778627242	0	MBC
200049_at	MYST2/KAT7	0.536067288	274.4143063	511.9027260	2.890895305	-0.565143168	-0.643873852	-0.516950446	-1.164927837	0	MBC
221820_s_at	MYST1/KAT8	0.407685375	169.8583730	416.6408298	2.203350639	-1.026155510	-0.926121094	-0.324062108	0.072988073	0	MBC
227527_at	MLL2/KMT2D	0.534714313	751.5040287	1405.430919	2.531038404	-0.833075196	-0.679953178	-0.271170714	-0.746839313	0	MBC
209984_at	JMJD2C/KDM	0.477853746	167.5727710	350.6779478	2.612380756	-1.737710084	0.139575107	-0.211688912	-0.802556866	0	MBC
1564521_x_a	SKB1/PRMT5	0.685489334	310.9408658	453.6042360	-2.826754957	2.119872232	0.888342191	1.532927897	-1.714387363	0	PrePBs
219408_at	PRMT7	0.525424392	130.5658258	248.4959351	-1.122100427	3.263311520	-0.702179576	0.718103180	-2.157134697	0	PrePBs
201833_at	HDAC2	0.792830164	529.8976184	668.3620806	-2.218586344	3.160752405	1.159049102	0.116328808	-2.217543971	0	PrePBs
209053_s_at	WHSC1	1.710836696	778.3181025	454.9341876	-2.245280691	9.436694967	-0.829969719	-2.713553693	-3.647890862	0	PrePBs
213320_at	HRMT1L3/PR	0.752104449	335.9152721	446.6338052	-0.343165715	4.136864958	-0.781730595	-1.777544149	-1.234424498	0	PrePBs
201209_at	HDAC1	0.945408960	968.3946891	1024.313000	0.961483238	4.013350923	-0.771737149	-1.574261342	-2.628835669	0	PrePBs
1554572_a_a	SUV39H2	1.045272493	430.4554737	411.8117298	-2.699058289	5.779612904	0.017050115	-1.454775994	-1.642828734	0	PrePBs
203358_s_at	EZH2	1.039508184	1604.677346	1543.688997	-0.711457640	4.789264764	-0.474920788	-1.343463159	-2.259423175	0	PrePBs
201697_s_at	DNMT1	1.179199114	878.8608215	745.3031558	-1.452515142	5.966370226	-1.093615912	-1.164721481	-2.255517690	0	PrePBs
206308_at	DNMT2/TRDI	0.382285636	58.55564464	153.1724949	-2.295921079	4.043019040	0.163521484	-1.031443206	-0.879176237	0	PrePBs
203138_at	HAT1	0.897788213	1204.629964	1341.775205	-1.248944268	4.138306811	-0.114137218	-0.793868547	-1.981356777	0	PrePBs
212348_s_at	AOF2/KDM1A	0.527299447	219.4244690	416.1287675	-0.471277335	2.525848603	0.688980250	-0.792883915	-1.950667604	0	PrePBs
216326_s_at	HDAC3	0.570626818	310.7530826	544.5819799	1.483302291	2.195960210	-1.443021626	-0.786136934	-1.450103940	0	PrePBs
218619_s_at	SUV39H1	0.284230339	109.7736347	386.2136429	-0.764802605	1.521018049	0.188644948	-0.715051585	-0.229808802	0.089425441	PrePBs
203098_at	CDYL	1.042759768	498.8551257	478.3988995	-2.925061443	0.808927137	4.003561811	1.777689729	-3.665117235	0	PB
211048_s_at	PDIA4	1.389228087	1874.516135	1349.322082	-4.968255705	0.181478182	5.043126203	1.522907860	-1.779256536	0	PB
203857_s_at	PDIA5	2.320908357	1061.620062	457.4157609	-10.05485986	-4.457918526	7.946395259	1.521841530	5.044541598	0	PB
220668_s_at	DNMT3B	0.298967237	52.41680951	175.3262664	-2.309686785	0.131760655	2.171144331	0.066364741	-0.059582943	0.089425441	PB
224928_at	SETD7	0.698674357	528.8125414	756.8798476	-1.061844222	0.337284608	3.178003993	-0.360774072	-2.092670307	0	PB
230777_s_at	PRDM15	0.745123124	650.3500374	872.8088232	-1.354773619	-1.732438559	1.485020591	2.928505731	-1.326314143	0	Early PC
203353_s_at	MBD1	0.870362738	1294.559555	1487.379339	-1.955820389	0.254089437	1.450968568	2.910570270	-2.659807887	0	Early PC
203845_at	PCAF/KAT2B	0.586926583	453.6871330	772.9878764	-0.189522577	-2.124146471	-0.557924361	2.469850236	0.401743174	0	Early PC

MBC
PrePBs
PB
Early PC
BMPC

202455_at	HDAC5	0.459937512	109.1502069	237.3152962	-0.290254082	-1.727689697	1.372688755	2.454570036	-1.809315011	0	Early PC
206846_s_at	HDAC6	0.507477565	173.8682388	342.6126606	-1.325700477	-1.826780359	-0.442807332	1.378209807	2.217078362	0	BMPC
220605_s_at	SIRT2	0.456711698	188.6242775	413.0051372	-1.283485895	-1.304719118	-0.352971111	0.809860115	2.131316009	0	BMPC
202326_at	EHMT2	0.166871822	35.94206894	215.3872864	-0.379010926	-0.023484325	-0.907408259	0.253761188	1.056142323	1.959583588	BMPC
212512_s_at	CARM1	0.373675427	147.5892613	394.9664621	-1.220856087	-0.340685556	-0.527969962	0.071604850	2.017906755	0	BMPC
244428_at	DNMT3A	0.474337124	128.8286104	271.5971485	1.231458447	-1.581035191	-0.955912350	-1.243312297	2.548801392	0	BMPC
206689_x_at	HTATIP/KAT5	0.473735608	113.7804257	240.1770600	1.058438212	-2.111992578	-1.041616072	-0.688893776	2.784064214	0	BMPC
220714_at	PRDM14	0.685178025	136.6683272	199.4639672	-2.172583422	-1.960797771	-0.800662213	-0.665719143	5.599762551	0	BMPC
220792_at	PRDM5	0.293636793	41.65870473	141.8715422	-0.947897350	-1.122981483	-1.094479415	-0.609944257	3.775302507	0.089425441	BMPC
214197_s_at	SETDB1	0.362136358	64.49323933	178.0910364	-1.318510259	-0.816979517	-0.652927405	-0.549808935	3.338226116	0	BMPC
202618_s_at	MECP2	0.141347700	35.42751021	250.6408679	0.159859578	-0.530500725	-0.429163432	-0.073216193	0.873020773	2.935925411	BMPC
228964_at	PRDM1	0.958393241	1867.584103	1948.661596	-3.997522944	-0.726457915	2.009559413	2.093830773	0.620590673	0	
207700_s_at	NCOA3	0.700427182	1996.446484	2850.326962	-0.308140342	-1.023551615	1.797405548	1.843460512	-2.309174103	0	
217937_s_at	HDAC7	0.206775584	46.63068860	225.5135138	-0.181679567	-0.487491775	0.699601917	1.062659759	-1.093090334	0.938967136150235	
243612_at	NSD1	0.210061354	45.30184631	215.6600699	0.319263785	-1.495104821	-0.032219281	1.009212027	0.198848290	0.938967136150235	
205659_at	HDAC9	0.320025350	224.4740768	701.4259231	-0.236908573	-1.194400368	0.852345131	1.004807792	-0.425843982	0	
222415_at	MLL3/KMT2C	0.371118686	430.4739616	1159.936098	0.260648634	-1.521987123	0.972241146	0.734606354	-0.445509012	0	
235338_s_at	SETDB2	0.424620172	218.4990207	514.5752245	-2.108372844	0.218195822	1.322450647	0.674311383	-0.106585008	0	
202484_s_at	MBD2	0.601070163	902.8283777	1502.034925	-0.071270303	1.617674097	-1.472266784	-1.690533861	1.616396851	0	
228813_at	HDAC4	0.179369141	34.60356513	192.9181620	0.342631702	-0.511950865	0.706028963	-1.258712265	0.722002469	1.95958358848745	
223908_at	HDAC8	0.195690884	40.61445653	207.5439369	-0.768000196	0.813996578	1.087535668	-0.727927590	-0.405604459	1.17721252890477	
225543_at	EHMT1/GTF3	0.268275202	114.9548781	428.4961007	0.869115078	0.340736000	0.597479385	-0.717809231	-1.089521232	0.176056338028169	
203205_at	JMJD2A/KDM	0.139793470	28.71373747	205.4011345	0.964768078	0.094807904	0.100134191	-0.547897366	-0.611812808	2.93592541162468	
218788_s_at	SMYD3	0.278653800	86.81357681	311.5463580	0.965839756	-1.393456486	-0.007678453	-0.477526678	0.912821862	0.0894254415381176	
212984_at	ATF2	0.280501111	390.8599298	1393.434512	-0.861394111	0.696250006	0.879230905	-0.319678393	-0.394408407	0.0894254415381176	
219515_at	PRDM10	0.287953444	126.7823785	440.2877654	0.331246768	1.089730508	0.231113103	-0.273008108	-1.379082272	0.0894254415381176	