Role of Histone Deacetylase HDAC7 in B Lymphocyte Biology

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Dedicated to Jorge

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Thesis Abstract

B lymphopoiesis is the result of several cell commitment, lineage choice and differentiation processes. Every differentiation step is characterized by the activation of a new, lineage specific, genetic program and the extinction of the previous one. To date, the central role of specific transcription factors in positively regulating these distinct differentiation processes to acquire a B cell specific genetic program is well established. However, the mechanisms by which B cell transcription factors mediate the process of gene silencing to acquire and maintain the cellular identity is poorly understood.

The main goal of this PhD Project is to investigate the mechanisms of gene transcriptional repression during B lymphocyte development. Since class IIa histone deacetylases (HDACs) are known as important modulators of gene transcription and are associated with developmental and differentiation processes, we decided to study their potential contribution in B lymphocyte Biology. Among all HDAC members, class IIa HDACs are potential candidates to participate in the gene transcriptional repression during B cell development for two reasons. First, they are expressed in a tissue-specific manner and are implicated in several differentiation and developmental processes. And second, they have an amino-terminal region that mediates their interaction with tissue-specific transcription factors, such as MEF2 family members, leading to the repression of their target genes.

To achieve our principal objective, we have made use of two main experimental approaches: an *in vitro* biochemical-genome wide assay by using a highly efficient immune reprogramming system, and an *in vivo* approach by generating conditional knock-out mouse models.

To study the potential contribution of class II HDACs in the reprogramming of B cells into macrophages, we have taken advantage of a cellular reprogramming system consisting of a genetically modified B cell line which is able to transdifferentiate to functional macrophages by addition of β-estradiol (Bussmann, 2009). We have demonstrated that among all class IIa HDACs, HDAC7 shows a

lymphoid lineage-specific expression pattern. HDAC7 expression is down-regulated during the conversion of pre-B cells into macrophages. Importantly, re-expression of HDAC7 interferes with the acquisition of the macrophage gene transcriptional program, blocks the induction of key genes for macrophage function, (such as immune, inflammatory response and phagocytosis), and abolishes crucial functions of macrophages, such as the ability to phagocytose bacteria and to respond to endotoxin by expressing major pro-inflammatory cytokines. Mechanistically, HDAC7 interacts with MEF2C and is recruited to the promoters of macrophage genes, leading to their transcriptional repression.

To study the role of HDAC7 during B cell development, we have generated conditional knock-out mouse models for specific deletion of HDAC7 in B cell progenitors (pro-B cells). Our results demonstrate that HDAC7-deficient mice show a significant block in B cell development at the pro-B cell stage, indicating that HDAC7 is an essential transcriptional repressor in B lymphopoiesis. Mechanistically, HDAC7 is recruited to MEF2 binding sites located at the promoters of lineage inappropriatege genes in pro-B cells. Importantly, we show that the absence of HDAC7 results in the up-regulation of relevant genes characteristic of macrophages and T lymphocytes.

In conclusion, we have identified HDAC7 as a novel master regulator in B lymphocyte Biology.

Resumen de Tesis

El desarrollo de células B es el resultado de varios procesos de especificación, compromiso y diferenciación, cada uno de los cuales se caracterizan por la activación de un nuevo programa de transcripcion génica y la extinción del anterior. Hasta la fecha, la regulación positiva durante el desarrollo de células B llevada a cabo por factores de transcripción específicos está bien establecida. Sin embargo, los mecanismos pro los cuales los factores de transcripción median procesos de represión de genes inapropiados de otros linajes celulares para adquirir y manetenrla identidad celular son vagamente conocidos.

El objetivo principal de este proyecto de tesis es investigar los mecanismos de represión transcripcional durante el desarrollo de linfocitos B. Las histonas desacetilasas (HDACs) de clase IIa han emergido como moduladores crucailes de la transcripción génica en numerosos procesos de desarrollo y diferenciación celular. De entre todas las enzymas que conforman esta familia, nos centramos en las clase IIa por dos razones fundamentales. En primer lugar, las HDACs de clase IIa se expresan de manera específica de tejido y están implicadas en numerosos procesos de diferenciación celular. En segundo lugar, las HDACs de esta sub-familia contienen un dominio N-terminal que media su interacción con factores de transcripción específicos de tejido (como miembros de la familia MEF2), mediando así su acción como co-represores transcripcionales.

Para lograr nuestro objetivo principal, hemos realizamos dos abordajes experimentales: una aproximación experimental *in vitro* mediante el uso de un sistema de reprogramación celular, y una aproximación *in vivo* mediante la generación de un modelo de ratón mutante para HDAC7.

Para estudiar la contribución potencial de las HDACs de clase IIa en la reprogramación de células pre-B a macrófagos, hemos utilizado un sistema de reprogramación celular que consiste en una línea de células B genéticamente

modificadas que se transdiferencian en macrófagos funcionales tras la adición de β-estradiol (Bussmann, 2009). Hemos demostrado que, a diferencia de las otras HDACs de clase IIa, HDAC7 presenta un patrón de expresión específico de linaje linfoide. Es importante destacar que la re-expresión de HDAC7 interfiere con la adquisición el programa transcripcional de genes característicos de macrófagos, tales como genes relacionados con la respuesta inmunológica y la fagocitosis, y suprime funciones cruciales de los macrófagos, como la capacidad para fagocitar bacterias y la respuesta celular a endotoxinas. Desde un punto de vista mecanístico, HDAC7 interacciona con MEF2C y es reclutado a los promotores de genes de macrófagos, dando lugar a su represión transcripcional.

Para estudiar el papel de HDAC7 durante el desarrollo de células B, hemos generado un modelo de ratón condicional para delecionar HDAC7 de manera específica en progenitores de células B (células por-B). Nuestros resultados demuestran que los ratones deficientes en HDAC7 muestran un bloqueo significativo del desarrollo de células B en el estadio celular pro-B, indicando que HDAC7 es un represor transcripcional esencial en la formación de linfocitos B. Desde un punto de vista mecanístico, HDAC7 es reclutada a sitos de unión para MEF2C localizados en los promotores de genes inapropiados de linaje en células pro-B. También hemos demostrado que la ausencia de HDAC7 resulta en la activación de genes relevantes característicos de macrófagos y linfocitos T.

En conclusión, hemos identificado HDAC7 como un nuevo regulador esencial en el desarrollo de linfocitos B.

PART I

INTRODUCTION AND GLOBAL HYPOTHESIS

Introduction

1. B lymphocyte development

1.1. Introduction and Overview

The hematopoietic system is one of the most intensively studied and best characterized paradigm in Developmental Biology. Hematopoietic stem cells (HSC) have the potential to self-renewal and also to differentiate into all mature blood cells. HSC give rise to lymphoid-primed multipotent progenitors (LMPPs) that can differentiate into common lymphoid progenitors (CLPs). CLPs will give rise to B and T lymphocytes, and also to natural killer (NK) cells (Kondo, 1997; Treiber, 2010; Banerjee, 2013; Hagman, 2012). LMPPs can also differentiate into common myeloid progenitors (CMP), which in turn branch into granulocyte/macrophage progenitors (GMP) and megakaryocyte/erythrocyte progenitors (MEP). GMP will give rise to mature granulocytes, macrophages and dendritic cells, while MEP will differentiate into mature megakaryocytes and erythrocytes (Molawi, 2013; Shortman, 2013) (Figure 1i).

According to the proposed model of hematopoiesis, B cells develop from hematopoietic precursor cells in the bone marrow in an organized differentiation and maturation process. B lymphocyte generation starts with the activation of the recombination activating genes *Rag1* and *Rag2* at the LMMP cellular stage, resulting in the generation of CLPs (Igarashi, 2002). CLP population express lymphoid specific surface antigens, such as CD127 and CD19, followed by a sequential heavy and light chain gene rearrangement that forms the pre-BCR (pre-B cell receptor) (initially expressing IgM), leading to its differentiation into early B cell progenitors and precursors, pro-B and pre-B cells, respectively. Pre-B cells will give rise to immature B cells which leave the bone marrow and circulate towards the spleen where they finally differentiate into plasma and memory B cells. This final differentiation step is determined by the expression of IgD on the cell surface and occurs in the absence of

any contact with exogenous antigen. This cell stage is known as antigen-independent B cell development (Rolink, 1996; Osmond, 1998).

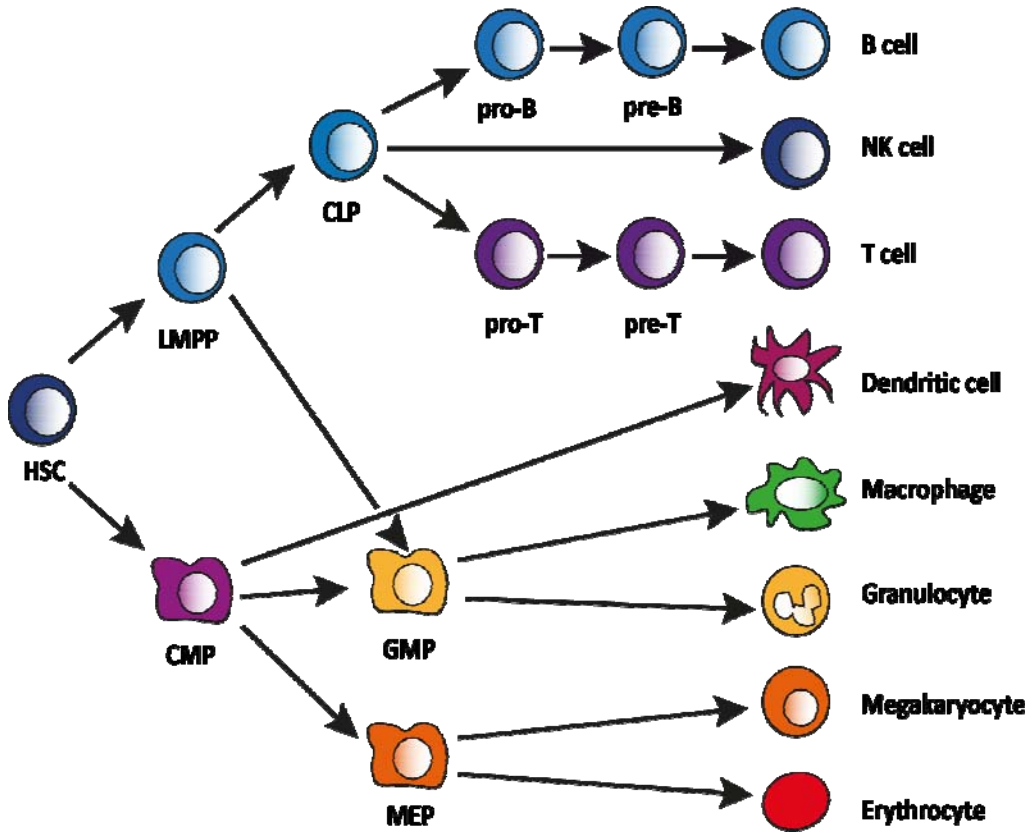

Figure 1i: Model for hematopoiesis. Lineage trees of adult hematopoiesis and lymphoid-myeloid branching points (Modified from Parra, 2009).

1.2 . Transcription factor networks in the generation of B lymphocytes

The processes of B cell lineage specification and commitment, which involve the initiation of B cell-specific gene expression, are dependent on the coordinated actions of signaling cascades and transcriptional networks. Elucidation of the molecular mechanisms behind these processes has extended our knowledge and understanding of how a new genetic program becomes activated whereas the previous one is silenced. At present, it is fully accepted the crucial role of

transcription factors in the specification and differentiation of B lymphocytes and it is well established the existence of networks of lymphoid lineage-specific transcription factors leading to the generation of mature B cells (Busslinger, 2004; Mandel, 2010; Laiosa, 2006).

Initially, at the LMPP stage the transcription factors IKAROS, PU.1 and MEF2C are essential for B lymphocyte development (Figure 2i). **IKAROS** is a transcription factor encoded by the *Ikzf1* gene (Georgopoulos, 1992; Hahm, 1994) and acts as a transcriptional co-activator or co-repressor by recruiting different chromatin remodeling complexes (CRC) to DNA regulatory elements, such as SWItch/Sucrose Non Fermentable (SWI/SNF) or Mi-2/Nucleosome Remodeling and Deacetylase (Mi-2/NuRD). It is also implicated in V(D)J recombination by mediating chromatin accessibility and regulates the expression of early B cell specific genes, such as *Igll1* (l5), by competing with the transcription factor EBF1 for DNA binding (O'Neill, 2000; Kim, 1999; Sridharan, 2007). IKAROS-deficient mice show a block in lymphoid differentiation at the LMPP stage (Georgopoulos, 1994; Wang, 1996). Moreover, it has been recently demonstrated that IKAROS-deficient LMPPs are not able to mature into B cells and, instead, they differentiate into myeloid lineage cells (Yoshida, 2006; Ng, 2009). **PU.1** is also an important transcription factor at the initial specification of LMPPs. It is encoded by the *Sfpi1* gene and its expression level determines whether the cells differentiate into the myeloid versus the lymphoid lineage. In this way, high levels of PU.1 conduct progenitor cells to the myeloid branch, while modest levels allows the cell for lymphoid differentiation (Scott, 1994; DeKoter, 2000). The absence of PU.1 in mice results in a drastic reduction of LMPPs, and in consequence, they do not generate B, T, NK or myelomonocytic cells, leading to a general sepsis and death around birth (Scott, 1994; McKercher, 1996; Karpurapu, 2011). The transcription factor **MEF2C** is a member of the myocyte enhancer factor 2 (Mef2) family which is characterized by a MADS-box domain that mediates its interaction with specific DNA sequences. It was originally identified in muscle where it specifically interacted with muscle genes (Gossett, 1989; Cserjesi, 1991). Later on, it has been demonstrated that

MEF2C is an essential regulator of lymphocyte differentiation and function and is specifically expressed in B cells within the lymphoyd lineage (Swanson, 1998). In particular, MEF2C specifically interacts with the promoter of key regulatory genes, such as *Ets1, Tcf7, Gata3, Pbx1, Il7r, Rag1, Ciita, Cd23, Cr1/Cr2 and Tnfsf4*, which contain MEF2C-binding sites at the promoter region. MEF2C-deficient LMMPs are unable to produce lymphoid-restricted progenitors such as CLPs, resulting in a low frequency of the CLP population (Stehling-Sun, 2009; Debnath, 2013).

Later on, commitment to the B cell lineage depends on the transcription factors E2A, EBF, PAX5 and BCL11A (Figure 2i). **E2A** is a transcription factor required for B cell development at pro-pre-B cellular stage and is encoded by the *Tcfe2a* gene (Bain, 1994). E2A is required for initiating and maintaining the expression of EBF, PAX5 and the B cell-specific program in pro-B cells. It regulates the expression of the recombination activating genes *Rag-1, Rag-2, Vpreb2, Vpreb3* and *Cd19* (Bain, 1994; Zhuang, 1994; O'Riordan, 1999; Sigvardsson, 1997; Månsson, 2004). In the absence of E2A, B lymphocyte development is totally blocked at the pro-pre-B cell stage, prior to immunoglobulin gene D_H-J_H rearrangement (Bain, 1997; Zhuang, 1994; Borghesi, 2005). The transcription factor **EBF** also has a crucial role in early B cell development. It has been demonstrated that EBF targets several components of the pre-B cell receptor complex, including *Cd79a* (*Mb-1*), *Cd79b*, *Blk*, *VpreB1, VpreB2, VpreB3, CD19* and *λ5* (*Igll1)*. EBF also interacts with a set of transcription factors, such as FOXO-1, which is critical in the activation of genes at the CLP stage (O'Riordan, 1999; Akerblad, 1999; Akerblad, 1999; Månsson, 2004). EBF-deficient CLPs show a reduction in IgH chain recombination and are defective in the transcription of B cell lineage-associated genes (O'Riordan, 1999; Zandi, 2008). Recently, it has been demonstrated that the dose of EBF1 is also important in the expansion of B cell progenitors. In this regard, the loss of one allele of *Ebf1* resulted in an accumulation of pro-B cells in G_1, whereas a complete deletion of the *Ebf1* gene in B cell progenitors led to a G_1 block (Ahsberg, 2013; Györy, 2012). **PAX5** is essential for maintaining B cell fate and is considered 'the guardian of B cell identity'. It is

expressed exclusively in the lymphoid lineage and is essential in B cell fate formation (Cobaleda, 2007; Adams, 1992; Li, 1996; McManus, 2011). PAX5 acts as a master regulator at different levels of B lymphopoiesis, increasing its expression from pro-B to mature B cellular stage. At early B cell development, PAX5 exerts its function by activating B cell-specific genes. Among them, it has been described that PAX5 specifically activates *cd19*, genes coding for the transcription factors LEF-1 and N-Myc, the signaling component mb-1 (Ig-α) of the pre-B and B-cell receptors and the anti-apoptotic regulator Bcl-x_L (Travis, 1991; DePinho, 1986; Sakaguchi, 1988; Boise, 1993). In addition, it has been described an essential role of PAX5 in late B lymphopoiesis. In particular, it has been demonstrated its implication in the generation of marginal zone, marginal center and memory B cells in the spleen and B1 cells in the peritoneum by the activation of the B cell markers genes *Cd19, Cd21, Cd22, Cd23, Cd40,* Cd72 and class II MHC (Horcher, 2001). Investigations in mice revealed that PAX5 deficiency results in a block in the differentiation of B lymphocytes at the early pro-B cellular stage, resulting in a dramatic accumulation of $B220^+$ pro-B cells in the bone marrow (Urbánek, 1994; Cobaleda, 2007; Zandi, 2012). Another transcription factor implicated in the regulation of EBF and PAX5 is **BCL11A.** BCL11A is a kruppel-related zinc finger protein which was originally identified as an oncogene related to different B cell malignancies (Liu, 2003; Satterwhite, 2001). Inactivation of BCL11A in mouse models revealed that this transcription factor is required for the development of B cell progenitors, such as CLPs, by regulating the expression of EBF, PAX5 and IL7R (Liu, 2003). Recently, it has been described that BCL11A promotes the transcription of *Rag1* and *Rag2* genes and a deletion of BCL11A in B cells resulted in an impaired V(D)J recombination, indicating that BCL11A is a key transcription factor in the regulation of B cell development by controlling V(D)J recombination (Lee, 2013).

B cells that reach to the pre-B cell stage require the function of the transcription factors **IRF4** and **IRF8**, in order to stop the expression of the components of the pre-BCR and activate the expression of *Rag1* and *Rag2* genes

(Johnson, 2008; Ma, 2006) (Figure 2i). **IRF4** and **IRF8** are members of the interferon-regulatory factor family of transcription factors characterized by a specific DNA binding domain and the ability to bind to regulatory elements in promoters of interferon-inducible genes (Mamane, 1999). IRF4 is highly expressed in plasma B cells and appears to have an important role in the initiation of plasma cell differentiation by promoting isotype switching. IRF4 induces the expression of activation-induced deaminase (AID) and class-switch recombination (CSR), and later on, activates BLIMP-1, which is essential for plasma cell differentiation (Falini, 2000; Klein, 2006; Sciammas, 2006). IRF4-deficient mice show very low titers of serum immunoglobulins, fail to induce BLIMP-1 and do not respond appropriately to immunization (Mittrücker, 1997; Lech, 2011). **IRF8** is also associated with the regulation of immunoglobulin light-chain gene transcription during B lymphocyte development (Eisenbeis, 1995; Pongubala, 1992; Shaffer, 1997; Brass, 1999). IRF8 is expressed at low levels in follicular B cells and at higher levels at germinal center B cells and is down-regulated in plasma B cells (Lee, 2006; Martinez, 2008). In germinal center B cells, IRF8 modulates the expression of BCL6 and AID (Lee, 2006). IRF8-deficient pre-B cells are highly proliferative and fail to rearrange L chain (Holtschke, 1996; Lu, 2003).

The internalization of the pre-BCR also depends on the phosphorylation of the transcription factor **FOXO1** (Figure 2i). In early pro-B cells, the pre-BCR signaling activity is low, allowing FOXO1 to stay in the nucleus where it inhibits the cell cycle progression by repressing the expression of cyclin D (Ramaswamy, 2002; Schmidt, 2002; Medema, 2000). The activation of protein kinase B (PKB) via IL7R signaling, promotes the phosphorylation of FOXO1, resulting in its nuclear export and degradation, allowing the progression of the cell cycle. The inactivation of FOXO1 also decreases IL7R signaling and is associated with lower expression of *Rag1, Rag2, Sell* (which encodes L-selectin (CD62L)) and *Aicda* (Baracho, 2011; Dengler, 2008). FOXO1-deficient mice show an impairment in B cell development at the pro-pre-B

cell stage and, as a consequence, FOXO1-deficient B cells do not express IgM or IgD (Dengler, 2008).

Finally, during the later B cell developmental stages, BCL6 and BLIMP-1 are crucial for the generation of germinal-center (GC) B cells and plasma cells, respectively (Figure 2i). **BCL6** is a transcriptional repressor required for mature B cells during the germinal center reaction (GC). It is involved in the immunoglobulin gene remodeling, avoiding pre-mature activation and differentiation of GC B cells. BCL6 acts as a transcriptional co-repressor by recruiting class I and II HDACs directly or through interaction with specific co-repressors, such as NCOR1 and 2, and BCOR, through its BTB domain (Fujita, 2004; Ranuncolo, 2007; Phan, 2005). BCL6-deficient mice do not form GCs and thus these B cells are unable to generate high-affinity antibodies (Fukuda, 1997; Barish, 2010). Finally, B-lymphocyte-induced maturation protein-1 **(BLIMP-1)** is an important transcription factor implicated in the terminal stage of B cell differentiation (plasma cell development). BLIMP-1 expression plays a critical role in establishing the plasma cell gene expression and is considered a master regulator in plasma cell differentiation Plasma cells differ significantly from activated cells, both morphologically and functionally; they express neither BCR nor class II MHC on their surface. DNA microarrays assays demonstrated that BLIMP1 blocks the expression of several keys genes directly (such as *Spi-B* and *Id3*, important for B cell receptor) or indirectly, through the inhibition of transcription factors (such as AID, KU70, KU86, DNA-PKCs, and STAT6), resulting in the inhibition of immunoglobulin class switching (Shaffer, 2002) and promotion of plasma B cell differentiation. Mice deficient in BLIMP-1 have normal B cell development but cannot form pre-plasma memory cells (Shaffer, 2002; Shapiro-Shelef, 2003).

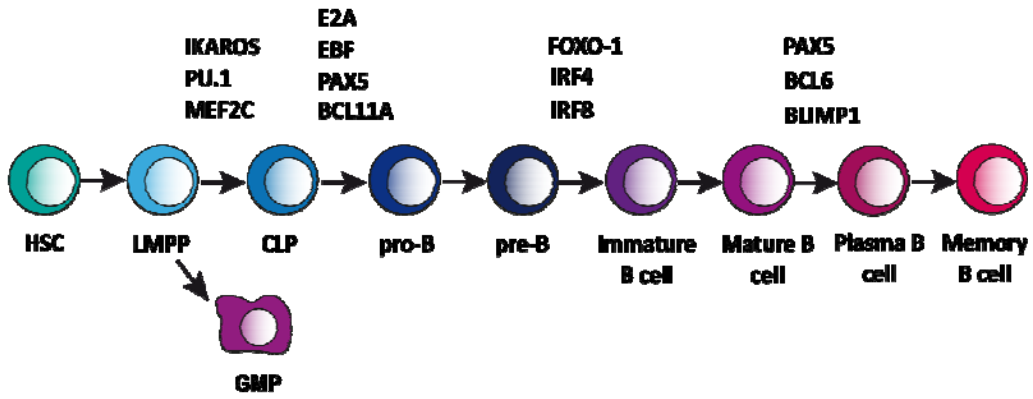

Figure 2i: Transcription factor expression during B lymphopoiesis. Transcription factors implicated in B cell development and differentiation (Modified from Ramirez, 2010).

1.3. Transcription factor-mediated gene silencing in B lymphocytes

The studies described above have elucidated many of the transcriptional mechanisms underlying the hierarchical regulation of B cell development. There is an increasing body of evidence indicating that transcriptional repression is also crucial for the proper differentiation of B lymphocytes.

In this context, transcription factors appear to play a dual role, acting as activators and repressors, in order to guarantee a correct B lymphocyte formation. A clear example of that is the transcription factor **PAX5**, which not only induces the expression of B cell specific genes, but also represses inappropriate genes of alternative lineages, ensuring the B cell identity and maturation. PAX5 is expressed throughout B cell development and conditional inactivation of PAX5 in the B cell lineage results in the down-regulation of B cell specific genes and reactivation of lineage inappropriate genes (Horcher, 2001; Delogu, 2006; Schebesta, 2007). Busslinger and colleagues demonstrated that deficiency of PAX5 in mature B cells results in the conversion of these cells into functional T cells by dedifferentiation into

uncommitted progenitors in the bone marrow (Cobaleda, 2007). Gene expression analyses of wild-type and PAX5-deficient B cells have identified 110 PAX5-repressed and 170 activated genes implicated in important regulatory and structural functions. In particular, PAX5 is involved in the transcriptional silencing of genes related to protein receptors and adhesion molecules (such as *Cd33, Notch1, Itgal* and *Ly6d*), secreted proteins (such as *Ccl3* and *Ccl9*), signal transduction (such as *Mpa21, Tes* and *Vav3*), cytoskeleton (such as *Myo11* and Tnni2), cellular metabolism (such as *Cmah* and *Acadm*), proteases and protease inhibitors (such as *Capn5* and Prrs16) and nuclear proteins (such as *Lmo2*, *Mef2c* and Stat2) (Delogu, 2006; Schebesta, 2007; Pridans, 2008). A recent investigation by Busslinger and colleagues has revealed the regulatory network of PAX5 in B cell development. By using genome-wide sequencing approaches, they have demonstrated that PAX5 represses two times the number of genes that it activates in pro-B and mature B cells. In this regard, PAX5 appears to regulate key transcriptional repressors, such as the histone deacetylase HDAC7. These results reinforce the importance of PAX5 for B cell specification and commitment (Revilla-I-Domingo, 2012).

Another transcription factor implicated in the transcriptional silencing of lineage inappropriate genes is **EBF1**. Although EBF1 promotes B cell linage specification, it is also an important factor in the down-regulation of myeloid important genes including *Cebpa* (encoding C/EBPα), *Pu.1*, *Sfpi1*, and *Id2*. Most of these genes were related to cell receptors, Akt signaling, cell adhesion and migration, and a third of them were also targets of PAX5 (Pongubala, 2008; Thal, 2009; Treiber, 2010). In the absence of EBF1, hematopoietic progenitor cells differentiate into CD11b+ cells and were morphologically identical to granulocytes and macrophages. These cells expressed the myeloid receptors M-CSFR and G-CSFR (Pongubala, 2008). Taken together, these results prompt the possibility that EBF1 could inhibit myeloid development in lymphoid progenitor cells by antagonizing the up-regulation of *Pu.1* and *Cebpa.*

IRF8 has also been identified as part of the transcriptional network that modulates B cell choice in hematopoietic stem cells. The transcription factor IRF8 can act as an activator or repressor depending on the formation of heterodimeric complexes with other factors. This includes Ets family members (PU.1, TEL), IRF members (IRF1, IRF2 and IRF4), and other transcription factor (E47, NFATc1 and MIZ1) (Brass, 1996; Kuwata, 2002; Rosenbauer, 1999; Nagulapalli, 1998; Tamura, 2005). More recent studies have shown that bone marrow of IRF8-deficient mice have lower numbers of B cell populations and an increase number of myeloid cells. Gene expression analyses of wild-type and IRF8-decicient myeloid progenitor cells revealed that 69 genes were altered in the absence of IRF8 during early differentiation. In particular, 62 genes were up-regulated in IRF8-deficient cells, whereas 7 were down-regulated. These genes were related to signal transduction, cell growth and antigen presentation, including cystatin C, cathepsin C, prosaposin, myc-c and lysozyme M, indicating that in the absence of IRF8, bone marrow progenitor cells differentiate into granulocytes and that IRF8 deficiency orchestrates HSCs differentiation in favor of the myeloid lineage (Tamura, 2005; Wang, 2008).

More recently, **MEF2C** has appeared as an important transcription factor implicated in the linear choice towards the lymphoid lineage. It is required for normal lymphoid gene expression and repression of myeloid-specific genes (Stehling-Sun, 2009; Debnath, 2013). Stehling-Sun *et al.* have recently revealed that MEF2C plays a critical role in lymphoid development at the LMPP stage, which is the branching point where cells have to decide between the lymphoid or the myeloid lineages. By using a conditional MEF2C knock-out mouse model, Stehling-Sun *et al.* have demonstrated that MEF2C-deficient LMPPs are not able to produce lymphoid cells properly. Instead, mutant LMPPs differentiate towards Gr1+ myeloid cells, in contrast to control LMPPs. From a mechanistic angle, they have demonstrated that MEF2C deficiency is associated with the down-regulation of key lymphoid transcriptional regulators and the up-regulation of the myeloid transcription factor C/EBPα. As a

result, mutant LMPPs could promote myeloid differentiation, suggesting that MEF2C may also antagonize myelopoiesis (Stehling-Sun, 2009).

All these studies demonstrate that transcriptional silencing or repression of lineage inappropriate genes is also essential for the proper differentiation of B lymphocytes, indicating that transcription factors must recruit both transcriptional co-activators and co-repressors to regulate their target genes.

1.4. Reprogramming of B cells

The conversion of cells from one lineage into another can be achieved by forced expression of tissue-specific transcription factors. The first to show a cellular transdifferentiation process were Schneuwly *et al.* in 1987. Experiments in *Drosophila melanogaster* they demonstrated that ectopic expression of the gene *Antennapedia* (*Antp*), under the control of a heat-shock gene promoter, led to the transformation of the body plan of the fruit fly resulting in the appearance of a second pair of legs in the thorax (Schneuwly, 1987). At the end of the same year, Davis *et al.* identified the first tissue-specific master transcription factor in mice. They discovered that over-expression of the myoblast determination protein 1 (MYOD1) in fibroblasts orchestrated myogenic transdifferentiation in a highly efficient manner (Davis, 1987). Later on, Graf and colleagues reported that primary B and T cells could be reprogrammed into macrophages by enforced expression of the myeloid transcription factors C/EBPα and C/EBPβ. C/EBPs repressed PAX5, resulting in the down-regulation of CD19. In combination with endogenous PU.1, the myeloid gene *Mac-1* became up-regulated, leading to the conversion of B cells into macrophages (Xie, 2004; Laiosa, 2006). Subsequently, an investigation by Hanna *et al.* revealed that, not only non-terminally differentiated B cells, but also mature B cells could be reprogrammed to a pluripotent state by either ectopic expression of C/EBPα or specific knock-down of PAX5 (Hanna, 2008). To decipher the molecular basis of lineage reprogramming, Graf and colleagues described a cellular reprogramming

system that allows the conversion of a pre-B cell line into macrophage-like cells at 100% efficiency within 2-3 days by expressing an estradiol-inducible form C/EBPα. The reprogrammed cells are phenotypically and functionally identical to normal macrophages (Bussmann, 2009). More recently, it has been demonstrated that most of human lymphoma and leukemia B cell lines can also be converted into macrophage-like cells, by over-expression of C/EBPα. Induction of C/EBPα also leads to a dramatically impairing of the cell tumorigenicity (Rapino, 2013). Using the cellular reprogramming system described above, it has been possible to analyze the methylation changes of specific promoters during the cellular conversion. Unexpectedly, no significant changes in the DNA methylation are observed during the reprogramming of pre-B cells into macrophages (Rodríguez-Ubreva, 2012). Specific enzymes can also play an important role in DNA modifications during cellular transdifferentiation. A clear example is Tet2 which becomes up-regulated during C/EBPα-mediated transdifferentiation of B cells into macrophages. A mutation in *C/ebpα* can result in the reduction of expression of Tet2, resulting in an impaired transdifferentiation of the cells (Kallin, 2012). Recently, it has been described that alterations in the structure of C/EBPβ led to differential expression of pro-inflammatory M1 and anti-inflammatory M2 genes, and key regulators of macrophage differentiation, resulting in different types of monocytes/macrophages, dendritic cells, and granulocytes (Stoilova, 2013).

Taken together, these studies indicate that the reprogramming of cells is mediated by the action of transcription factors and its cooperation with specific enzymes is essential to chromatin remodeling and the correct transdifferentiation of one differentiated cell type into another.

2. Histone Deacetylases

2.1. Introduction and overview

In eukaryotes, DNA is organized into a highly ordered nucleoprotein assembly called chromatin, whose fundamental unit is the nucleosome. The nucleosome is composed of an octamer of four core histones (H3, H4, H2A and H2B, organized into two dimmers, H3-H4 and H2A-H2B) with 146 bp of DNA wrapped around them (Luger, 1997). Post-translational modifications (PTM) of histone tails protruding the nucleosome determine the structural conformation of the chromatin. These modifications influence gene expression pattern by allowing or restricting the accessibility of transcription factors to the nucleosomes or to the DNA itself (Reynolds, 2013).

PTM include acetylation, methylation, phosphorylation, ADP-ribosylation, ubiquitination, sumoylation, carbonylation, glycosylation, among others. Transcriptional co-regulators play an important role in epigenetic regulation by recruiting chromatin-modifying enzymes. These co-factors bind to transcription factors which are recruited to the regulatory region of genes, controlling their transcriptional activation or repression (Mottis, 2013). Recently, microRNAs (miRNAs) have emerged as new regulators of gene expression by repressing the transcription of target genes and are involved in different cellular processes including cell growth, differentiation, and lineage commitment (de Yébenes, 2013; Vigorito, 2013). In this context, long non coding RNAs (lncRNAs) have also emerged as modulators of gene expression by regulating different processes, such as epigenetic control of transcription, mRNA stability, and protein localization (Paralkar, 2013).

Acetylation and deacetylation of histone and non-histone proteins are regulated by two groups of enzymes: histone acetyltransferases (HATs) and histone deacetylases (HDACs). From the initial discovery of the first HAT and HDAC involved in gene activation, it became apparent that the antagonistic activities of HATs and

HDACs regulated gene expression through chromatin modification (Brownell, 1996; Taunton, 1996). The acetylation neutralizes the positive charge of the histone lysine residues, relaxing the chromatin conformation and enabling greater accessibility of the transcription machinery. Protein acetylation is therefore generally associated with gene activation. In contrast, the removal of acetyl groups from histones induces chromatin condensation and gene transcriptional repression (Figure 3i) (Haberland, 2009).

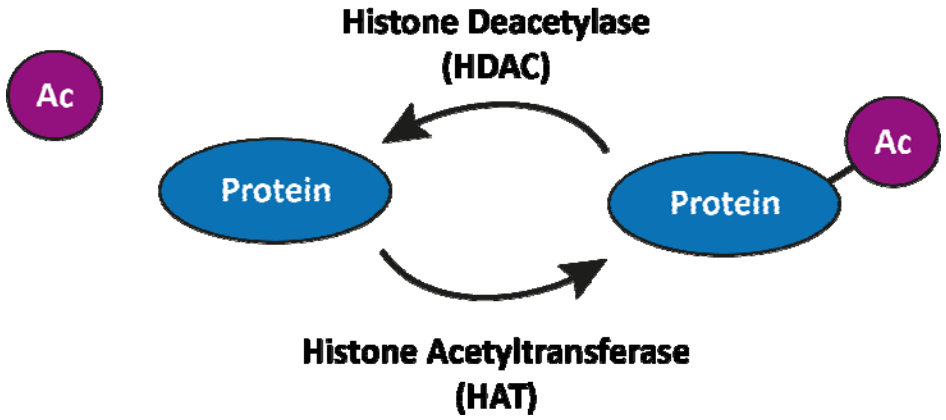

Figure 3i: Protein acetylation. Protein acetylation is an important posttranslational modification regulated by histone acetyltransferases (HATs) and histone deacetylases (HDACs) (Adapted from Alamdari, 2013).

In addition to their chromatin-related functions, HDACs also deacetylase an increasing list of non-histone proteins, consisting of a co-translational Nα-terminal acetylation of eukaryotic proteins. Some of these non-histone proteins include transcription factors (such as p53, STAT3 and STAT6, among many others) and other cytoplasmic proteins (such as α-Tubulin and Importin-α), and affect gene transcription and other cellular processes (Peng, 2011; Tang, 2013).

HDACs are known as modulators of gene transcription and are associated with proliferation and differentiation of a variety of cell types and the pathogenesis

of some diseases. HDACs have come to be considered crucial targets in various diseases, including cancer, interstitial fibrosis, autoimmune and inflammatory diseases, and metabolism disorders (Tang, 2013).

2.2. Structure

The identification of the enzymes that regulate acetylation of histones and non-histone proteins has revealed the key role of dynamic acetylation and deacetylation in various cellular processes (Martin, 2007). In this context, HDACs have emerged as crucial transcriptional co-repressors in highly diverse physiological and pathological systems (Barneda-Zahonero, 2012).

Mammalian HDACs are grouped into four different classes, on the basis of their similarity to yeast counterparts (Martin, 2007). Class I HDACs (HDAC1, 2, 3 and 8), which are related to yeast Rpd3 (reduced potassium dependency 3) (Fu, 2007); Class II HDACs, which are divided into two subclasses, class IIa (HDAC4, 5, 7, and 9) and class IIb (HDAC6 and 10), both homologous with the yeast gene Hda1 (histone deacetylase 1) (Mihaylova, 2013); Class III, also known as sirtuins (SIRT1, 2, 3, 4, 5, 6 and 7) are homologous with yeast Sir2 (silent information regulator 2) (Blander, 2004; Trapp, 2006); and Class IV (HDAC11), which contains conserved residues in catalytic regions shared by both class I and II HDAC enzymes (Figure 4i) (Gao, 2002).

Class I HDACs comprise HDAC1, 2, 3 and 8 and are the most abundant and widely expressed HDACs. They are primarily localized in the nuclear compartment of the cell and exert a strong catalytic effect on histone and non-histone lysine residues. They are almost entirely comprised of a conserved deacetylase domain and have minimal N- and C-terminal domains (Richardson, 2013; Tang, 2013) (Figure 4i). HDAC1 and HDAC2 are found in large transcriptional repressor multiprotein complexes, such as the nucleosome remodeling and deacetylation complex (NuRD) and the Sin3 complex. HDAC3, in contrast, is found in other complexes such as the SMRT/N-CoR complex (silencing mediator of retinoic and thyroid receptors (Nuclear

receptor co-repressor) (Gräff, 2013). Recently, it has been demonstrating that HDAC8 is implicated in the deacetylation of cohesin (Deardorff, 2012).

Class II HDACs can be localized in both the nucleus and the cytoplasm. Class IIa HDACs, which comprises HDAC4, 5, 7 and 9, are expressed in a tissue-specific manner and are involved in cell differentiation and development (Figure 4i). They exert their transcriptional repressive function in skeletal, cardiac, and smooth muscle, bone, the immune system, the vascular system, and the brain among others. A peculiarity of class IIa HDACs is that, in addition to the conserved deacetylase domain, they possess a long regulatory N-terminal domain that mediates their interactions with tissue-specific transcription factors, such as MEF2, CtBP or HP1. This N-terminal domain also contains highly conserved serine residues that are subject to phosphorylation, which is a critical event that determines whether class IIa HDACs are localized in the nucleus or the cytoplasm and, therefore, their ability to act as transcriptional co-repressors in the nuclear compartment (Parra, 2010; Yang, 2008; Clocchiatti, 2011). Class IIa HDACs have been found as part of the repressor complex SMRT/N-CoR (Fischle, 2002; Huang, 2000). Class IIb HDACs, which comprises HDAC6 and HDAC10, have less well established functions, although HDAC6 is considered the major cytoplasmic deacetylase and is the only HDAC that contains two deacetylase domains in addition to a ubiquitin binding domain at its C-terminus (Mihaylova, 2013) (Figure 4i). HDAC6 regulates the deacetylation of α-tubulin, cortactin, chaperones, and IFNαR (Haberland, 2009; Matsuyama, 2002; Tang, 2007; Zhang, 2007; Kovacs, 2005; Kaluza, 2011), and has recently been implicated in regulating autophagy as well as hepatic metabolism (Lee, 2010). HDAC10 has similar structural and pharmacological properties than HDAC6. It contains two catalytic domains but, unlike HDAC6, the second one is not functional. HDAC10 can be found in both nucleus and cytoplasm. In the nucleus, it specifically binds to HDAC2, HDAC3 and SMRT (silencing mediator for retinoid and thyroid hormone receptors), resulting in gene transcriptional repression. More recently, it has been described to associate with metalloproteinases (MMP) 2 and 9, leading to their transcriptional silencing and

cervical cancer metastasis suppression (Guardiola, 2002; Fischer, 2002; Tong, 2002; Song, 2013).

Class III HDACs, mainly referred as **sirtuins,** comprise seven members (SIRT 1-7) and are localized in different cellular compartments. SIRT1, 6 and 7 are nuclear, SIRT2 is localized in the cytosol and SIRT 3, 4 and 5 are mitochondrial (Bosch-Presegué, 2011; Saunders, 2007) (Figure4i). Sirtuins are NAD+ dependent enzymes and thus they are related to cellular energy consumption. In this regard, they associate chromatin re-modeling and transcriptional regulation under oxidative stress with cellular homeostasis, inflammation and numerous aging-related disorders, such as cancer (Rajendran, 2011). Sirtuins are also related to the regulation of DNA damage in eukaryotic cells. In particular, they recognize and bind to proteins with DNA damage repairs functions, including various FOXO family members, Ku70, p73, pRb, and Werner helicase (WRN), leading to their transcriptional repression. In brief, sirtuins play also a role in DNA repair and the maintenance of genomic stability (Motta, 2004; Uhl, 2010; Dai, 2008; Wong, 2007; Rajendran, 2011).

Finally, HDAC11 forms itself the **class IV HDACs** and has not been widely studied (Figure4i). It is specifically expressed in brain, heart, kidney and skeletal muscle. HDAC11 plays a role in immunosuppression, DNA replication and neuron and oligodendrocyte development, by targeting interleukin-10, the OX40 ligand, the ARH1 tumor suppressor, myelin basic protein, and proteolipid protein (Bagui, 2013).

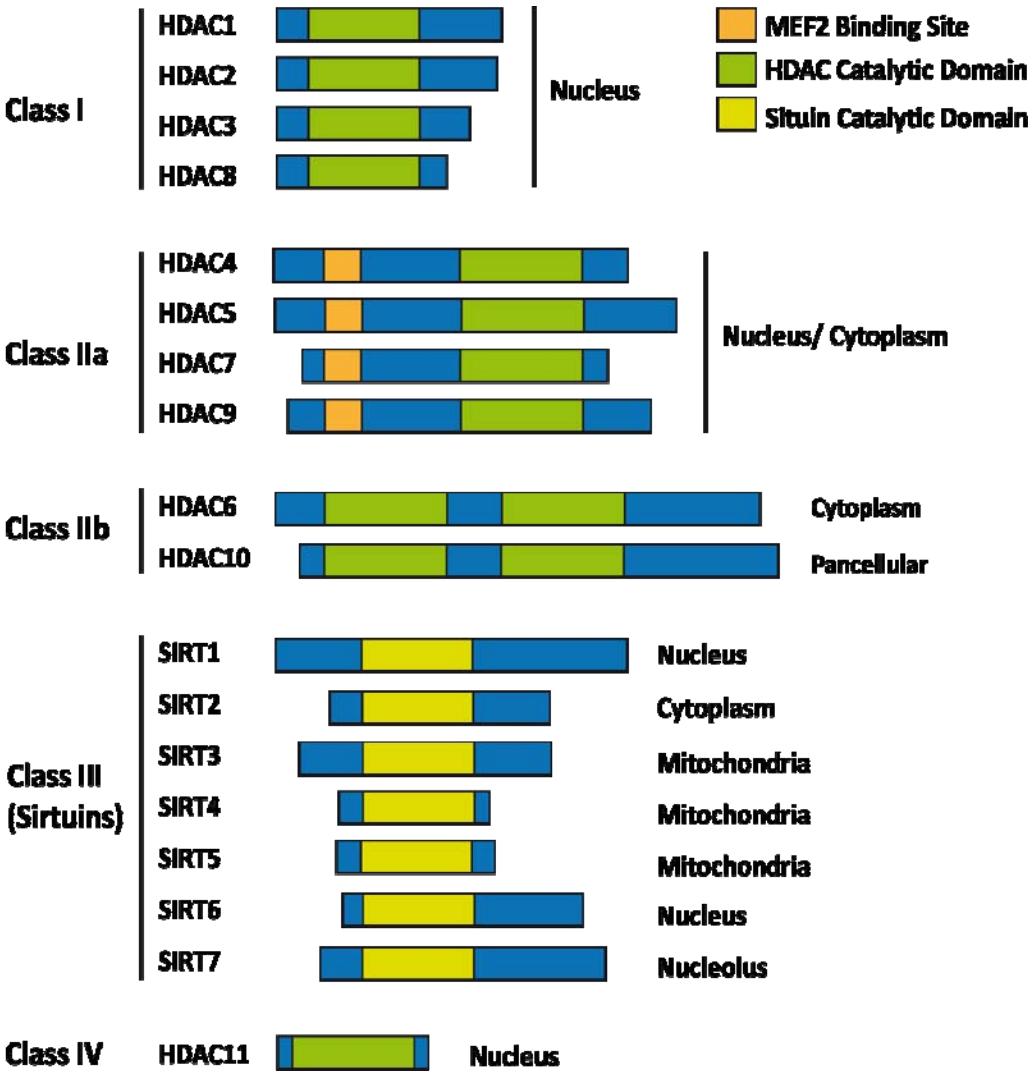

Figure 4i: The histone deacetylase family. The histone deacetylase family is subdivided into different subfamilies according to homologies to yeast prototypes (Modified from Barneda-Zahonero, 2012).

2.3. Function

In order to assert their repressive potential, HDACs are recruited to the chromatin by interaction with specific transcription factors. The best characterized is

MEF2, which is involved in cell differentiation, cell growth and survival (McKinsey, 2000; Arnold, 2007; Chang, 2004; Chang, 2006; Bolger, 2005). SRF and RUNX2 are other transcription factors that are modulated by the association with histone deacetylases (Davis, 2003; Chen, 2006; Margariti, 2009; Jeon, 2006; Vega, 2004). The ability of HDACs to interact with different binding partners forming macromolecular structures has an important role in the correct chromatin remodeling and modification. In this regard, class IIa HDACs associate with HDAC3 in order to reveal their deacetylase activity (Fischle, 2002). Class IIa subfamily also interact with the methyltransferase SUV39H1 and HP1, which is essential for the correct DNA packaging (Zhang, 2002). In addition to their chromatin-related functions, HDACs also deacetylase an increasing list of non-histone proteins, including transcription factors (such as p53, STAT3 and STAT6) and other cytoplasmic proteins (such as α-Tubulin and Importin-α) (Peng, 2011).

2.3.1. Histone deacetylation

Histone modifications can affect chromatin structure in multiple ways. The basic charge of histones is due to the high proportion of positively charged lysine and arginine residues that are found in all histone proteins. Negatively charged DNA interacts electrostatically to regions of the histone that contain these amino acids. PTM of either lysines or arginines have the potential to disrupt DNA/protein and protein/protein interactions by changing the charge of the modified amino acid (Kouzarides, 2007; Shahbazianm, 2007). Histone acetylation occurs at multiple lysine residues on histones and is controlled by histone acetyltransferases (HATs) and histone deacetylases (HDACs), which add or remove, respectively, acetyl groups to histones (Serrano, 2013) (Figure5i). In particular, HDAC1 deacetylases H3K9 and thus promotes memory formation (Bahari-Javan, 2012). HDAC2 is implicated in the deacetylation of H3K56 preventing mouse brain from DNA damage (Hagelkruys, 2013). HDAC3-deficient cells show high levels of acetylation marks on H4K5 and H4K12, resulting in a loss of heterochromatin, an increase in DNA double-strand

breaks and less proliferation (Bhaskara, 2010). Class IIa HDACs, which comprises HDAC4, 5, 7 and 9, have not identified substrates and they only show histone deacetylase activity when interacting with HDAC3 (Ruijter, 2003). HDAC6 and HDAC10 have less well established functions, although HDAC6 is considered the major deacetylase enzyme in the cytoplasm by deacetylating non-histone proteins (Mihaylova, 2013). Among sirtuins, only SIRT2 and SIRT6, and more recently, SIRT1, show histone deacetylase activity; in particular, SIRT2 deacetylase histone H4K56, which is implicated in the regulation of cell cycle, and both SIRT1 and SIRT2 deacetylase H3K56 and this acetylation marker is increased in multiple types of cancer (North, 2007; Das, 2009); SIRT6 deacetylase H3K9, which is important in metabolism and base excision repair (Van Gool, 2009). Finally, class IV HDACs, which comprises HDAC11, specifically deacetylases H3K9/K14 and this is implicated in the regulation of oligodendrocyte-specific gene expression, and thus in cell growth and oligodendrocyte development (Liu, 2009).

As mention above, histone acetylation plays an important role in the regulation of gene expression. In this regard, hyperacetylated chromatin is transcriptionally active, and hypoacetylated chromatin is silent. The strong correlation between gene activation and histone acetylation was noticed 50 years ago (Allfrey, 1964).

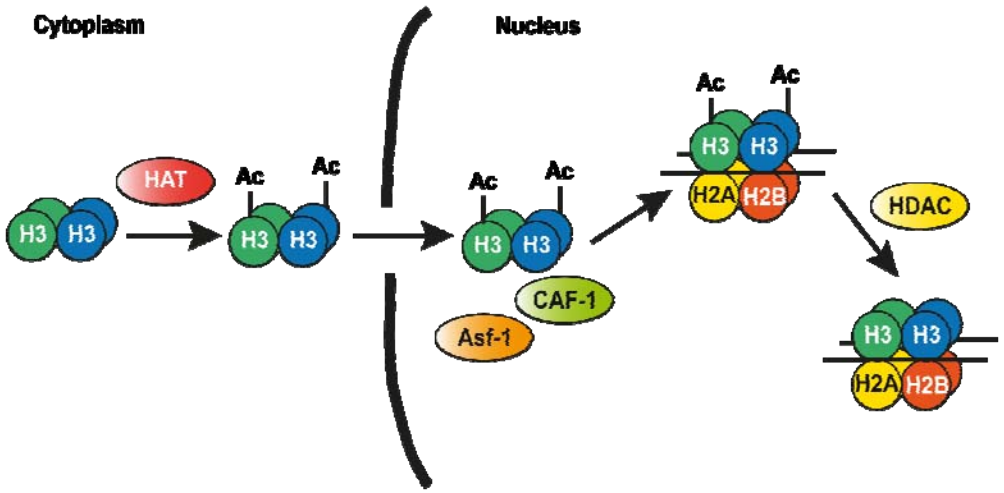

Figure 5i: Role of histone acetylation and chaperones in nucleosome assembly. Histones H3 and H4 are acetylated in the cytoplasm by Hat1 and Hat2. Chaperones such as CAF-1 and Asf-1 recognize the H3-H4 acetylated and deposit the histones onto newly replicated DNA along with histone H2A-H2B. Once assembled into nucleosomes, the histones are promptly deacetylated by histone deacetylases (HDACs) (Modified from Shahbazian, 2007).

2.3.2. Deacetylation of non-histone proteins

Genealogical studies have demonstrated that deacetylation of non-histone proteins is also a primary function of HDACs. It is in this context where HDACs are being found to target a variety of non-histone proteins, some of them are important transcription factors and co-regulators (such as p53 and STAT3) and other cytoplasmic proteins (such as α-Tubulin and PTEN) (Gregoretti, 2004; Lee, 2013; Peng, 2011; Pirola, 2012).

One of the most important examples of deacetylation of non-histone proteins is the transcription factor p53, which becomes deacetylated by SIRT1. Since p53 is an important tumor suppressor in a large number of tumors, the significance of SIRT1 deacetylation on p53 suggest that this interaction is an important target to

regulate p53 tumor suppressor activity (Luo, 2001; Vaziri, 2001) (Figure 6i). SIRT1, and in a minor extent, HDAC3, also deacetylases the transcription factor STAT3. STAT3 is acetylated at Lys-685 and this is critical for STAT3 phosphorylation, dimerization, nuclear translocation, and transactivation (Hu, 2013). Deacetylation by SIRT1 impairs its dimerization ability, and thus STAT3 cannot bind to the DNA resulting in the inhibition of the transcription of STAT3 target genes (Yuan, 2005). PTEN is another non-histone target of HDACs, which is an important phosphatase involved in the cellular signaling. Acetylation of PTEN by the histone acetyltransferase p300/CBP-associated factor (PCAF) can repress its activity; on the contrary, deacetylation by SIRT1 and HDAC1 can stimulate PTEN activity (Pirola, 2012). Another clear example of deacetylation of non-histone proteins is the deacetylation of lysine 40 in α-tubulin by HDAC6. In this regard, HDAC6 has been identified to have an important role in serotonergic neurotransmission in a manner that is dependent on GSK3b activity (Hagggarty, 2003; Chen, 2010). Other examples of HDACs implicated in the deacetylation of non-histones are SIRT3 and SIRT4, which are implicated in the deacetylation of the glutamate dehydrogenase (GDH) in the mitochondria, which controls the amino acid flux into the tricarboxylic acid cycle (TCA) (Shi, 2005; Lombard, 2007; Hallows, 2006). HDAC1, 2 and 3 have been identified in the deacetylation of the Yin Yang 1 protein (YY1), leading to the transcriptional repression of its target genes (Shi, 1997; Thomas, 1999; Yao, 2001). HDAC5 is involved in the deacetylation of GATA-1 and restrains its ability to promote erythroid differentiation (Watamoto, 2003). More recently, SIRT6 has been found to interact with the p65 component of the transcription factor NF-κB, resulting in the repression of NF-κB target genes (Kawahara, 2009).

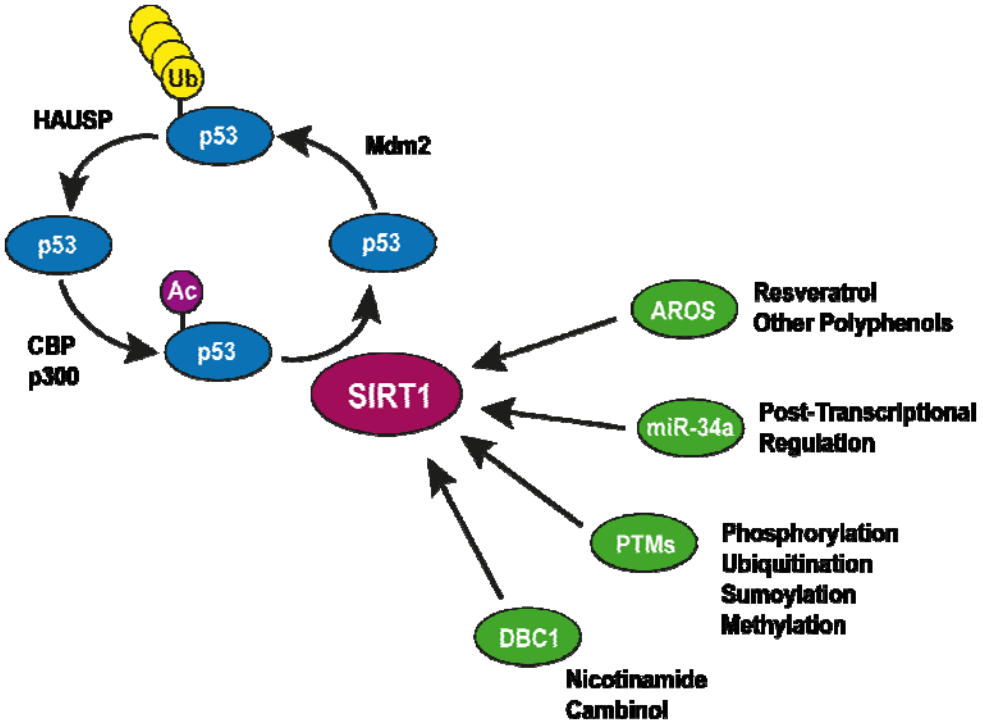

Figure 6i: SIRT1- p53 pathway regulators. Since p53 is an important tumor suppressor, its deacetylation by SIRT1 plays an important role in p53 tumor suppressor activity (Modified from Lee, 2013).

2.4. HDACs in physiology and pathology

Having shown that HDACs interacts with multiple complexes that contain DNA binding sites, their physiological roles should be considered in the chromatin manipulation context. Classically, HDACs were thought to be recruited by transcriptional repressors in order to facilitate local histone deacetylation and transcriptional repression. More recently, genome-wide assays have mapped HDAC1 and 2 and their associated proteins to transcriptionally active loci, providing specific functions in alternative contexts, whereas their repressing functions are exerted to balance transcriptional activation and repression. HDAC1 and HDAC2 are implicated

in many different tissues and cells, such as skin, brain, heart, B and T cells (Kelly, 2013). Class IIa HDACs show a tissue-specific restriction pattern, are also expressed in many different tissues (they are expressed in skin, brain, skeletal muscle, cardiovascular and hematopoietic system) and are implicated in different differentiation processes (such as chondrocyte, B and T cell differentiation) (Cocchliattti, 2011).

Today, it is widely accepted that HDACs are promising therapeutic targets in cancer and other diseases. Increased expression of HDAC1, 2 and 3 is related to the expansion of the tumor to the lymphatic nodules and is considered as a prognostic marker for gastric cancer (Weichert, 2008). Elevated expression of HDAC6 is associated to tumor invasion in breast cancer (Park, 2011), and low expression of HDAC5 and 10 is correlated to poor prognosis in lung cancer (Osada, 2004). In addition to their role in cancer, HDACs are also involved in different diseases, such as tissue fibrosis, autoimmune and inflammatory diseases, and metabolic disorders. In this context, HDAC inhibitors (HDI) have emerged as potential anti-cancer agents, as well as agents dissecting HDAC roles (Khan, 2010). The mechanism of action of HDIs involves inhibiting the deacetylation of histone and non-histone proteins, including p53, GATA and NFκB (Tang, 2013). Most of the HDIs developed target classes I, II and IV HDACs while, although interest in the sirtuin family is increasing (New, 2012). A classical example of HDIs is the trichostatin A (TSA), one of the first HDIs identified (Finnin, 1999). Today, there are many different evidences indicating that HDACs are implicated in different biological functions and therefore consequences of HDAC inhibition. However, the mechanisms by which HDACs exert their actions are poorly understood and remain unclear. Different HDACs are implicated in different pathways and the possibility of crosstalk between different them is elevated. In this regard, more studies are needed to reveal the different functions of HDACs and determine their interaction networks and cell substrates.

3. Class IIa HDACs

3.1. Introduction and overview

Among all HDACs, class IIa HDACs (HDAC4, 5, 7 and 9) has three unique features. First, they are expressed in a tissue-specific manner and exert their transcriptional repressive function in a tissue-restricted manner. They are expressed in skeletal, cardiac, and smooth muscle, bone, the immune system, the vascular system, and the brain, among others. Second, class IIa HDACs contains a regulatory N-terminal domain that mediates their interactions with tissue-specific transcription factors such as members of the MEF2 family of transcription factors. And third, they are signal-dependent co-repressors and become phosphorylated at two or three conserved serine residues in the regulatory N-terminal domain (Parra, 2010).

3.1.1. Class IIa HDACs in physiology and pathology

In order to study the functions of class IIa HDACs in physiology and pathology, gene knock-out mice have been generated for all class IIa HDACs. Mice lacking HDAC4 possessed numerous skeletal abnormalities because of premature endochondral ossification. Initially, the phenotypic effect of HDAC4 was explained by a loss of function of Runx2 (Vega, 2004). Later, a careful examination of the transcription factor MEF2 demonstrated that a correct balance between HDAC4 and MEF2C is required for the correct bone marrow development (Arnold, 2007). HDAC4 also plays an important role in neuronal homeostasis. In this context, it acts under the control of miR-206 and controls the genetic response to denervation (Tang, 2009; Cohen, 2007; Williams, 2009; Choi, 2012). More recently, it has been reported a role of HDAC4 in synaptic plasticity, with implications in learning and long-term memory formation (Kim, 2012). It has been also related to Huntington Disease (HD) and is presented as a novel therapeutic mechanism against HD cytoplasmic aggregation (Mielcarek, 2013).

Mice deficient in HDAC5 and/or HDAC9 showed a cardiac hypertrophic response to stress induced by chronic β-adrenergic stimulation, suggesting that HDAC5 and 9 act as modulators of cardiac response to stress conditions (Chang, 2004). Another role proposed for HDAC5 is a regulatory function in the skeletal muscle. In this system, it blocks the differentiation of myoblasts towards myotubes by suppressing the activity of the transcription factor MEF2 (Lu, 2000). HDAC5 also controls osteoblastic differentiation. In this context, it forms macromolecular complexes with RUNX2 and SMAD3, promoting RUNX2 degradation, which is a critical transcription factor is osteoblastic maturation (Harada, 2003; Kang, 2005). Another way to control osteoblastic differentiation is by the action of miR-2861 which targets HDAC5, resulting in the down-regulation of HDAC5 (Li, 2009). More recently, HDAC5 has been implicated in the regulation of erythropoiesis through protein kinase D activation, revealing a molecular pathway involved in cytokine regulation of hematopoietic differentiation (Delehanty, 2012). In muscle, it has been described a pathway that connects HDAC5, the cellular FLICE-like inhibitory protein (cFLIP) and caspases regulating the down-regulation of the polypyrimidine tract binding protein (PTB), in the control of contraction and transcription during cardiac muscle development (Ye, 2013).

HDAC7 is highly expressed in $CD4^+CD8^+$ thymocytes, heart, and lung and in vascular endothelial cells (Kao, 2000; Dequiedt, 2003; Kasler, 2011). During early embryogenesis, HDAC7 is essential for vascular cell homeostasis and angiogenesis. HDAC7 deficient mice are lethal at day 10 of embryonic development, due to a failure in cell junctions and a rupture of blood vessels. HDAC7, via MEF2 interaction, repressed MMP10 (a matrix metalloproteinase which is an essential regulator of blood vessel development), demonstrating that HDAC7 plays an important role in the maintenance of vascular integrity (Chang, 2006) (Figure 7i). HDAC7 is also important in other differentiation pathways. In osteoblast differentiation, it specifically interacts with RUNX2 (which is an essential regulator of bone formation) resulting in its transcriptional repression (Jensen, 2009; Jensen, 2008).

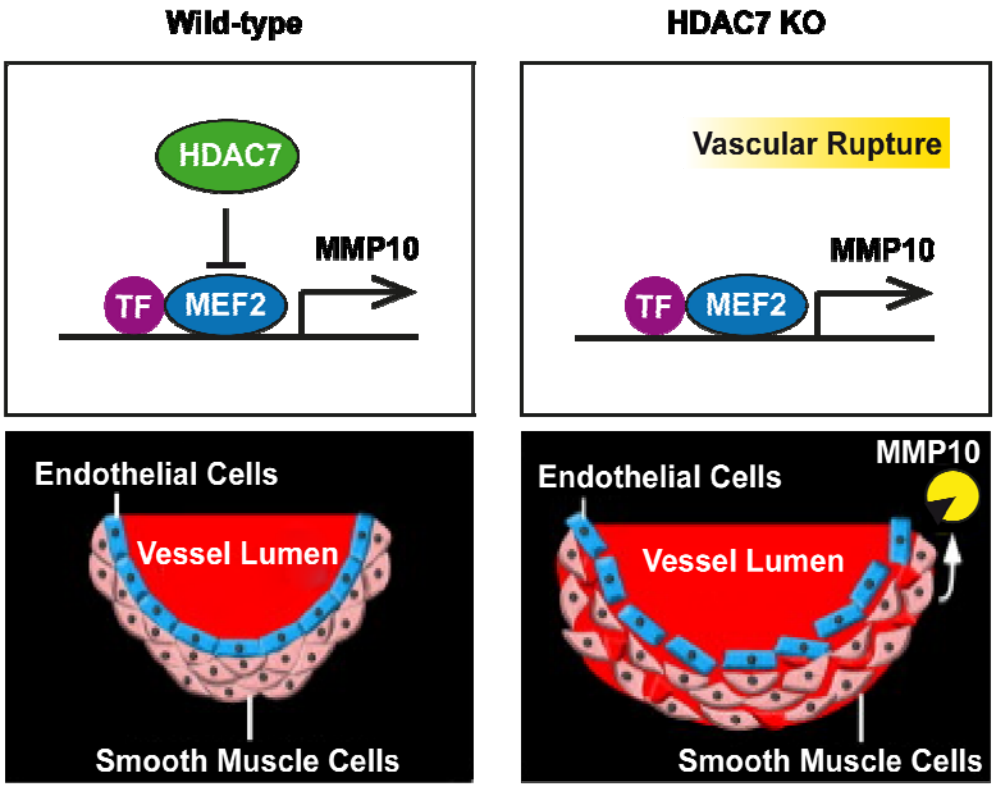

Figure 7i: HDAC7 and MEF2 control MMP10 expression. Model for modulation of MMP10 by HDAC7 and MEF2 in endothelial cells (ECs). In normal cells, HDAC7 inhibits MEF2, resulting in the repression of MMP10. In the absence of HDAC7, MMP10 is overexpressed and thus, the extracellular matrix is greatly disrupted, leading to the rupture of blood vessels (Modified from Chang, 2006).

Finally, HDAC9 alternative splicing that gives rise to different isoforms involved in different pathways in brain and skeletal muscle (Zhou, 2001; Petrie, 2003; Sparrow, 1999). During muscle differentiation, HDAC9 specifically associates with MEF2 proteins, resulting in their transcriptional silencing (Haberland, 2007). Mice deficient in HDAC9 presented cardiomyocyte hypertrophy due to an activation of MEF2 pathways (Zhang, 2002). In the neural system, HDAC9 is involved in the

regulation of gene expression and dendritic growth in cortical neurons. HDAC9-deficient neurons are hypersensitive to denervation and present a decrease in the total length of dendritic branches (Mejat, 2005; Sugo, 2010). In the immune system, it has been reported that HDAC9 is implicated in the regulation of the transcription factor FOXP3, which is essential for Treg cell functions. In systemic autoimmunity HDAC9-deficiency leads to a decreased inflammation and cytokine and chemokine production (Tao, 2007; Yan, 2011).

Class IIa HDACs exert their action in many different tissues and differentiation processes, such as the skeletal muscle physiology and metabolism, adipogenesis, liver metabolism and hematopoiesis, among others, where they act as important transcriptional co-repressors (Parra, 2010; Mihaylova, 2013). The role of class IIa HDACs in muscle physiology and metabolism has been well studied over the years and these studies have shown that HDAC4, 5, 7 and 9 are regulated via calcium/calmodulin-dependent protein kinase (CaMK) and Protein kinase D (PKD), and more recently via 5' adenosine monophosphate-activated protein kinase (AMPK) (Potthoff, 2007; McGee, 2008; Grégoire, 2007). In cardiac muscle, the DNA-binding domain of MEF2 confers sensitivity to CaMK signaling through and mediates the repression of its target genes through interaction with HDAC4 and HDAC5. In response to CaMK signaling, HDAC4 and 5 are released from MEF2-HDACs association, leading to the activation of MEF2 and its target genes. The association of MEF2 with HDACs determines the potential function of MEF2 as a transcriptional activator or repressor, depending on intracellular signaling and interactions with other transcription factors. In this regard, in cells expressing high levels of HDACs (such as cadiomyocytes) and in the absence of CaMK, MEF2 is expected to act as a transcriptional repressor. In contrast, in cells expressing low levels of HDACs, MEF2 is expected to be activated and to show less responsiveness to CaMK signaling (Lu, 2000). The phosphorylation of class IIa HDACs in myotubes allows the de-repression of genes mediated by MEF2 and induces the activation of Glut4, resulting in the glucose uptake in the muscle (McGee, 2008). In adipogenesis, it has been reported

recently that HDAC9 acts as a negative regulator whose down-regulation is essential for the correct adipocyte development (Chatterjee, 2011).

3.2. Regulation of class IIa HDACs function: N-terminal domain

The N-terminal region of class IIa HDACs is a hallmark of this subfamily of HDACs. It contains specific conserved amino acid motifs that are specialized for binding an array of proteins, such as DNA-binding transcription factors, transcriptional co-repressors and chaperone proteins (Martin, 2009). The N-terminal domain in class IIa HDACs consists of 450-600 amino acids, which only 30-45% of the sequence shares homology with the other HDAC members (Martin, 2007). The N-terminal domain contains conserved binding motifs which mediate their interactions with tissue-specific transcription factors. The most important and best characterized interacting transcription factor is MEF2, which mediates its interaction with class IIa HDACs via a conserved motif consisting of 17 amino acids (Lu, 2000; Dequiedt, 2003; Han, 2005). The adaptor domain of class IIa HDACs also contains various motifs that are specialized in their regulation of subcellular localization (McKinsey, 2001; Wang, 2001). In addition, several conserved residues are implicated in post-translational modifications, such as ubiquitination, sumoylation, phosphorylation and proteolytic cleavage (Martin, 2009) (Figure 8i).

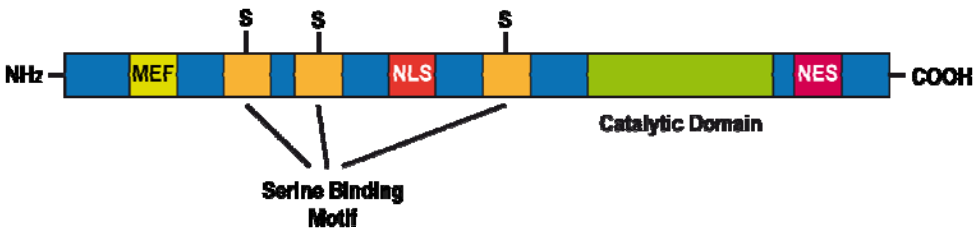

Figure 8i: Class II HDACs domain organization. Class II enzymes possess a conserved deacetylase domain and a long regulatory N-terminal domain which mediates their interactions with specific transcription factors, such as MEF2, CtBP

or HP1, and several serine residues that are subjected to phosphorylation, which in turn determines their sub-cellular localization and thus, their transcriptional repressor activity (Modified from Clocchiatti, 2011).

3.2.1. Regulation of function via MEF2 interaction

In order to exert their repressive function, class IIa HDACs must be in the nucleus. Since class IIa HDACs does not bind directly to the DNA, they interact with sequence-specific DNA binding proteins resulting in the repression of target genes (Martin, 2009). By far, the best characterized transcription factor is MEF2 which interacts with class IIa HDACs via a sequence of 17 amino acids highly conserved in all class IIa members (Lu, 2000; Dequiedt, 2003; Han, 2005) (Figure 8i). MEF2 proteins are a family of transcription factors that are involved in cellular differentiation by controlling the expression of their target genes. They contain a MADS-Box and a MEF2 DNA-binding domains, which mediates dimerization, DNA binding and interaction with specific transcription factors (Shore, 1995; Black, 1998; McKinsey, 2002). The association with other transcription factors is essential to drive the functions of MEF2 and thus the expression of its target genes. MEF2 proteins are expressed in many cell types, including neurons, chondrocytes, muscle cells and lymphocytes, and the balance between the transcription-activating functions of MEF2 and the repressive function of class IIa HDACs are essential to regulate the development and differentiation of these tissues. The interaction between class IIa HDAC and MEF2 is highly dynamic and the association and dissociation of this complex is essential to regulate MEF2 functions. (Arnold, 2007; Chang, 2004; Chang, 2006; Lu, 2000; Verzi, 2007; Youn, 2000). The first association between class IIa HDACs and MEF was described in myocytes, and this interaction resulted in the repression of MEF2 activity (Black, 1998; McKinsey, 2002). The phosphorylation of HDACs by CaMK resulted in the dissociation of HDACs from MEF2 and the activation of MEF2 (McKinsey, 2000; Lu, 2000). During chondrocyte differentiation, the maturation of immature chondrocytes to hypertrophic chondrocytes is controlled by

parathyroid hormone-related peptide (PTHrP), which induces the dephosphorylation of HDAC4 and its translocation to the nucleus, resulting in the repression of MEF2 trancriptional activity (Vega, 2004; Kozhemyakina, 2009). In cancer, there is an increasing interest in targeting the axis between MEF2 and HDACs, in order to decipher the functional relationship between them and use this knowledgemet in the treatment of cancers. In breast cancer, the repressive activity of class IIa HDACs on transcription factors via MEF2 interaction has been related to poor prognosis in these tumors (Clocchiatti, 2013).

3.2.2. Regulation of subcellular localization by phosphorylation

The N-terminal domain of class IIa HDACs also contains several serine residues that are subject to phosphorylation, which is critical for their subcellular localization (McKinsey, 2001; Wang, 2001) (Figure 8i). The phosphorylation status of class IIa HDACs determines whether these deacetylases are localized in the nucleus or in the cytoplasm, and thus, the repression activity in the nuclear compartment (Parra, 2010). In the nucleus, class IIa HDACs specifically interacts and form a complex with the MEF2, the serum response factor (SRF) and the transcription factor Runx2, leading to their recruitment to the DNA and resulting in the repression of the target genes (Clocchiatti, 2011). However, in response to different physiological signals, class IIa HDACs becomes phosphorylated at conserved serines in the N-terminal domain and then they interact with 14-3-3 proteins, resulting in the dissociation of the binding of the specific transcription factors. This dissociation leads to the de-repression of their target genes, while the phosphorylated HDACs are translocated to the cytoplasm where they become inactive. The action of specific phosphatases can lead to the de-phosphorylation of class IIa HDACs and thus, to their nuclear re-localization and repression of their target genes (Martin, 2009; Parra, 2010; Clocchiatti, 2011) (Figure 9i).

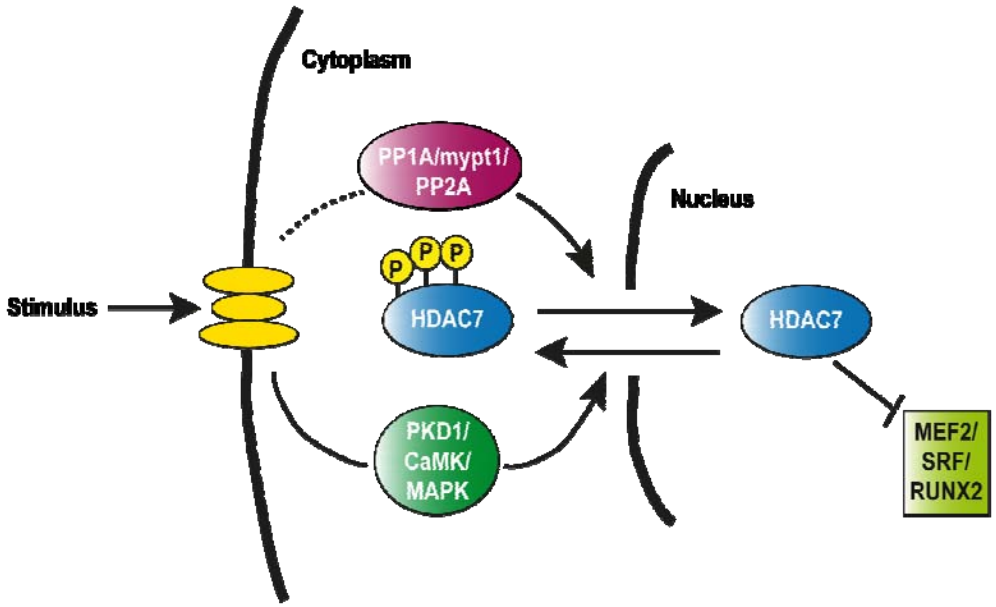

Figure 9i: Regulation of subcellular localization by phosphorylation. Extracellular stimuli can activate the kinases PKD1, CaMK and MAPK leading to the phosphorylation of class IIa HDACs and thus their resulting in their nuclear export. In contrast, the phosphatases PP1A and PP2A can lead to the de-phosphorylation of class IIa enzymes and thereby, to their nuclear re-localization and repression of target genes (Modified from Clocchiatti, 2011).

3.3. Class IIa HDACs catalytic activity

The c-terminal domain of class IIa HDACs shows the highest homology to yeast Hda1 (51-53% similarity) and is highly conserved among the members of this sub-family of HDACs (around 80% sequence similarity). This domain contains 400-450 amino acids (Martin, 2007). However, compared to other members of HDAC family, class IIa enzymes are very inefficient histone deacetylases (Fischle, 2002; Hu, 2000). An explanation for this weak activity of class IIa HDACs could be a substitution of a catalytic tyrosine residue in their active site, which is highly conserved in class I enzymes, for a histidine, leading to a low basal activity on acetylated lysines (Lahm,

2007). Importantly, these findings are consisting with the possibility that class IIa HDACs might no to target acetylated proteins, but their substrates still have not been identified (Lobera, 2013). For example, it is proposed that HDAC9 targets TRIM29 protein, but it has been only identified in engineered over-expression systems (Yuan, 2010). In addition, class IIa HDACs can repress transcription independently of their C-terminal catalytic domain. Indeed, the isolated N-terminal region of class IIa HDACs regulates the repression activity by recruiting specific transcription co-repressors (McKinsey, 2000; Zhang, 2002). Thus, until an endogenous enzymatic substrate is discovered for class IIa HDACs, the most accepted hypothesis is that the catalytic domain, analogous to bromodomains, acts as a domain that recognizes the acetylated lysine residues, which is essential for the protein-histone association and chromatin remodeling (Lobera, 2013).

3.4. Class IIa HDACs in lymphocyte Biology

HDAC7 and HDAC9 appear to play a role at different stages of T cell development. HDAC7 and HDAC9 have been identified as key functional regulators of the function of a subset of T cells; the regulatory T cells (Tregs). Tregs are crucial for maintaining self-tolerance. The transcription factor FOXP3 acts as a repressor of transcription in these cells and is essential in their development and function. Strikingly, HDAC7 and HDAC9 are part of a repressor complex that interacts with FOXP3 and presumably mediates its gene transcriptional repressive activity (Tao, 2007; Li, 2007). Reduced expression of HDAC9 in mice induced FOXP3 expression and this resulted in Treg suppression (de Zoeten, 2010). HDAC7 is the primary class IIa HDAC expressed in thymocytes at the CD4+CD8+ double-positive stage. It is localized in the nucleus of resting thymocytes, where it represses expression of an extensive cassette of genes involved in positive and negative selection. Among these genes, HDAC7 represses the orphan nuclear receptor *Nur77,* which is involved in the negative selection of thymocytes (Kasler, 2007; Dequiedt, 2007; Parra, 2005). In CD4/CD8 double-positive thymocytes, the events that mediate TCR engagement are

regulated by HDAC7. In the absence of HDAC7, thymocytes are not correctly positively selected and present a truncated TCR, resulting in a reduced life span (Kasler, 2011). Moreover, the serine/threonine kinase PKD1 phosphorylates HDAC7 in response to T-cell receptor (TCR) activation. This phosphorylation event leads to the nuclear export of HDAC7, de-repression of *Nur77,* and induction of apoptosis of T cells (Parra, 2005; Dequiedt, 2005). In contrary, the myosin phosphatase PP1B/mypt1 and PP2A dephosphorylates the 14-3-3 binding sites in HDAC7 in order to control its functions as a regulator of T cell apoptosis and endothelial cell functions (Martin, 2008; Parra, 2007). Similarly, in the DT40 B-cell line, PKD1 and PKD3 are required for the signal-dependent phosphorylation and nuclear export of HDAC5 and HDAC7 in response to B cell receptor (BCR) signaling (Matthews, 2006). Recently, the laboratory of Cornelis Murre has applied a ChIP-seq approach and has identified HDAC7 as a target of E2A, EBF and Foxo1 in pro-B cells postulating that it could be an important factor in B cell development (Lin, 2010). Finally, it has been reported that HDAC7 and HDAC9 are highly expressed in childhood acute lymphoblastic leukemia (ALL), with the highest expression levels correlating with poor prognosis (Moreno, 2010). Moreover, by using a PiggyBac transposon mutagenesis screening in mice, HDAC7 has been identified as a target gene in hematopoietic cancers (Rad, 2010). Overall, these studies reveal the importance of the epigenetic regulation exerted by members of the class IIa HDACs sub-family in lymphocyte Biology and represent a valuable resource for investigating their functions and molecular mechanisms in health and disease. This encompasses an increasing interest in the regulation and the functional understanding of HDAC complex stability and activity.

Hypothesis

Through the hematopoietic system, all the distinct mature blood cell types are generated, thereby constituting one of the best-studied paradigms for cell lineage commitment and differentiation in Biology. The processes of B cell lineage specification, commitment and differentiation, which involve the activation of B cell-specific gene expression and repression of alternative lineage determinants, are dependent on the coordinated actions of signaling cascades and transcriptional networks. The complexity of the diverse developmental stages that comprise the hematopoietic system reflects the necessity for tight regulation. Such regulation may take place at the transcriptional level. In fact, every differentiation step is characterized by the activation of a new lineage-specific genetic program and the extinction of the previous one. To date, the central role of lineage-specific transcription factors in positively regulating these distinct developmental steps is well established. However, there is an increasing body of evidence indicating that transcriptional repression is crucial for the proper differentiation of hematopoietic cells, suggesting that transcription factors must recruit transcriptional co-activators but also co-repressors to regulate their target genes.

According to this, our global hypothesis is that **gene transcriptional repression is critical to acquire a particular cell fate in the hematopoietic system.** Transcriptional repressors may be essential for the extinction of lineage inappropriate genes in B lymphocytes. This hypothesis provides a novel conceptual basis for cell lineage commitment and differentiation within the hematopoietic system and creates a whole new paradigm for understanding how this complex developmental system is regulated.

PART II

OBJECTIVES AND METHODOLOGY

Objectives

The main goal of the project is **to investigate the role of the histone deacetylase HDAC7 in B lymphocyte Biology**. To achieve this, we have applyed a unique research concept based on two experimental approaches: First, *in vitro* biochemical-genome wide experimental approach (cell lines and primary cells from mice) by using an immune reprogramming system (Bussmann, 2009), and second, *in vivo* experimental approach (conditional knock-out mouse models).

Specific objectives:

1. Role of HDAC7 during the reprogramming of pre-B cells into macrophages:

1.1. Analysis of class IIa HDACs expression during reprogramming of pre-B cells into macrophages.

1.2. Study of the effect of HDAC7 re-expression in C10 cells and its possible interference with the acquisition of the macrophage gene transcription program during cellular reprogramming.

1.3. Analysis of the physiological function of HDAC7 in pre-B cells.

1.4. Study of the interaction of HDAC7 with particular sequence-specific transcription factors in pre-B cells.

1.5. Analysis of the effects of HDAC7 re-expression on the functional properties of the reprogrammed macrophages.

1.6. Elucidation of the contribution of the interaction of HDAC7 with ME2C and its catalytic activity on the repression of Mac-1.

2. Role of HDAC7 in B cell development :

2.1. HDAC7 expression pattern in the murine hematopoietic system.

2.2. Generation of HDAC7 conditional knockout mice in B cell progenitors.

2.3. Analysis of the hematopoietic cell population profile in the bone marrow of HDAC7 knockout mice.

2.4. Analysis of the hematopoietic cell population profile in peripheral lymphoid organs of HDAC7 knockout mice.

2.5. Gene expression profiling of HDAC7 deficient pro-B cells.

2.6. Molecular mechanisms underlying HDAC7 repression in pro-B lymphocytes.

2.7. Analysis of the responsiveness of HDAC7-deficient pro-B cells *ex-vivo.*

Methodology

1. Role of HDAC7 during the reprogramming of pre-B cells into macrophages

The study of the role of HDAC7 in B lymphocytes was first performed using a cellular reprogramming system that allows the transdifferentiation of pre-B cells into macrophage-like cells. This inducible cellular reprogramming system includes C10 and C11 cell lines (Bussmann, 2009).

1.1. Cell culture and β-estradiol treatment

The pre-B cell line HAFTL was grown at 37°C in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM glutamine, and 50 U/ml streptomycin/penicillin. C10 cells (HAFTL cells transduced with a MSCV-GFP-C/EBPα retroviral vector) and C11 cells (HAFTL cells transduced with a MSCV-hCD4-C/EBPα retroviral vector) were cultured at 37°C in RPMI 1640 without phenol red supplemented with 10% of charcoal treated fetal bovine serum, 2 mM glutamine, and 50 U/ml streptomycin/penicillin. RAW 264.7 macrophages were grown at 37ºC in DMEM supplemented with 10% fetal bovine serum, 2 mM glutamine, and 50 U/ml streptomycin/penicillin. B-cell precursors and macrophages were obtained from mouse bone marrow as described (Di Tullio, 2011). For transdifferentiation induction, cells were treated with 100 nM β-estradiol in the presence of 10 nM of IL-3 and 10 nM of mCSF1 for the indicated periods of time.

1.2. Analysis of Class IIa HDACs expression during the reprogramming of pre-B cells into macrophages

In order to study the potential role of class IIa HDACs during the transdifferentiation of pre-B cells into macrophages we first analyzed their expression levels by RT-qPCR, followed by western-blots analysis.

RT–qPCR experiments

RNA from C10 cells un-induced and induced with β-estradiol for 72 hours, as well as from HAFTL and RAW 264.7 cells, was extracted by Trizol extraction (Qiagen) and cDNA synthesyzed using the High Capacity cDNA Reverse Trancription Kit (AB Applied Biosystems). RT-qPCRs were performed in triplicate using SYBR Green I Master (Roche). PCR reactions were run and analyzed using the LightCycler 480 Detection System (Roche).

Primers

The sequences of the primers used are given below:

Rag1 FW	GACATTCTAGCACTCTGGCCGGG
Rag1 RW	TAAGCTACCTTGCTCCACAGGCC
Pax5 FW	GCCTGGGAGTGAATTTTCTGGA
Pax5 RW	GGGCTGCAGGGCTGTAATAGTAT
C/Ebpβ FW	ACTTCAGCCCCTACCTGGAG
C/Ebpβ RW	GAGGTCGGAGAGGAAGTCGT
Csf1r FW	GCCGTCTCCCTAGGACAAA
Csf1r RW	CAGCAGTAGCACCAGCAGAG
Hdac4 FW	GCCATCTGTGATGCTTCTGA
Hdac4 RW	ATTGGCATTGGGTCTCTGAT
Hdac5 FW	AGCCATGGGATTCTGCTTCT
Hdac5 RW	AGTCCACGATGAGGACCTTG
Hdac7 FW	TGTGGTCAGGGTGCACAG
Hdac7 RW	AGTCTGGACAGGAGGCCAAG
Hdac9 FW	CCCCTATGGGAGATGTTGAG
Hdac9 RW	CAATGCATCAAATCCAGCAG

Western blot analysis

C10 cells were un-induced or induced with β-estradiol for 72 hours. HAFTL and RAW 264.7 cells were used as control. Total cellular extracts were prepared in PLB buffer (0.5% Triton X-100, 0.5 mM EDTA, 1 mM DTT in PBS) supplemented with

protease inhibitors (Complete, Roche Molecular Biochemicals). SDS-PAGE and Western blot experiments were performed by standard procedure.

Antibodies

Anti-HDAC7 (H-273), anti-HDAC7 (C-18), anti-HDAC4 (H-92), anti-E2A (V-18), anti-PAX5 (C-20) and anti-RUNX1 (DW71) were purchased from Santa Cruz Biotechnology. Anti-IKAROS (ab26083) was purchased from Abcam; anti-MEF2C (D80C1) XP from Cell Signaling Technology; and anti-α-Tubulin (T61999), from Sigma-Aldrich.

1.3. Study of the effect of HDAC7 re-expression in C10 cells and its possible interference with the acquisition of the macrophage gene transcription program during cellular reprogramming

To test whether the presence of HDAC7 could interfere with the acquisition of a macrophage-specific gene program during transdifferentiation of pre-B cells into macrophages, we performed a gain of function experimental approach followed by genome-wide microarray analysis.

Plasmids

MSCV-puro-HDAC7 construct was generated by cloning the HDAC7-Flag cDNA PCR amplificated from the pcDNA3-HDAC7 into the MSCV-puro vector (Clontech).

Retroviral supernatant generation and cellular transduction

For retrovirus generation MSCV-puro and MSCV-puro-HDAC7 retroviral vectors were transfected into the packaged cell line Platinum-E and supernatants were collected at 48-72 hours post-transfection. For the generation of C10-MSCV and C10-HDAC7 cells, C10 cells were spin after infected with the collected viral

supernatant and 48 hours after were selected in the presence of 3ug/ml of puromycin.

Western blot analysis

C10-MSCV and C10-HDAC7 cells were un-induced or induced with β-estradiol for 72 hours and total cellular extracts were prepared in PLB buffer. Western blots experiments were performed as described above.

Antibodies

Anti-HDAC7 (H-273) was purchased from Santa Cruz Biotechnology; anti-Flag (M2) and anti-α-Tubulin (T61999), from Sigma-Aldrich.

Microarray experiments

Biological duplicates of C10-MSCV and C10-HDAC7 cells were un-induced or induced to transdifferentiate for 48 and 72 hours (12 samples in total). Total RNA from cultured cells was extracted by Trizol and then purified. PCR amplified RNAs were hybridized against Affymetrix mouse arrays chip (Mouse Genome 430 PM strip) at the IRB Genomics Facility. Affymetrix raw CEL files and processed (normalized) data have been deposited in GEO database under accession number GSE36827.

Microarray data analysis

Affymetrix CEL files were background corrected, normalized using Bioconductor, package 'affy' (version 1.28.1) using 'expresso' algorithm (Gautier, 2004; Kauffmann, 2009). Since the Affymetrix chip version used in this study contains only perfect match (pm) probes, for normalization and acquiring raw probe intensities to expression values we used the following parameters: background correction method 'rma'; normalization method 'constant'; pm correct method 'pmonly'; and summary method 'avgdiff'. Quality of microarray experiment (data not

shown) was verified by Bioconductor package 'arrayQualityMetrics' (version 3.2.4 under Bioconductor version 2.7; R version 2.12.1) (Kauffmann, 2009). To determine genes that are differentially expressed (DE) between two experimental conditions, Bioconductor package Limma was utilized to generate contrast matrices and fit the corresponding linear model (Smyth, 2004). Probe to gene annotation were performed using microarray vendor's annotation data. When more than one probe were annotated to same gene, highest absolute expression value was considered (maximizing). To consider a gene is differentially expressed, besides multiple test corrected, FDR p-value ≤0.05 as cut off, we also applied Log2 fold change (Log2FC) cut off 0.5 for β-estradiol treatment. We used Log2FC cut off 0.5 for genes that are affected by the expression of HDAC7 in β-estradiol treated cells. Expression data on Mef2c deficient multipotent progenitor cells were obtained from GEO database (accession No. GSE13686) (Stehling-Sun, 2009). Data were analyzed using the limma package from Bioconductor. Spots were not background corrected before within array loess normalization. After array normalization using the quantile method log2 ratios (mutant/control) was calculated. To define a gene up-regulated, we used Log2FC ≥1.0.

Functional and pathway enrichment analysis

Functional annotation of differentially expressed genes is based on Gene Ontology (GO) (http://www.geneontology.org) as extracted from EnsEMBL and KEGG pathway database. Accordingly, all genes are classified into three ontology categories (i) biological process (BP), (ii) cellular component, (CC) and molecular function (MF) and KEGG pathways when possible. We have taken only the GO/pathway categories that have at least 10 genes annotated. We used Gitools for enrichment analysis and heatmap generation (www.gitools.org). Resulting p-values were adjusted for multiple testing using the Benjamin and Hochberg's method of False Discovery Rate (FDR).

RT–qPCR experiments

C10-MSCV and C10-HDAC7 cells were un-induced or induced with β-estradiol for 72 hours. RNA was extracted by Trizol extraction (Qiagen) and cDNA synthesyzed using the High Capacity cDNA Reverse Trancription Kit (AB Applied Biosystems). RT-qPCR analyses were performed as described above.

Primers

The sequences of the primers used are given below:

Cxcl10 FW	CCCGAGCCAACCTTCCGGAA
Cxcl10 RW	TTGCAGCGGACCGTCCTTGC
Fcgr1 FW	TAGCCACGGAGGACAGCAGTGT
Fcgr1 RW	TGACTGGGGACCAAGCACTTGGAG
Ifi35 RW	ACGCTGCTGGAGGCTGAGGA
Ifi35 FW	AGAGGACAGTTTGCAGGGTCACA
Ifi47 FW	CTTCTTGGATCTGAGCTTCCCTGGT
Ifi47 RW	TCTGAAGCACCCTTCAGGAAGGC
Tlr3 FW	TCCTTGCGTTGCGAAGTGAAGA
Tlr3 RW	GGAAACACCCCGGGGAGAACTCT
Tlr9 FW	TGCCGCCCAGTTTGTCAGAGG
Tlr9 FW	CCTTCGACGGAGAACCATGTTGGG
Il18 FW	GGCCGACTTCACTGTACAACCGC
Il18 RW	TGGTCTGGGGTTCACTGGCACT
C3 FW	ACTGGAGGCCCACGATGCTCA
C3 RW	GGCTGGAATCTTGATGGAGACGC
Pax5 FW	GCCTGGGAGTGAATTTTCTGGA
Pax5 RW	GGGCTGCAGGGCTGTAATAGTAT

1.4. Analysis of the physiological function of HDAC7 in pre-B cells

In order to analyze the physiological function of HDAC7 in pre-B cells, we performed a loss-of-function experimental approach, by using small interference RNA (siRNA) to knock down HDAC7 in both HAFTL B cell line and primary B cells.

siRNA depletion

Dharmacon siRNA control and on-target smartpools targeting transcript of the mouse HDAC7 gene were used to knockdown its expression in HAFTL cells and primary B cell precursors. siRNA were transfected using Lipofectamine RNAiMAX Transfection Reagent (Invitrogen).

RT-qPCR

mRNA levels were examined 72 hours after siRNA transfection by RT-qPCR.

Primers

The sequences of the primers used were the following:

Hdac7 FW	TGTGGTCAGGGTGCACAG
Hdac7 RW	AGTCTGGACAGGAGGCCAAG
Itgam FW	GTGTCCCTTGCCTCGAGGGCAGA
Itgam RW	AGAGCTTCACACTGCCACCGTGC
Fcgr1 FW	TAGCCACGGAGGACAGCAGTGT
Fcgr1 RW	TGACTGGGGACCAAGCACTTGGAG
Ccl3 FW	ACAGCCGGAAGATTCCACGCC
Ccl3 RW	TCAGGAAAATGACACCTGGCTGGG

1.5. Study of the interaction of HDAC7 with sequence-specific transcription factors in pre-B cells

To determine whether HDAC7 interacts with particular sequence-specific transcription factors in pre-B cells, we performed co-immunoprecipitation experiments. Next, we performed chromatin-immunoprecipitation (ChIP) experiments in order to investigate if HDAC7 is recruited to MEF2 binding sites located at the promoters of macrophage genes.

Co-immunoprecipitation and Western blot analysis

Total cellular extracts were prepared in PLB buffer (0.5% Triton X-100, 0.5 mM EDTA, 1 mM DTT in PBS) supplemented with protease inhibitors (Complete, Roche Molecular Biochemicals). Immunoprecipitation with specific antibody for HDAC7, SDS-PAGE and Western blot experiments were performed as described above.

Chomatin immunoprecipitations assays (ChIP)

C10 cells un-induced or induced for 72 hours were fixed by adding formaldehyde to a final concentration of 1% for 30 min at RT. The reaction was quenched with 125 mM glycine, cells washed with buffer B (0.25% Triton-X 100, 1 mM EDTA, 0.5 mM EGTA, 20 mM Hepes, pH 7.6), buffer C (150 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 20 mM Hepes, pH 7.6), and resuspended in ChIP incubation buffer (0.3% SDS, 1% Triton-X 100, 0.15 M NaCl, 1 mM EDTA, 0.5 mM EGTA, 20 mM Hepes, pH 7.6). To shear chromatin to an apparent length of ~500 bp, chromatin was sonicated using a BioRuptor sonicator (Cosmo Bio Co., Ltd) with either 40 45-s pulses (uninduced cells) or 30 45-s pulses (induced cells) at maximum setting. More than 20 million cells were used per IP, and 5 µg of HDAC7 or MEF2C antibodies was incubated with the chromatin and BSA-blocked protein G beads overnight at 4°C. IPs were washed twice with each buffer 1 (0.1% SDS, 0.1% deoxycholate, 1% Triton-X 100, 150 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 20 mM Hepes pH 7.6), buffer 2 (0.1% SDS, 0.1% deoxycholate, 1% Triton-X 100, 0.5 M NaCl, 1 mM EDTA, 0.5 mM EGTA, 20 mM Hepes pH 7.6), buffer 3 (250 mM LiCl, 0.5% deoxycholate, 0.5% NP-40, 1 mM EDTA, 0.5 mM EGTA, 20 mM Hepes, pH 7.6), and buffer 4 (1 mM EDTA, 0.5 mM EGTA, 20 mM Hepes, pH 7.6). Immunoprecipitated complexes were eluted in elution buffer (1% SDS, 0.1 M $NaHCO_3$) for 20 min at RT, and decrosslinked overnight at 65°C in presence of 200 mM $NaCl_2$. DNA was phenol:chloroform extracted, chroloform:isoamyl alcohol extracted, ethanol precipitated, and resuspended in 100

ml H_2O by shaking at 37°C. Input and immunoprecipitated DNA (5 µl) were subjected to qPCR with specific primers.

qPCR

qPCR experiments were performed as described above.

Antibodies

Anti-HDAC7 (H-273) was purchased from Santa Cruz Biotechnology and anti-MEF2C (D80C1) XP from Cell Signaling Technology.

Primers

The sequences of the primers used in the ChIP experiments are the following:

Cxcl10

PCR Amplicon

1 F: GCTCACAGTATTGGTTAGGCTCAG
R: AAGGAACGAAGAAGGGAAGAAAGG
2 F: GATTTCTTCCTGCTTCCCTCTTC
R: TCCTGCTGGGTCTGAGTGG
3 F: ACTCAGACCCAGCAGGATG
R: TCAGCCAATCAGGACTCAGG
4 F: AACAGTTAGAGCAGGCATTCAG
R: ACAAAGAAGACAATCAAAGCATCC
5 F: TTCAAAAGACTGGCAGGAACC
R: GCTTTCATAAGGCTCATAGCATAG
6 F: TCCAATGTCCTATGTCAGCAAC
R: TGGCAGGGAGTGAAGAGC

Itgam

PCR Amplicon

1 F: ATCTGGGCATTTAGGCTTCC

R: AACTCACTCTGTAGACAAGGC
2 F: TTACTGCTGTATGACTGTGACC
R: GGCTCCATTTCCCGTTTCTC
3 F: GCTGGGGAGGTTGAGGCAGGAG
R: GGTCAGGTCCTCAGGCTTGGTG
4 F: CACTATGGCTAGACGCCGATGG
R: GCTGACCACCTGTGTCCAATCC
5 F: CGGAGGAAGGCTACAGAGG
R: GCACCACAGACACTTTGAGG

Fcgr1

PCR Amplicon

1 F: CGTAGAAGGAGAAGTGAAGC
R: AACAGTGACAGAGTGAATACC
2 F: GGTATTCACTCTGTCACTGTTC
R: CTGAGGCTCTATGGCTTCG
3 F: GAACCGTGCTGGATACTTG
R: TAGGATTATAGTCTTGTGCTACC
4 F: TAGGATTATAGTCTTGTGCTACC
R: GAACCGTGCTGGATACTTG
5 F: ACCCTGACCTGTAAGCATTTC
R: CTGGATGAGTGTGGTGAAGG

Venn Diagrams

In order to find a potential overlap between the set of genes whose up-regulation was impaired in the presence of HDAC7 during the conversion of pre-B cells into macrophages with the set of genes repressed by MEF2C in LMPPs reported in Stheling-Sun, 2009 we applied a Venn diagram analysis and a Chi-square test to analyse the significance of the overlap.

1.6. Analysis of the effects of HDAC7 re-expression on the functional properties of the reprogrammed macrophages

In order to address the potential effects of HDAC7 re-expression on the functional characteristics of the converted cells, we undertook different experimental approaches. We first analyzed the expression kinetics of CD19 and Mac1 during the cellular reprogramming, followed by phagocytosis and immune response assays.

Flow cytometry

C10-MSCV and C10-HDAC7 cells were un-induced or induced to transdifferentiate. 48 hours later, cells were stained with fluorochrome conjugated antibodies against Mac-1 and CD19 (BD Pharmingen). Mac-1 and CD19 expression were monitored on a Gallios Flow Cytometer (Beckman Coulter) and analyzed by FlowJo software (Tree Star, Inc.).

Phagocytosis assays

C10-MSCV and C10-HDAC7 cells were induced or not for 48 h and subjected to the pHrodo *E.coli* phagocytosis KiT (Invitrogen) following the manufacturers protocol.

LPS induced inflammatory cytokines

C10-MSCV and C10-HDAC7 cells were un-induced or induced to transdifferentiate. 48 h after induction the cells were incubated or not with LPS (1 µg/mL Sigma) for 6 hours. RNA extraction, cDNA synthesis and RT-qPCR were performed as described above. Primers sequences upon request.

Quantification of pro-inflamatory genes

The sequences of the pro-inflamatory genes analyzed in this assay were the following:

*Tnfalpha*_RT_F	CGCTCTTCTGTCTACTGAACTT
*Tnfalpha*_RT_R	GATGAGAGGGAGGCCATT
*Il1b*_RT_F	CCAAAATACCTGTGGCCTTGG
*Il1b*_RT_R	GCTTGTGCTCTGCTTGTGAG
*Il6*_RT_F	GAGGATACCACTCCCAACAGACC
*Il6*_RT_R	AAGTGCATCATCGTTGTTCATACA

1.7. Elucidation of the contribution of the interaction of HDAC7 with ME2C and its catalytic activity on the repression of Mac-1

In order to elucidate the molecular mechanisms behind HDAC7 transcriptional repression in pre-B cells, we generated retroviral vectors for expression of different HDAC7 mutants, targeting either its MEF2 binding site or the catalytic domain.

Plasmids

MSCV-GFP-HDAC7, MSCV-GFP-HDAC7(ΔMEF), MSCV-GFP-HDAC7(K86A/K88A), MSCV-GFP-HDAC7(H657A), MSCV-GFP-HDAC7(1–487) and MSCV-GFP-HDAC7(438–915) construct were generated by cloning the HDAC7-Flag cDNAs PCR amplified from the pCDNA3-HDAC7 wild-type and mutant plasmids into the MSCV-GFP vectors (Clontech).

Retroviral supernatant generation and cellular transduction

For retrovirus generation the MSCV-GFP, MSCV-GFP-HDAC7, MSCV-GFP-HDAC7(ΔMEF), MSCV-GFP-HDAC7(K86A/K88A), MSCV-GFP-HDAC7(H657A), MSCV-GFP-HDAC7(1–487) and MSCV-GFP-HDAC7(438–915) plasmids were transfected into

the packaged cell line Platinum-E and supernatant was collected at 48–72 hours post-transfection. C11 cells were spin infected with MSCV-GFP, MSCV-GFP-HDAC7, MSCV-GFP-HDAC7(ΔMEF), MSCV-GFP-HDAC7(K86A/K88A), MSCV-GFP-HDAC7(H657A), MSCV-GFP-HDAC7(1–487) and MSCV-GFP-HDAC7(438–915) and 48 hours after treated with β-estradiol and analyzed the expression of Mac-1 by flow cytometry, as described before.

2. Role of HDAC7 in B lymphocyte development

To definitive demonstrate the role of HDAC7 in B lymphocyte Biology, we evaluated its function *in vivo,* in whole organisms. To do so, we have generated conditional HDAC7 knockout mouse models for specific deletion of HDAC7 in B cell progenitors.

Mice and animal care

$HDAC7^{loxP/ko}$ (Chang, 2006) mice were kindly provided by Dr. Eric N. Olson (University of Texas Southwestern, USA), and $Mb1\text{-}Cre^{ki/+}$ (Hobeika, 2006) mice were obtained from Dr. Michael Reth (Max Palnck Institute of Immunology and Epigenetics, Freiburg, Germany). Animal housing and handling, and all procedures involving mice, were approved by the Bellvitge Biomedical Research Institute (IDIBELL) ethics committee, in accordance with Spanish national guidelines and regulations.

DNA extraction from mice punch

To isolate genomic DNA from mice punch, we added 300uL of Punch buffer (10mM NaOH; 1mM EDTA) and heated 11min at 99ºC. Once finished, we spin down the samples at 12000rpm.

Genotyping PCR

Using the DNA extracted, the genotyping PCRs conditions were the following:

Mb1-Cre$^{ki/+}$:

Mix per Reaction (ul): ***Mb1-Cre***		Final Concentration	Conditions		
2,5	Buffer 10X	1X	95ºC	15'	
2,5	dNTPs (2mM)	0,2mM	94ºC	30''	
1	MgCl2 (50mM)	2mM	60ºC	30''	38 Cycles
2,5	DMSO	10%	72ºC	1'	
1	Primer F (5uM)	0,2uM	72ºC	10'	
1	Primer R (5uM)	0,2uM			
0,2	Taq DNA pol				
12,3	H20				

Sample per Reaction (ul): 2

WT band 500bp
KI band 530bp

HDAC7$^{LOXP/KO}$:

Mix per Reaction (ul): ***HDAC7/loxP***		Final Concentration	Conditions		
2,5	Buffer 10X	1X	95ºC	15'	
2,5	dNTPs (2mM)	0,2mM	95ºC	30''	
0,75	MgCl2 (50mM)	1,5mM	60ºC	30''	37 Cycles
2,5	DMSO	10%	72ºC	45''	
0,45	Primer SA5' (10uM)	0,18uM	72ºC	5'	
0,55	Primer LacZ3' (10uM)	0,22uM			
1	Primer SA3' (10uM)	0,4uM			
0,2	Taq DNA pol				
12,55	H20				

Sample per Reaction (ul): 2

WT band 400bp
Floxed 475bp

Null band 640bp

2.1. Generation of HDAC7 conditional knockout mice in B cell progenitors

Since HDAC7-deficient mice are lethal at a very early embryonic stage (Chang, 2006) we have worked with conditional knockout mice to specifically delete its expression in the hematopoietic system. In collaboration with the group of Dr. Eric N. Olson (UT Southwestern), we obtained mice with exons 2-10, encoding the entire N-terminal regulatory domain of the HDAC7 gene, flanked by loxP sites, which are recognized by the Cre recombinase (*HDAC7*$^{+/loxP}$).

Experimental design

HDAC7$^{loxp/ko}$ mice were crossed with the transgenic mouse line, *mb1*-Cre$^{ki/+}$ to delete HDAC7 expression in B cell progenitors, since mb1 is an early promoter expressed in pro-B cells. *HDAC7*$^{loxP/ko}$ and *mb1*-Cre$^{ki/+}$ mice were first crossed to generate *HDAC7*$^{+/loxP}$*;mb1-Cre*$^{ki/+}$ and *HDAC7*$^{+/ko}$*;mb1-Cre*$^{ki/+}$ mice, which were then crossed to obtain *HDAC7*$^{loxp/ko}$*;mb1-Cre*$^{ki/+}$ knockout mice and their *HDAC7*$^{+/ko}$*;mb1-Cre*$^{ki/+}$ littermate control mice.

2.2. Analysis of the hematopoietic cell population profile in the bone marrow of the HDAC7 knock out mice

The hematopoietic cell population profile in *HDAC7*$^{loxp/ko}$*;mb1-Cre*$^{ki/+}$ mutant mice was determined by staining bone marrow cells with a combination of antibodies recognizing specific surface markers to distinguish the different cell types. Cells from *HDAC7*$^{+/ko}$*;mb1-Cre*$^{ki/+}$ littermate mice were used as control. Cells were analyzed by flow cytometry on a Gallios flow cytometer (Gallios, Beckman-Coulter) and the data analyzed with FlowJo software.

Bone marrow extraction

- The clean bones are crushed with mortar and pestle in dissection medium. An efficient way to do it is to crush tibias, femurs and hips of each mouse, then 2 spines at a time. Use Labeling Buffer: PBS + 4% serum + 2mM EDTA (240ml PBS 1x, 10ml FBS, 1ml 0.5M EDTA).
- It is usually advisable to crush the leg bones twice to obtain white (with no bone marrow) bone fragments and spine fragments 2 or 3 times.
- The cell mixture obtained from each mouse is kept separate and filtered through a 40µm filter into a 50ml falcon tube.
- Fill up the tubes to have them balanced and spin 5min at 300g.
- Aspirate the supernatant and loosen the pellet by dragging the tubes on the tube rack (this step is important after each centrifugation to minimize clump formation and cell loss).
- Resuspend in ≈20ml ACK lysing buffer (8.3g NH_4Cl, 1g $KHCO_3$ or 0.7g K_2CO_3, 250µl EDTA 0.5M. Fill up till 1L H_2O and adjust the pH at 7.2). Spin 5min at 300g. Resuspend in ≈20ml Labeling buffer.
- Count the cells using Turk staining.

Cell staining

- Centrifuge 10min at 300g and resuspend in Labeling Buffer (10^7 cells/ml).
- Add Fc Block (0.1µg/10^6cells) and incubate for 10min dark ice.
- Spin 1200rpm 5min.
- Add 100µl PBA (0.1% BSA, 0.02% Sodium Azide in PBS) + staining antibodies.
- 30min dark ice.
- Spin 1200rpm 5min.
- Wash PBS.
- Spin 1200rpm 5min.
- Add 500µl PBA or 4% PFA in PBS.

The different hematopoietic cell populations analyzed were the following:

Pro-B cells ($B220^{+}CD43^{+}IgM^{-}$)

Pre-B cells ($B220^{+}CD43^{-}IgM^{-}$)

Immature B cells ($B220^{+}CD43^{-}IgM^{+}IgD^{-}$)

Mature recirculating B cells ($B220^{+}CD43^{-}IgM^{+}IgD^{+}$)

Granulocytes ($Gr1^{+}CD11b^{+}$)

Macrophages ($Gr1^{-}CD11b^{+}$)

Erythrocytes ($Ter119^{+}$)

2.3. Analysis of the hematopoietic cell population profile in peripheral lymphoid organs of HDAC7 knockout mice

To investigate whether HDAC7 is required at later developmental stages in both B and T lineages, we analyzed the hematopoietic cell population profile in peripheral organs, such as the spleen, thymus and also the peripheral blood from *HDAC7*$^{loxp/ko}$*;mb1-Cre*$^{ki/+}$ mice and *HDAC7*$^{+/ko}$*;mb1-Cre*$^{ki/+}$ control mice. Cells were analyzed by flow cytometry and the data analyzed with FlowJo software.

Spleen and thymus extraction

- Place the spleen or the thymus in a 6-well plate in 1ml PBS and prick it several times with a needle.
- Push slowly with the piston of a syringe.
- Transfer the supernatant to a 50ml falcon and filter the cells in a 40µm strainer.
- Use clean PBS again and repeat the last two steps until the capsule is clean.
- Fill up the tube up to 10ml PBS.
- Spin 5min at 400g.
- Resuspend in ≈10ml ACK buffer.
- Spin 5min at 400g.
- Resuspend in ≈10ml Labeling buffer.

Count the cells using Turk staining.

Peripheral blood extraction

Peripheral blood from HDAC7$^{loxp/ko}$;*mb1*-Cre$^{ki/+}$ mice and HDAC7$^{+/ko}$;*mb1*-Cre$^{ki/+}$ control mice was extracted from the tail. A little cut was done at the end of the tail and the blood recollected drop by drop in tubes containing EDTA. Once extracted, we continued with the ACK protocol described before.

Cell staining

Cells were stained with specific antibodies as described above and the different hematopoietic cell populations analyzed were the following:

Immature B cells (B220^{+}CD43^{-}IgM^{+}IgD^{-})
Marginal zone (MZ) B cells (B220^{+}CD21^{+}CD23^{Low}CD93^{-})
Follicular (FO) B cells (B220^{+}CD21^{Low}CD23^{+}CD93^{-})
Transitional (T) B cells (B220^{+}CD21^{Low}CD23^{+}CD93^{+})
Double positive (DP) T cells (CD4^{+}CD8^{+})
Single positive CD4 (SP4) T cells (CD4^{+}CD8^{-})
Single positive CD8 (SP8) T cells (CD4^{-}CD8^{+})
Granulocytes (Gr1^{+}CD11b^{+})
Macrophages (Gr1^{-}CD11b^{+})

2.4. Immunohistochemistry of spleens from HDAC7 mutant mice.

Spleens from *HDAC7$^{loxp/ko}$;mb1-Cre$^{ki/+}$* mice and *HDAC7$^{+/ko}$;mb1-Cre$^{ki/+}$* control mice were fixed in 4% formaldehyde overnight at 4ºC, embedded in paraffin wax and sectioned at 4µm. For immunofluorescence staining, antigen retrieval was performed in 10mM sodium citrate (pH 6.0). Spleen sections were blocked with 5% horse serum in phosphate-buffered saline for 1 hour at room temperature and incubated with primary antibodies overnight at 4ºC. The primary antibodies used

was anti-F4/80 and anti-CD3. Spleen sections were then incubated with secondary antibodies for 1 hour at room temperature. Samples were imaged on a Leica TCS SP5 spectral confocal microscope (Leica).

2.5. Gene expression profiling of HDAC7 deficient pro-B cells

We performed microarray experiments to determine whether the gene expression profile was altered in the absence of HDAC7. We purified pro-B cells from *HDAC7$^{loxp/ko}$;mb1-Cre$^{ki/+}$* mice and *HDAC7$^{+/ko}$;mb1-Cre$^{ki/+}$* control mice by cell sorting.

RNA extraction

RNA was extracted from purified pro-B cells with Trizol (Invitrogen) and hybridized against Affymetrix 430.2 mouse arrays at the Functional Genomics Core Facility (FGC) in the Institute for Research in Biomedicine (IRB) in Barcelona. Data analysis was performed as described in section 1.3.

Microarray validation by RT-qPCR

The microarray data was validated in a large number of selected genes by RT-qPCR described in section 1.2. The sequences of the primers used were the following:

Klf4-F	AGAACAGCCACCCACACTTG
Klf4-R	GTGGTAAGGTTTCTCGCCTGT
Jund FW	TCCGAGTAGGGGCTCTAAGG
Jund RW	AATAGGGCGGAATCGGACAC
Cebpz FW	CGTCCCAAACAGGTGACAGA
Cebpz RW	AATCTTGCTTTGTGCCTCCG
Malt1 FW	GCCAAGGCTCATGAACTTCC
Malt1 RW	TGGAGAACTCTGCGGCAAAT
Sykb FW	CCCCGAAGCAAAACAACGTC
Sykb RW	AGTGTATGGAGAAGTACCGCAT
CD69 FW	ACAACCAAGAGTTGGGCCTT
CD69 RW	CGCTTCAGAAACGTCATGTCC

Map3k8 FW	GAATGGCGTGCAAACTGATCC
Map3k8 RW	CTCGCCGGCTTCCATAAAGA
Nfkbid FW	AGACTTCGAAGGCCTTACCC
Nfkbid RW	CACACACTAGCTGGGAGCAT
Usp7 FW	AAGGGGATGGCAAATGGTGT
Usp7 RW	GGCATTTGTGCAGTGTCGAA
Junb FW	CAGGCGCATCTCTGAAGCTA
Junb RW	TTGCTGTTGGGGACGATCAA
Nfkbiz FW	GACGGAGATGGTGACACGTT
Nfkbiz RW	CCACCTGAAAGGCACTCTGT
Crebzf FW	TCCCCTTGTTCAGAGTTGCG
Crebzf RW	TCATCCAAGGCCATGTCCAC
Fosb FW	CCCGAGAAGAGACACTTACCC
Fosb RW	TTCCGCCTGAAGTCGATCTG
Egr1 FW	TGAGCACCTGACCACAGAGTC
Egr1 RW	GAAGCGGCCAGTATAGGTGA
TET2 FW	GCTGCCCTGTAGGATTTGTT
TET2 RW	GGGCAAGCTGCTGAATGTAT
C/Ebpβ FW	ACTTCAGCCCCTACCTGGAG
C/Ebpβ RW	GAGGTCGGAGAGGAAGTCGT
CD28 FW	GGAGGCCTGGGCTCACTCGA
CD28 RW	CAGGGGCGGTACGCTGCAAA
Mdm2 FW	AAGTCAGCAAGACTCTGGCAC
Mdm2 RW	TTCTCTTCTGGTGGCGCTTG

Venn Diagrams

In order to find a potential overlap between the genes up-regulated in HDAC7-deficient pro-B cells with the genes whose up-regulation was impaired in the presence of HDAC7 during the conversion of pre-B cells into macrophages we applied a Venn diagram analysis and a Chi-square test to test the significance of the overlap.

2.6. Recruitement of HDAC7 to lineage inappropriate genes in pro-B cells

In order to determine whether HDAC7 is recruited to the promoters of lineage inappropraite genes in pro-B cells we performed ChIP experiments using pro-B cell from wild type mice. We first extracted hematopoietic cells from the bone marrow using the protocol described before, and then purified pro-B cells using Miltenyi microbeads specific for B220 (CD45R) and CD43. We performed ChIP experiments as described in section 1.5.

Purification by anti-PE multisort kit

- Count cells.
- Spin 300g 10min.
- Resuspension: 90µL Buffer/ 10µL B220 antibody.
- Mix and incubate 20min at 4ºC
- Resuspension: 90µL Buffer/ 10µL anti-PE microbeads/ 10^7 cells.
- Mix and incubate 15min at 4ºC
- Wash with 1-2ml Buffer/ 10^7 cells.
- Spin 300g 10min.
- Resuspension: 500µL Buffer/ 10^8 cells.

Magnetic separation (LS columns)

- Rinse with 3ml buffer.
- Apply cell suspension onto the column.
- Collect unlabeled cells with 3x3ml buffer.
- Remove column and add 5ml buffer and flush out.

Removal of multisort microbeads using multisort release reagent

- Count cells.
- Add 20µL release reagent/ 1ml suspension.

- Mix and incubate 10min at 4ºC.
- Wash with 1-2ml buffer/ 10^7 cells.
- Spin 300g 10min.
- Resuspension: 50µL Buffer/ 10^7 cells.
- Add 30µL multisort stop reagent/ 10^7 cells.
- Add until 100uL buffer.
- Mix and incubate 15min at 4ºC.

Magnetic labeling with CD43 microbeads

- Count cells.
- Spin 300g 10min.
- Resuspension: 90µL Buffer/ 10µL CD43 microbeads/ 10^7 cells.
- Mix and incubate 15min at 4ºC.
- Wash with 1-2ml buffer/ 10^7 cells.
- Spin 300g 10min.
- Resuspension: 500µL Buffer/ 10^8 cells.

Magnetic Separation (LS columns)

- Rinse with 3ml buffer.
- Apply cell suspension onto the column.
- Collect unlabeled cells with 3x3ml buffer. Collect total effluent (CD45R+CD43-).
- Remove column and add 5ml buffer and flush out (CD45R+CD43+).

2.7. Molecular mechanisms underlying HDAC7 repression in pro-B lymphocytes

Finally, we performed experiments to analyze the response of HDAC7-deficient cells when they are purified and cultured *ex-vivo.* To do so, we first extracted the hematopoietic cells from the bone marrow of *HDAC7$^{loxp/ko}$;mb1-Cre$^{ki/+}$*

mice and *HDAC7*$^{+/ko}$*;mb1-Cre*$^{ki/+}$ control mice and purified pro-B cells by the Miltenyi protocol described above. These pro-B cells were culture in S17 stromal cells and the specific cytokines (IL-7, SCF, Flt3, mCsf-1 and IL-3). We then analyzed the cells by their ability to proliferate, as well as the expression of *IL-7r* and *Mac-1*.

Culture in S17 stroma

S17 cells were grown at 37°C in DMEM supplemented with 20% fetal bovine serum, 2mM glutamine, and 50 U/ml streptomycin/penicillin. Purified pro-B cells were grown on S17 stromal cells at 37°C in αIMIM supplemented with 20% fetal bovine serum, 2 mM glutamine, and 50 U/ml streptomycin/penicillin.

Mitomycin-C treatment

In order to stop the cell cycle of S17 cells, we treated the cells as following:

- Add 10µL Mitomycin-C (1mg/ml)/ 500µL DMEM.
- 2h at 37ºC.
- 2x Washes with PBS.
- 1ml of pro-B cells in αIMIM.

MTT assay

- Add 50µL MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)).
- 24h at 37ºC.
- Spin cells 4000rpm 1min.
- Resuspension in 100µL DMSO (dimetilsulfóxido).
- Absorbance at 560nm.

PART III

RESULTS

1. Role of HDAC7 during the reprogramming of pre-B cells into macrophages

In order to investigate the potential role of class IIa HDACs in B lymphocytes, we have taken advantage of a cellular reprogramming system consisting of pre-B-cell lines modified to express a β-estradiol-inducible form of the myeloid gene C/EBPα, which enables the conversion of pre-B cells into macrophage-like cells with 100% efficiency (Figure 1R) (Bussmann, 2009). These pre-B cell lines were named C10 and C11 and consisted of pre-B cells (HAFTL cell line) transduced with either an MSCV-C/EBPα-GFP retroviral vector (C10 cells) or an MSCV-C/EBPα-hCD4 retroviral vector (C11 cells). Using this cellular reprogramming system we have demonstrated that during the conversion of pre-B cells into macrophages, HDAC7 expression is dramatically down-regulated and that this down-regulation is crucial for the proper reprogramming of pre-B cells into macrophages. Moreover, reintroduction of HDAC7 interferes with the acquisition of the macrophage transcriptional program. In pre-B cells, HDAC7 specifically interacts with the transcription factor MEF2C and is recruited at the promoters of key macrophage genes (Barneda-Zahonero, 2013).

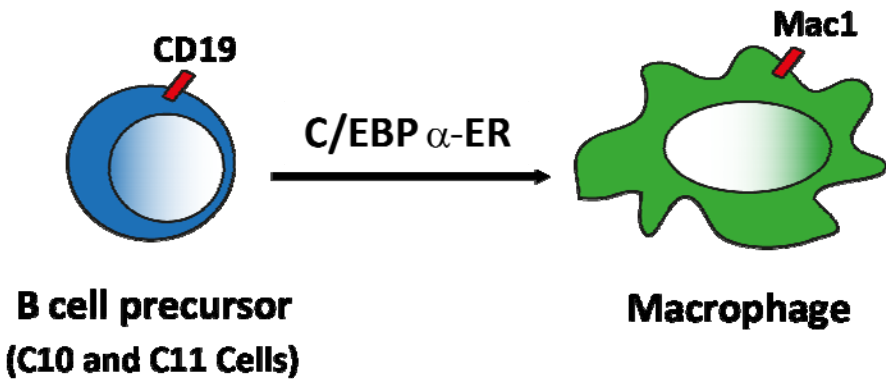

Figure 1R: Model for reprogramming B cells into macrophage-like cells. Cellular reprogramming system consisting of a B-cell line modified (C10 and C11 cells) to

express an estradiol-inducible form of the myeloid gene C/EBPα, allowing the conversion of B cells into macrophage-like cells with 100% efficiency (Bussmann, 2009).

1.1. Analysis of class IIa HDAC expression during reprogramming of pre-B cells into macrophages

First, we used RT-qPCR to analyze the expression of the four class IIa HDAC members (HDAC4, 5, 7 and 9) during the reprogramming of pre-B cells into macrophages. We observed that HDAC7 was dramatically down-regulated during the conversion of pre-B cells into macrophages after 72 hours of β-estradiol treatment (Figure 2R). In contrast, HDAC4, 5 and 9 underwent no changes in their expression levels. In agreement with a previous report (Bussmann, 2009), lymphoid-specific genes such as *Rag1* and *Pax5* became down-regulated during cellular reprogramming, whereas genes like *c/Ebpβ* and *Csfr1*, which are characteristic of myeloid cells, became up-regulated.

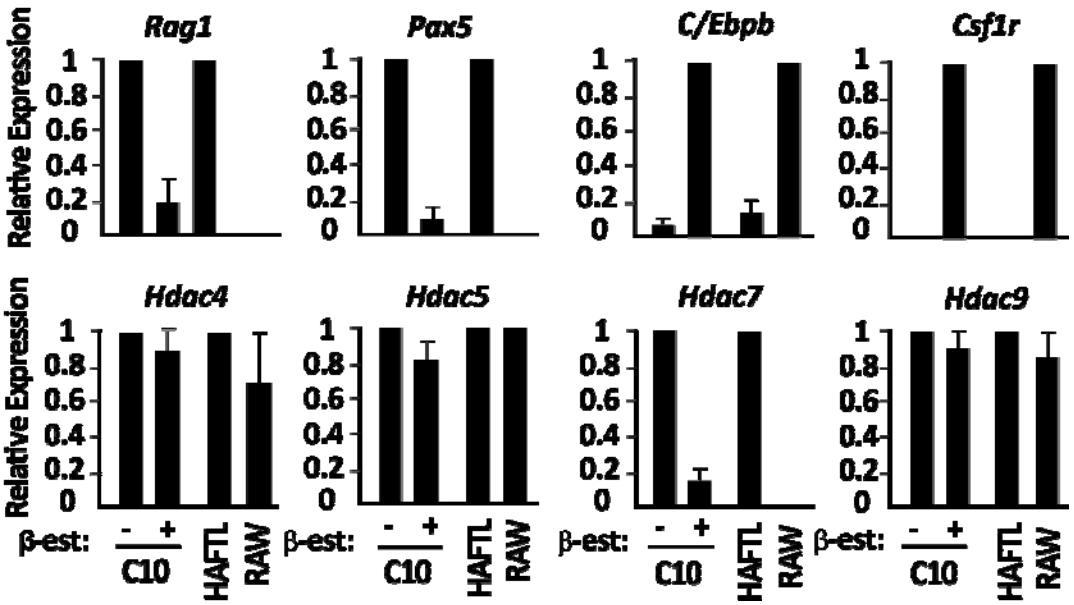

Figure 2R: HDAC7 expression is down-regulated during the transdifferentiation of pre-B cells into macrophages. RT-qPCR experiments for gene expression changes of class IIa HDACs, B cell and macrophages genes (uninduced cells (-) and β-estradiol induced C10 cells (+) for 72 hours).

The down-regulation of HDAC7 was confirmed at the protein level by western blotting under the same experimental conditions. As expected, we found that the protein levels of IKAROS, E2A and PAX5, which are important B cell transcription factors, became down-regulated during cellular transdifferentiation. In contrast, the protein levels of the transcription factors MEF2C and RUNX1 did not change during the conversion of pre-B cell into macrophages (Figure 3R). Neither HDAC7 mRNA nor the corresponding protein was detected in RAW 264.7 macrophages (Figures 2R and 3R).

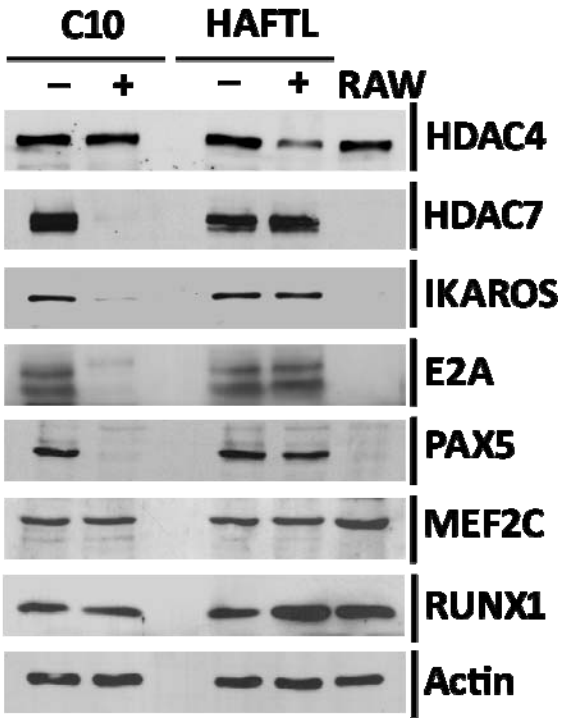

Figure 3R: HDAC7 and B cell transcription factors are down-regulated during transdifferentiation of pre-B cells into macrophages. Western blot of class IIa HDACs and B cell transcription factors during the reprogramming of B cells into macrophages (uninduced cells (-) and β-estradiol-induced C10 cells (+) for 72 hours).

To confirm our results, we analyzed HDAC7 expression during the reprogramming of pre-B cells into macrophages in previously reported microarray experiments (Bussmann, 2009). We observed that the HDAC7 mRNA expression level indeed diminished during cellular conversion in a similar way as observed with the transcription factor PAX5 (Figure 4R).

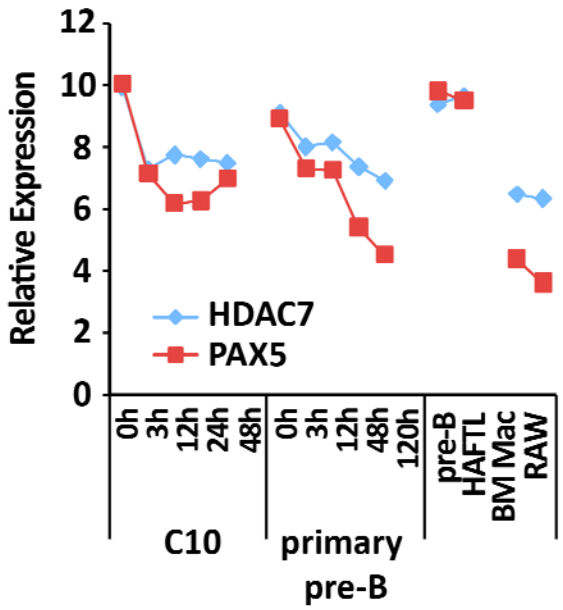

Figure 4R: HDAC7 is down-regulated during transdifferentiation of pre-B cells into macrophages. Kinetics of regulation (log_2 Affymetrix expression values) of Hdac7 and Pax5 genes in C10 cells and primary pre-B cells transduced with a retroviral vector to induce expression of C/EBPα. Cells were treated with β-estradiol for the times indicated. HAFTL cells, primary pre-B cells, primary bone marrow macrophages and RAW264.7 cells were used as a control.

Since HAFTL cells are a fetal pre-B cell line transformed by the Ha-ras virus, we next wanted to confirm the relevance of our findings and extend our analysis to primary cells. We took advantage of a recently reported microarray analysis (Di Tullio, 2011) and examined the expression of HDAC7 in primary pre-B cells and primary macrophages. We observed that, similarly to PAX5, HDAC7 was more highly expressed in B cell precursors than in primary macrophages (Figure 5R). Consistent with our results from the C10 cell line, HDAC7 was down-regulated during the transdifferentiation of primary pre-B cells into macrophages (Figure 5R). Together, these results indicate that, of the members of the class IIa HDAC sub-family, HDAC7 shows a lymphoid-lineage specific expression pattern within the hematopietic system. Our findings also suggest that HDAC7 might be crucial for maintaining B cell functions and identity by repressing lineage-inappropriate genes in pre-B cells, such as genes charcteristic of myeloid cells.

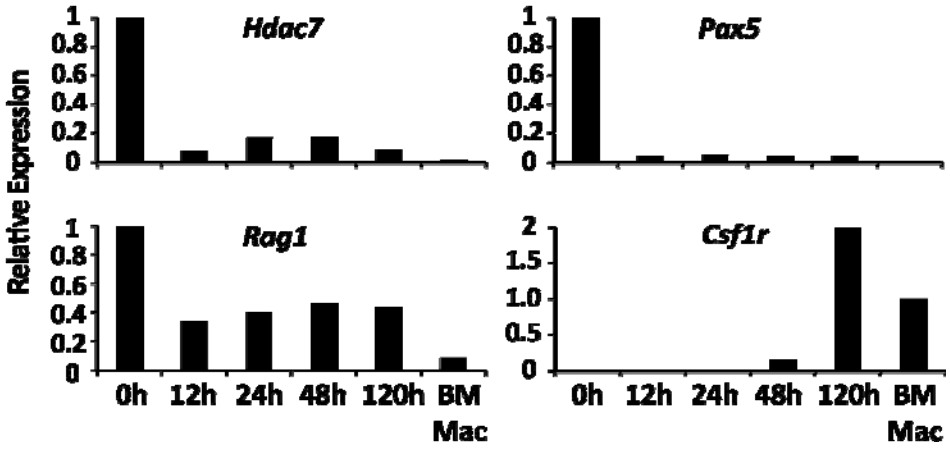

Figure 5R: RT-qPCR experiments for gene expression changes of *Hdac7, Pax5, Rag1* and *Csf1r* genes during reprogramming. RT-qPCR assays in uninduced (0) and β-estradiol-induced primary pre-B cells transduced with a retroviral vector for C/EBPα expression for the indicated times. Primary macrophages were used as a control.

1.2. Study of the effect of HDAC7 re-expression in C10 cells and its possible interference with the acquisition of the macrophage gene transcription program during cellular reprogramming

In order to determine whether the down-regulation of HDAC7 is essential for the correct reprogramming of pre-B cell into macrophages, we performed a gain-of-function experimental approach. We transduced C10 cells with a retroviral vector for HDAC7-Flag expression (C10-HDAC7 cells). As a control, we transduced the cells with an empty retroviral vector (C10-MSCV cells). After 72 hours of β-estradiol cell treatment, the expression of the endogenous form of HDAC7 protein was completely down-regulated during cellular reprogramming, whereas the exogenous HDAC7-Flag continued at the same level (Figure 6R).

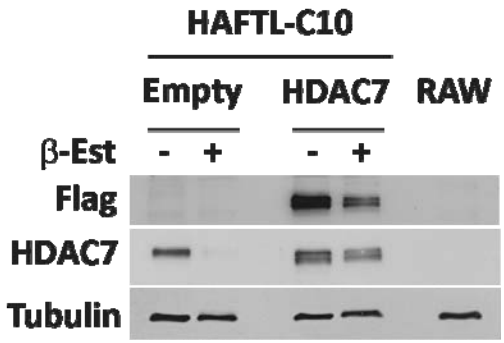

Figure 6R: Down-regulation of endogenous HDAC7 protein levels during reprogramming. Western blot showing the protein levels of endogenous and exogenously expressed HDAC7 in C10 cells untreated or treated with β-estradiol for 72 hours.

To test whether HDAC7 re-expression could interfere with the transcriptional program of the converted macrophages, we adopted a genome-wide experimental approach. Microarray experiments were conducted in both C10-MSCV and C10-HDAC7 cells un-induced and induced with β-estradiol for 48 and 72 hours. The addition of β-

estradiol to C10-MSCV cells resulted in the up- and down-regulation, respectively, of 1609 and 1798 genes at 48 hours and of 1531 and 1567 genes at 72 hours after treatment, in agreement with a previous report (Bussmann, 2009). Remarkably, the re-expression of HDAC7 (C10-HDAC7 cells) resulted in a partial or a total block of the up-regulation of 988 and 866 genes, after 48 and 72 hours respectively. As described by Bussmann et al*,* most of these up-regulated genes are associated with important macrophage functions, such as genes involved in inflammatory responses or in phagocytosis, whereas the down-regulated genes were related to cell cycle regulation and important B cell functions (Bussmann, 2009). To determine the type of genes affected by the re-expression of HDAC7 during cellular reprogramming, we performed a gene set enrichment analysis. To test whether these genes were enriched in the gene ontology (GO) categories corresponding to Biological Processes, Cellular Components and Molecular Functions, as well as in the KEGG pathways, we used the Gitools software (Perez-Llamas, 2011). This provides a framework for analyzing and visualizing multidimensional genomic data using interactive heat-maps. Importantly, we found that the types of genes whose up-regulation was affected by the presence of HDAC7 were involved in biological processes that correspond to important macrophage functions such as inflammatory response, phagocytosis, cellular response to interferon and positive regulation of interleukins, among others. Similar results were observed when we analyzed the enrichment of genes in KEGG pathways (Figure 7R). Gene ontology analysis of Cellular Components and Molecular Functions confirmed these results (Figure 8R).

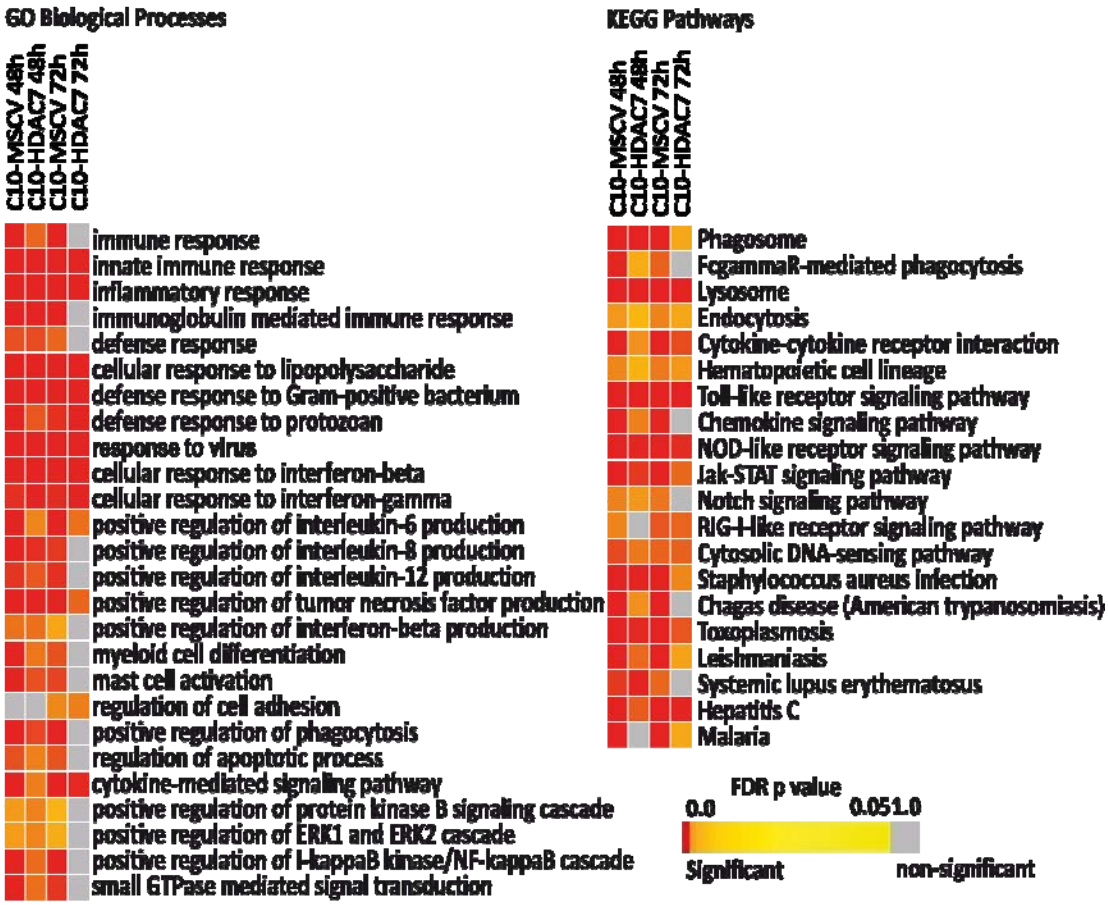

Figure 7R: HDAC7 re-expression interferes with the gene transcriptional program of the converted macrophages. Heat-maps showing significantly (corrected p-value < 0.05) enriched GO Biological Process and KEGG Pathways categories among the up-regulated genes affected by the re-expression of HDAC7 during transdifferentiation of pre-B cells into macrophages. Red and yellow indicate high and low statistical significance, respectively; gray indicates no statistical significance.

Analyzing the type of genes affected by the re-expression of HDAC7 during transdifferentiation, we found that some of these genes (such as *Cxcl10, Ccl9, Tlr4* and *Il18*) were related to the immune response, some were important for phagocytosis (such as

Fcgr1, C3 and *Mbp*) and others were related to the positive regulation of Il-6 production (such as *Tlr3, Tlr9* and *Ccr5*) (Figure 9R).

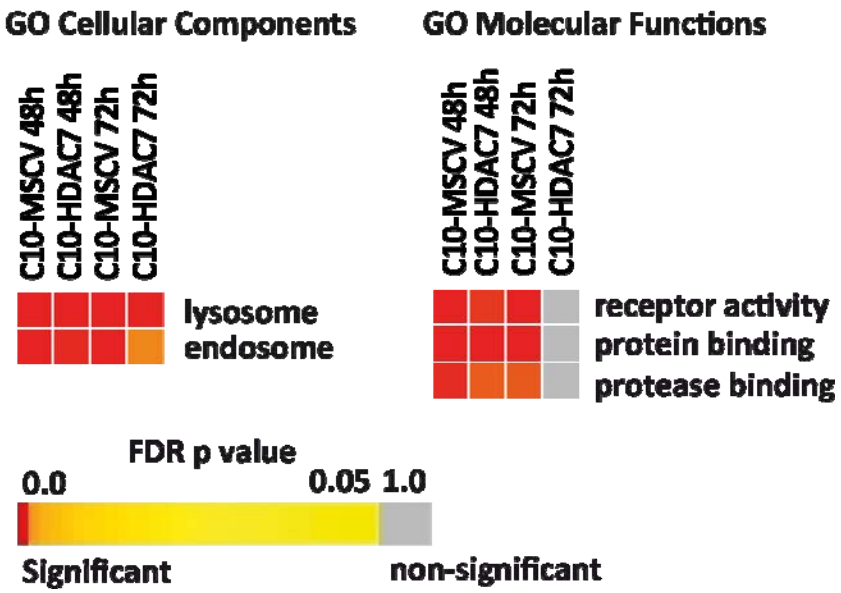

Figure 8R: Gene ontology analysis corresponding to Cellular Components and Molecular Functions. Heat-maps showing significantly (corrected p-value < 0.05) enriched GO Cellular Components and Molecular Functions categories among the up-regulated genes affected by the re-expression of HDAC7 during transdifferentiation of pre-B cells into macrophages. Red and yellow indicate high and low statistical significance, respectively; gray indicates no statistical significance.

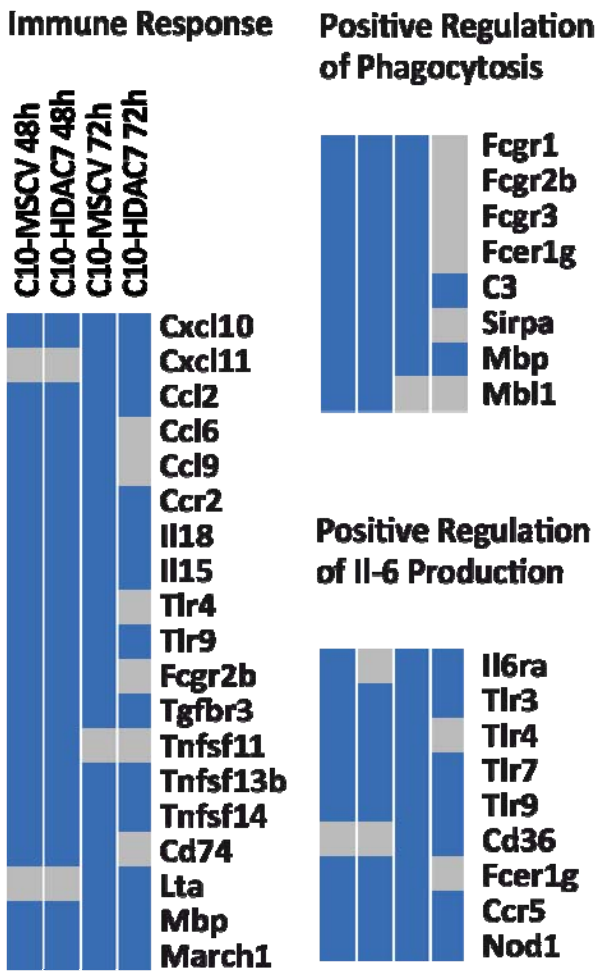

Figure 9R. HDAC7 re-expression interferes with the gene transcriptional program of the converted macrophages. Heat-maps showing genes expressed differentially for selected enriched GO categories. Blue indicates positive events while gray indicates that the gene was not observed differentially expressed under that experimental condition.

We next validated a selected set of genes by qPCR, and confirmed that many of the myeloid genes that became up-regulated during cellular transdifferentiation, such as *Cxcl10, Fcgr1, Tlr3* and *C3*, were partially or totally blocked in the presence of HDAC7. Importantly, no significant changes were observed in the down-regulated genes related to B cell Biology, such as the transcription factor PAX5 (Figure 10R). These data demonstrate that HDAC7 interferes with the acquisition of the macrophage transcriptional program and indicate that HDAC7 may be an important transcription repressor of inappropriate genes in pre-B cells.

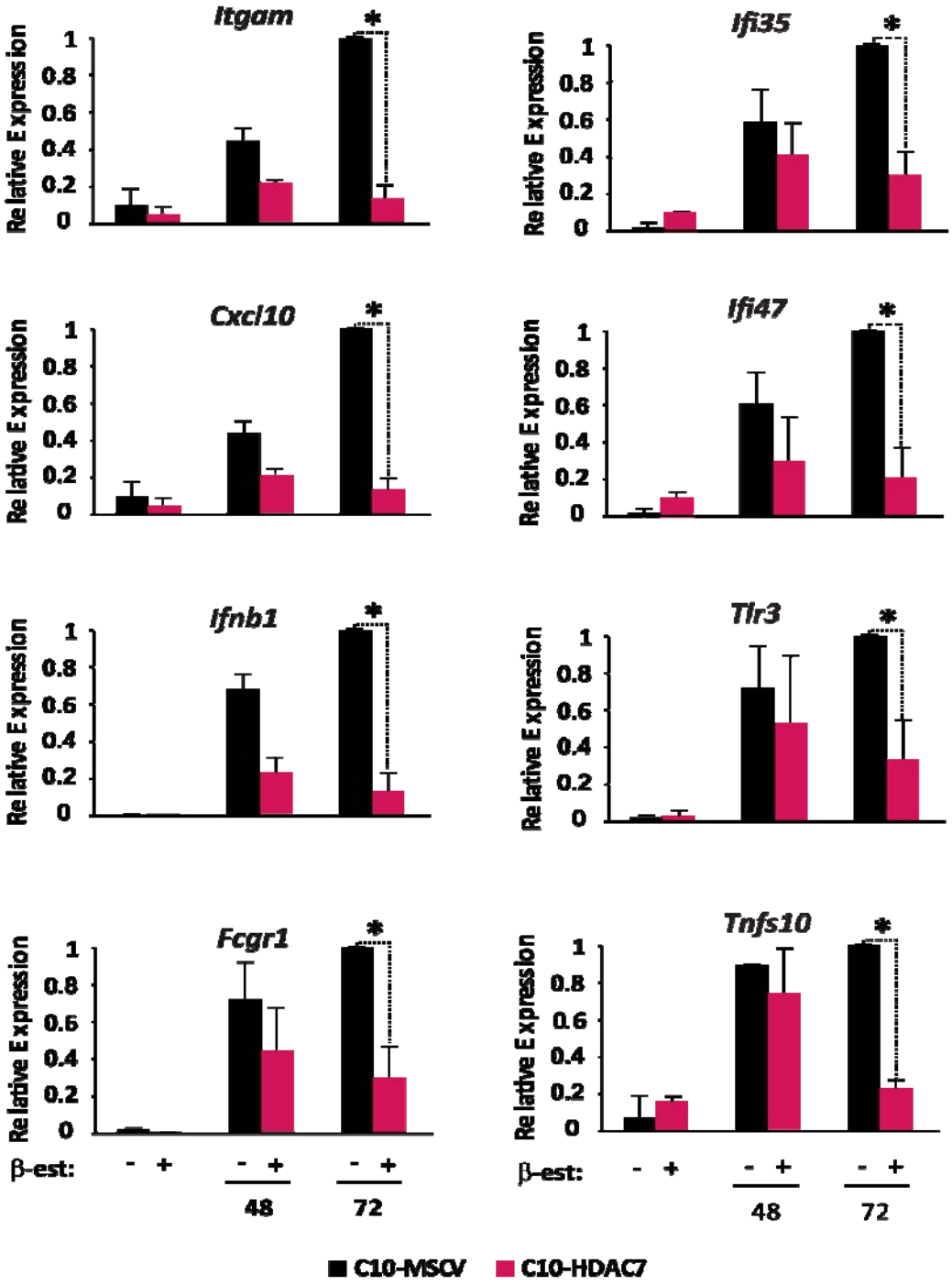
Itgam
Ifi35
Cxcl10
Ifi47
Ifnb1
Tlr3
Fcgr1
Tnfs10
Relative Expression
1
0.8
0.6
0.4
0.2
0
*
β-est:
- + - + - +
48
72
C10-MSCV
C10-HDAC7

Figure 10R: RT-qPCR for microarray validation of selected genes. RT-qPCR experiments for gene expression changes for 8 up-regulated genes, Cxcl10, Fcgr1, Ifi35, Ifi47, Tlr3, Tlr9, Il18 and C3, in the absence or presence of HDAC7 48 hours after β-estradiol treatment. Importantly, HDAC7 re-expression does not interfere with the down-regulation of Pax5. Data are represented as the mean ± standard error of the mean (SEM) of three independent experiments. A two-way ANOVA was used to identify significant differences between the indicated groups. *$p < 0.001$.

According to Bussmann *et al.,* during the reprogramming of pre-B cells into macrophages, the converted macrophages stop the cell cycle and many genes involved in this process become down-regulated (Bussmann, 2009). Our microarray data showed that down-regulation of these genes was also affected by the presence of HDAC7, resulting in a partial block of the down-regulation of these genes during the cellular reprogramming process (Figure 11R).

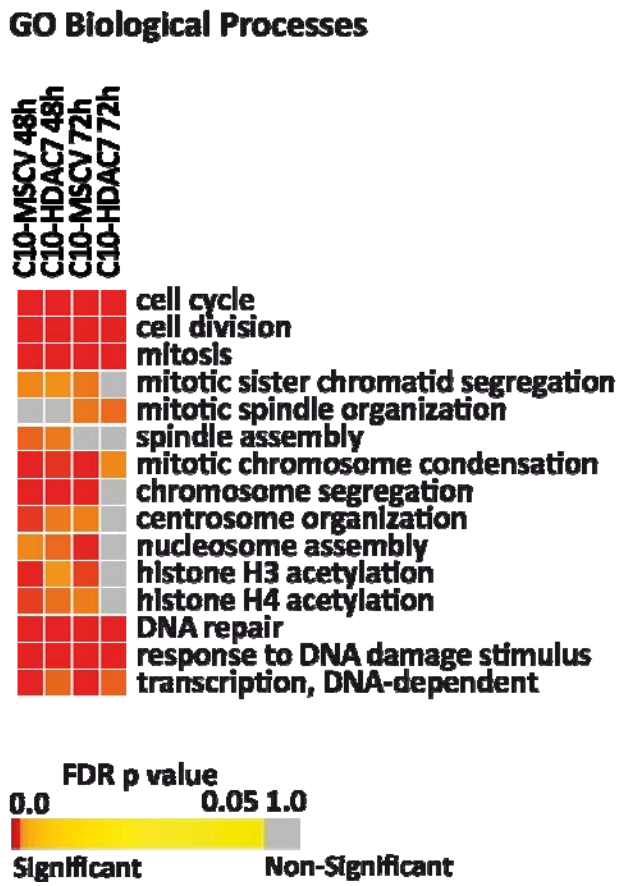

Figure 11R: HDAC7 re-expression interferes with the gene transcriptional program of the converted macrophages. Heatmap statistics showing significantly (FDR p-value ≤ 0.05) enriched GO Biological Processes categories among the down-regulated genes affected by the re-expression of HDAC7 during transdifferentiation of pre-B cells into macrophages. Red and yellow indicate high and low statistical significance, respectively; gray indicates no statistical significance.

1.3. Analysis of the physiological function of HDAC7 in pre-B cells

In order to analyze the physiological function of HDAC7 in pre-B cells, we adopted

a loss-of-function experimental approach. We used small interference RNA (siRNA) to knock down HDAC7 in the HAFTL B cell line and in primary pre-B cells. Strikingly, knockdown of HDAC7 resulted in the expression of myeloid genes related to important macrophage functions, such as *Itgam, Fcgr1* and *Ccl3*, in both types of cell (Figure 12R). These data clearly demonstrate that HDAC7 is a transcriptional repressor of lineage-inappropriate genes in pre-B cells.

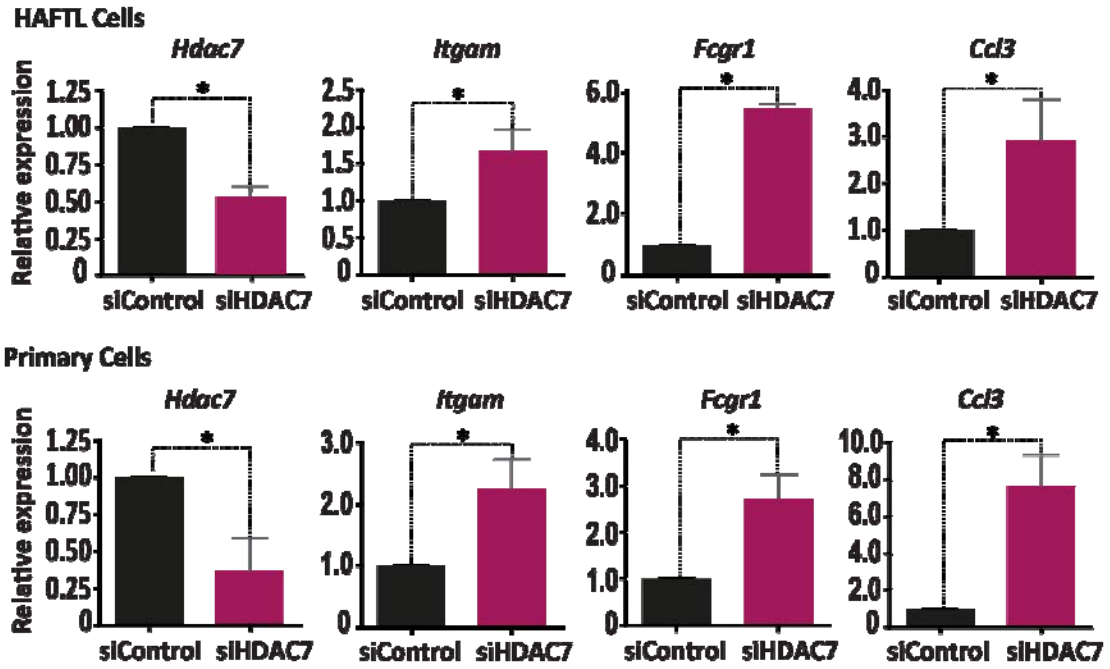

Figure 12R: HDAC7 knock down leads to the de-repression of macrophage genes. HAFTL cells and primary B cell precursors were transfected with control siRNA or siRNAs targeting HDAC7 72 hours after transfection. Hdac7, Itgam, Fcgr1 and Ccl3 mRNA levels were determined by RT-qPCR experiments.

1.4. Study of the interaction of HDAC7 with particular sequence-specific transcription factors in pre-B cells

From a mechanistic persepective, class IIa HDACs appear to interact with tissue-specific transcription factors, leading to their recruitment to target genes (Parra, 2010; Martin, 2009). To determine whether HDAC7 interacts with particular transcription factors in pre-B cells, we performed a series of coimmunoprecipitation experiments. We tested the interaction of endogenous HDAC7 with crucial transcription factors in the B lymphocytes. We found that HDAC7 specifically interacted with the transcription factor MEF2C in pre-B-cells, whereas no binding was observed with other B-cell transcription factors, such as E2A, IKAROS and PAX5 (Figure 13R). Interestingly, Stehling-Sun *et al.* have reported that MEF2C is critical in the cellular choice towards the lymphoid versus the myeloid lineage in LMPPs and the absence of MEF2C resulted in the up-regulation of genes related to myeloid cell functions such as immune and inflammatory response and cellular response to infections (Stehling-Sun, 2009).

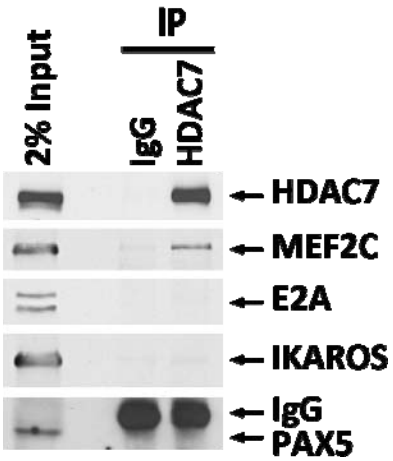

Figure 13R: HDAC7 binds to the transcription factor MEF2C. Coimmunoprecipitation experiments showing the specific binding of HDAC7 with MEF2C in pre-B cells. HDAC7 does not bind to IKAROS, PAX5 or E2A.

Therefore, we wondered whether there was an overlap between the myeloid genes repressed by MEF2C in LMPPs and the macrophage genes whose up-regulation was blocked by the presence of HDAC7 during cellular conversion into macrophages.

Constructing a Venn diagram to compare the genes whose up-regulation was totally or partially impaired in the presence of HDAC7 during the reprogramming of pre-B cells into macrophages with the set of MEF2C repressed genes reported in Stheling-Sun, 2009, we found a significant overlap, as determined by a chi-square test of 46 ($p < 10^{-6}$) and 30 ($p = 0.00044$) genes, respectively (Figure 14R). Interestingly, several genes related to phagocytosis (such as *Fcgr1*, *Fcgr2b* and *Fcgr3*) were targets for both HDAC7 and MEF2C. These results suggest that HDAC7 may be the natural corepressor partner of MEF2C for silencing inappropriate genes in both LMPPs and pre-B cells.

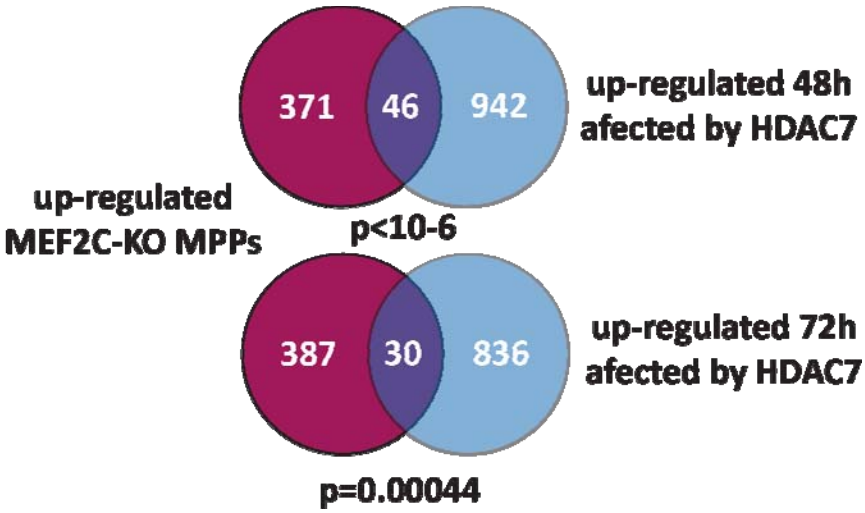

Figure 14R: HDAC7 interacts with MEF2C and alters the up-regulation of several genes. Venn diagrams showing the total number of genes up-regulated in MEF2C deficient LMPPs, genes up-regulated at 48 and 72 hours of β-estradiol treatment affected by HDAC7 and the overlap between the two conditions.

We next used chromatin immunoprecipitation (ChIP) experiments to test whether HDAC7 is recruited to the promoters of macrophage-specific genes in pre-B cells. To design specific primers for HDAC7 in these genes, we took advantage of the bioinformatic

tool TFconsite to detect putative MEF2 binding sites in genes related to important macrophage functions, such as *Fcgr1, Cxcl10* and *Itgam*. We designed specific primers in the upstream regulatory region, the gene body and downstream regulatory regions of mouse *Fcgr1, Cxcl10* and *Itgam* genes. We prepared chromatin from C10 cells uninduced and induced with β-estradiol for 72 hours and subjected it to ChIP with specific antibodies for HDAC7 and MEF2C. qPCR analysis of the immunoprecipitated material revealed that HDAC7 and MEF2C were both enriched at the MEF2 binding sites identified in control cells, whereas non-enrichment of HDAC7 was observed in C10 cells induced for 72 hours with β-estradiol (Figure 15R). These results demonstrate that, in pre-B cells, HDAC7 is recruited to the MEF2 sites at the promoter of important macrophage genes resulting in their transcriptional repression.

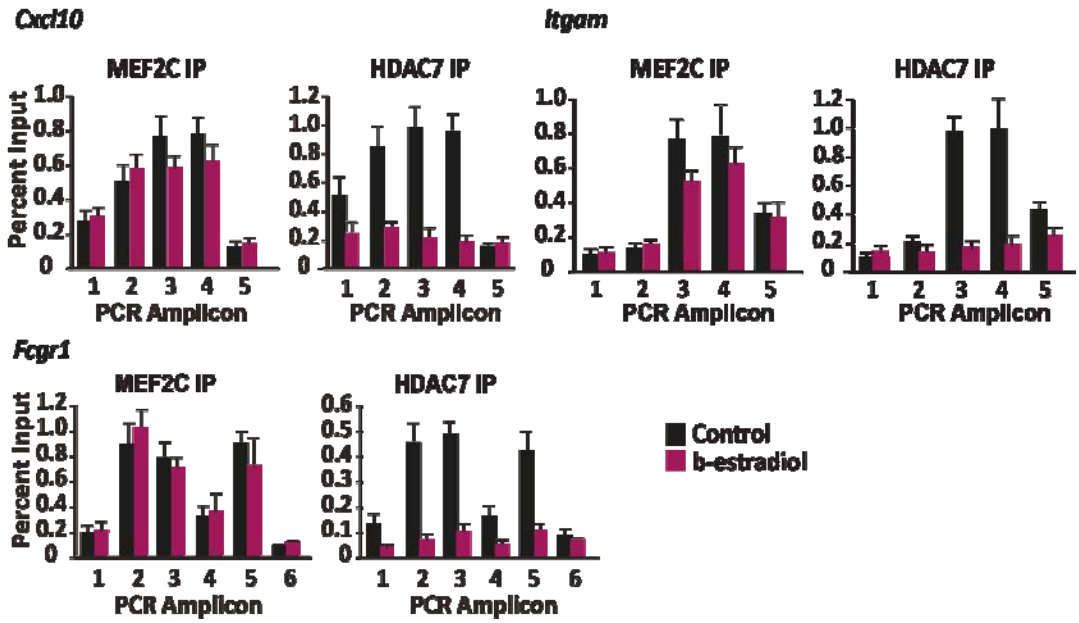

Figure 15R: HDAC7 binds to the transcription factor MEF2C and is recruited to the promoter of key genes for macrophage function in pre-B cells. Chromatin immunoprecipitation experiments showing the enrichment of HDAC7 and MEF2C to putative MEF2 binding sites on the *Cxcl10*, *Fcgr1* and *Itgam* gene loci in pre-B cells.

Results are presented as percentage immunoprecipitation over input and are representative of three independent experiments.

1.5. Analysis of the effects of HDAC7 re-expression on the functional properties of the reprogrammed macrophages

As previously reported by Bussmann *et al.* (2009) during the transdifferentiation of pre-B cells into macrophages, the myeloid cell surface marker Mac-1 (*Itgam*) is up-regulated, while the B cell-specific marker CD19 becomes down-regulated. Remarkably, the converted macrophages are phagocytic and respond to LPS treatment by producing pro-inflammatory cytokines (Bussmann, 2009). To analyze the effects of HDAC7 re-expression on the functional properties of the reprogrammed macrophages, we adopted several experimental approaches in both C10-MSCV and C10-HDAC7 cells. We first analyzed Mac-1 and CD19 expression during the cellular reprogramming of both cells by flow cytometry. The addition of β-estradiol resulted in the up-regulation of Mac-1 and down-regulation of CD19 in both C10-MSCV and C10-HDAC7 cells. However, illustrating the results as histograms and mean fluorescence intensity (MFI) shows that the presence of exogenous HDAC7 resulted in a significant block in Mac-1 expression. No significant changes were observed in the expression of CD19, whose decrease was similar to that of C10-MSCV (Figure 16R).

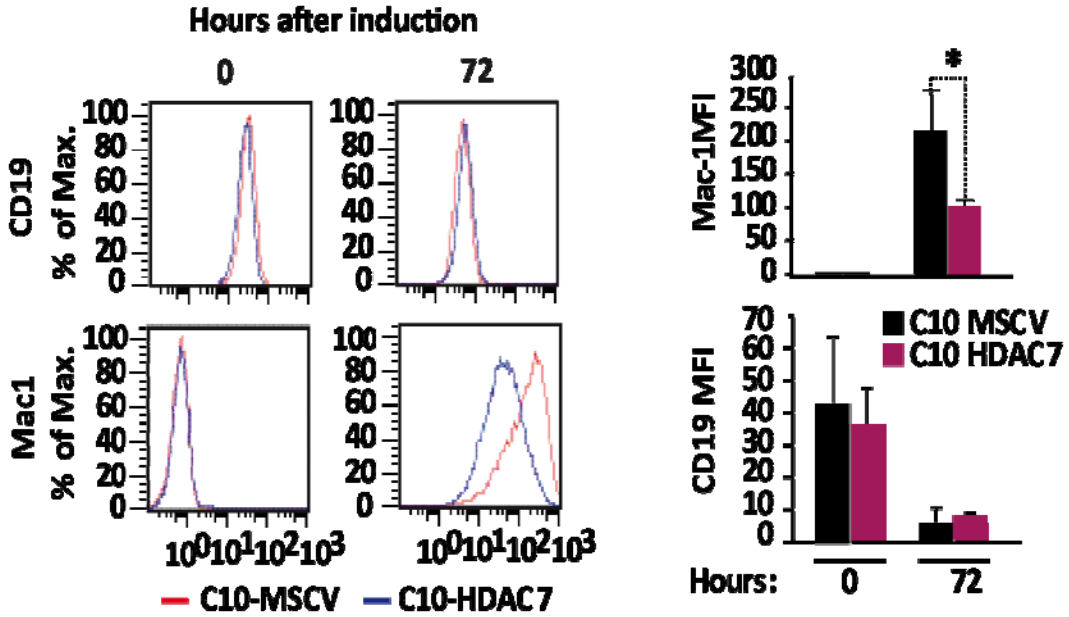

Figure 16R: HDAC7 re-expression interferes with the functional properties of the converted macrophages. Percentage and MFI of Mac-1 and CD19-positive cells in C10-MSCV and C10-HDAC7 cells untreated or treated with β-estradiol for 72 hours.

Exogenous expression of HDAC7 in primary B cell precursors also interfered with the up-regulation of Mac-1 in transdifferentiation experiments (Figure 17R). These results demonstrated that in the presence of HDAC7 the reprogrammed cells are not able to express the levels of Mac-1 protein that are normally present in macrophages. Next, we examined whether HDAC7 re-expression could also interfere with the phagocytic activity of the reprogrammed macrophages. Importantly, we found that in the presence of red-fluorescent bacteria β-estradiol-treated C10-HDAC7 cells were not able to phagocytose them properly and thus their phagocytic activity was lower in comparison to β-estradiol-treated C10-MSCV cells (Figure 17R).

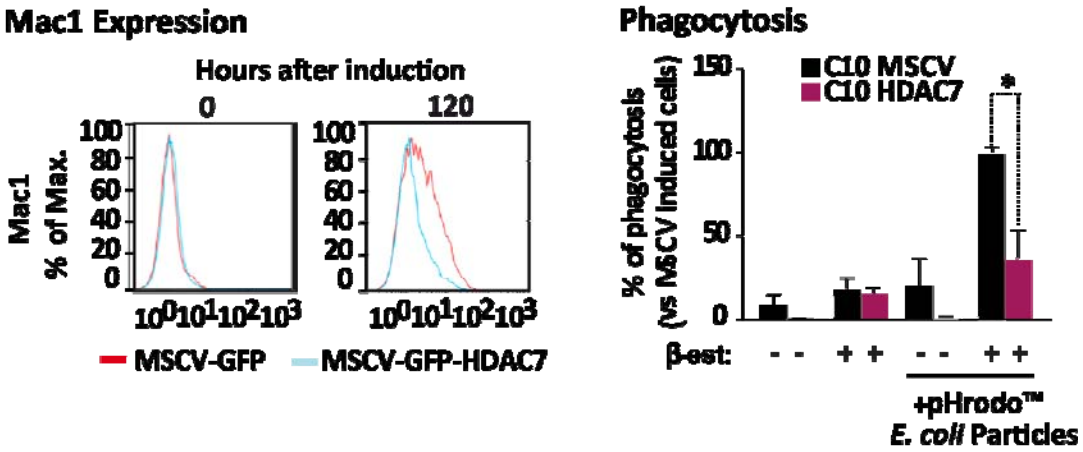

Figure 17R: HDAC7 re-expression interferes with Mac1 expression and the phagocytic activity of the converted macrophages. Mac1 expression in primary pre-B cells transduced with the indicated retroviral vectors and induced to transdifferentiate for 48 hours, and capacity of C10-MSCV and C10-HDAC7 cells untreated or treated with β-estradiol for 48 hours to phagocytose red fluorescence bacteria.

Finally, we examined whether the re-expression of HDAC7 could interfere with the inflammatory response of the reprogrammed macrophages when cultured in the presence of LPS, a bacteria surface marker. Importantly, we observed that the presence of HDAC7 significantly blocked the inflammatory response to LPS by inhibiting the expression of the pro-inflammatory cytokines *Tnfα*, *Il-1β* and *Il-6* (Figure 18R). Together, our findings demonstrate that the expression of HDAC7 interferes with functional properties of the reprogrammed macrophages.

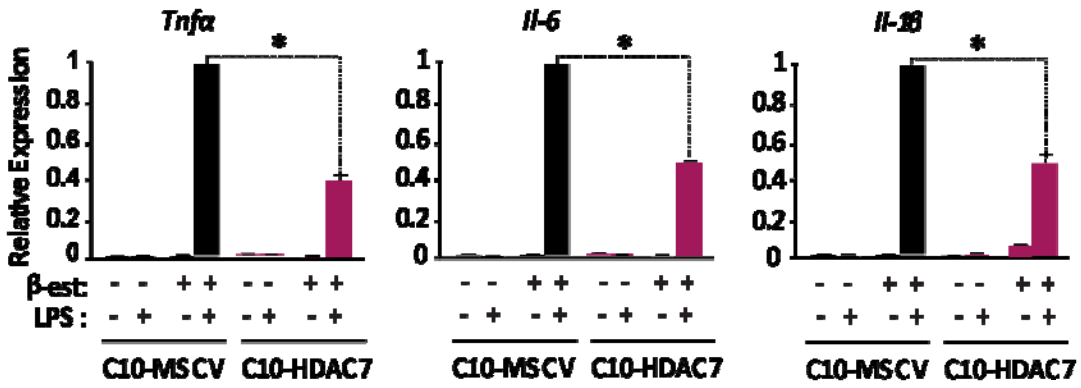

Figure 18R: HDAC7 re-expression interferes with the inflammatory response of the converted macrophages. Effect of HDAC7 expression on LPS-mediated *Tnfa, Il-1b* and *Il-6* gene expression. C10-MSCV and C10-HDAC7 cells were treated or not with β-estradiol for 48 hours. Then, the cells were incubated or not with LPS for 6 hours and RNAs analyzed by RT-qPCR.

Next, we wondered whether, once the cells have been transdifferentiated into macrophages, the exogenous expression of HDAC7 could interfere with the expression of Mac-1. To examine this we transduced β-estradiol-treated C10 cells for 72 hours with a retroviral vector carrying HDAC7-Flag and tested Mac-1 expression by flow cytometry. We observed that 24 hours after re-expression of HDAC7, the protein levels of Mac-1 became down-regulated in comparison with the control cells (Figure 19R). However, when performing the same experiments with RAW cells, we found no significant effect on either the expression of myeloid genes or their phagocytic activity (Figure 20R).

In conclusion, this set of results demonstrates that HDAC7 down-regulation is crucial for the proper reprogramming of B-cells to macrophages and the presence of HDAC7 significantly interferes with important macrophage functions of the reprogrammed cells, such as phagocytosis and the inflammatory response.

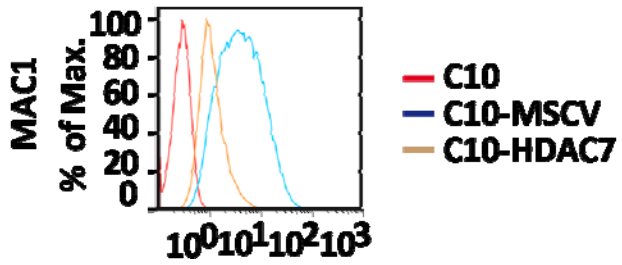

Figure 19R: HDAC7 re-expression decreases Mac1 protein levels in the reprogrammed macrophages. Histograms for Mac-1 protein levels in reprogrammed macrophages transduced with an empty vector or a retroviral vector for HDAC7 expression.

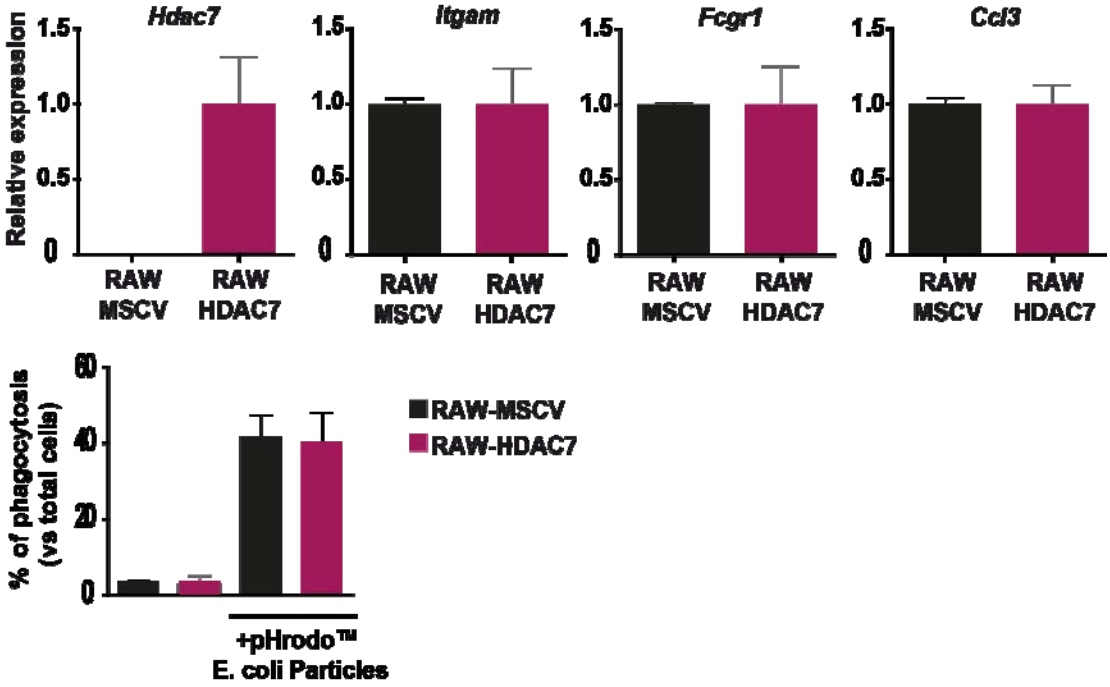

Figure 20R: HDAC7 re-expression interferes with functional properties of reprogrammed macrophages. RT-qPCR experiments for gene expression changes for Hdac7, Itgam, Ccl3, and Fcgr1 genes in RAW-MSCV and RAW-HDAC7 cells, and capacity of these cells to

phagocytose red fluorescence bacteria.

1.6. Elucidation of the contribution of the interaction of HDAC7 with ME2C and its catalytic activity on the repression of Mac-1

As previously reported, class IIa HDACs contain an MEF2-interacting domain consisting of 17 amino acids located at the amino terminal region, and a catalytic domain located at the carboxyl terminal region of the proteins (Verdel, 1999; Wang, 1999; Lu, 2000; Kao, 2001; Dequiedt, 2003; Han, 2005). To investigate the contributions of the interaction between HDAC7 and MEF2C, and HDAC7 catalytic activity to the repression of Mac-1, we generated retroviral vectors to express different HDAC7 mutants and GFP as a marker of infection. Specifically, we generated constructs consisting of a deletion of the MEF2 binding motif (HDAC7-ΔMEF), a complete deletion of the N-terminal domain lacking the MEF2 interaction motif (HDAC7-438-915), the catalytic domain deletion (HDAC7-1-487), and point mutations in the MEF2 binding site (HDAC7-K86AK88A) or in the catalytic domain (HDAC7-K86AK88A and HDAC7-H657A). We transduced C11 cells with the indicated retroviral constructs and analyzed the expression of Mac-1 after β-estradiol treatment. As expected, expression of wild type HDAC7 significantly interfered with the up-regulation of Mac-1 during the conversion of pre-B cells into macrophages. However, expression of the HDAC7 mutants had no significant effect. These results demonstrate that HDAC7-MEF2 and the catalytic site of HDAC7 are both necessary for repressing Mac-1 during the reprogramming of pre-B cells into macrophages (Figure 21R).

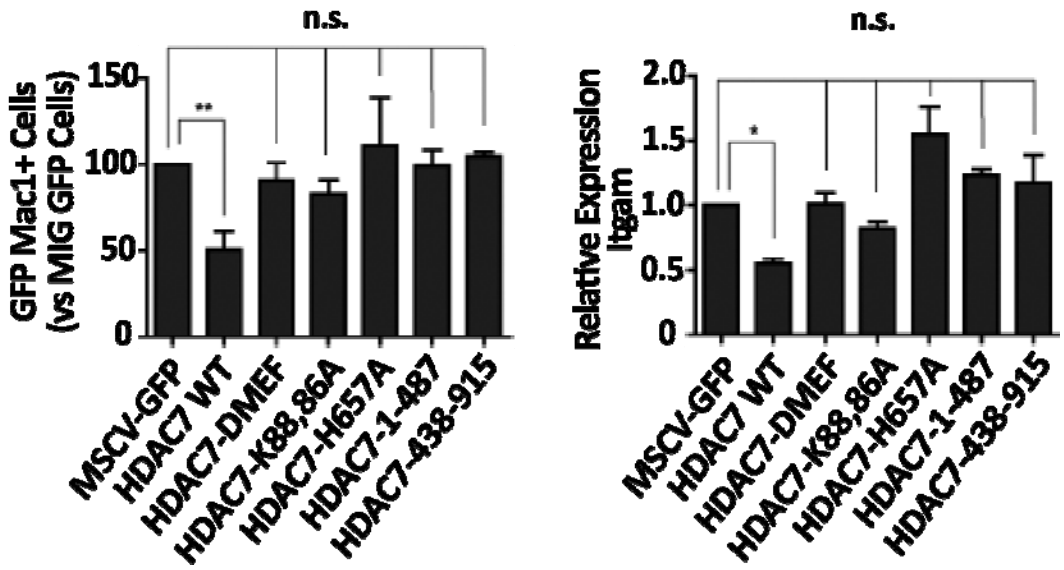

Figure 21R: Interaction of HDAC7 with MEF2C and its catalytic activity are essential for repression of Mac-1. Percentage of Mac-1-positive cells (left) and RT-qPCR experiments for Itgam gene expression changes (right) in C11 cells infected with MSCV-GFP, MSCV-GFP-HDAC7, MSCV-GFP-HDAC7(DMEF), MSCV-GFP-HDAC7(K86A/K88A), MSCV-GFP-HDAC7(H657A), MSCV-GFP-HDAC7(1-487) and MSCV-GFP-HDAC7(438-915) viruses and induced with β-estradiol for 48 hours. Data are given as mean ± SEM of values obtained from three independent experiments. *p< 0.0001.

2. Role of HDAC7 in B lymphocyte development

2.1. HDAC7 expression pattern in the murine hematopoietic system

To understand the function of HDAC7 in B lymphopoiesis better, we analyzed its expression pattern in the hematopoietic system. To this end, we used the Immunological Genome Project Database (Immgen) (http://www.immgen.org/). Immgen is a collaborative scientific research project that is currently building a gene-expression

microarray database for all characterized immune cells in the mouse. Using this database we found HDAC7 to be highly expressed in lymphoid cells but not in cells from the myeloid lineage (Figure 22R). This corroborates our findings that HDAC7 is a lymphoid-lineage-specific transcriptional repressor within the hematopoietic system.

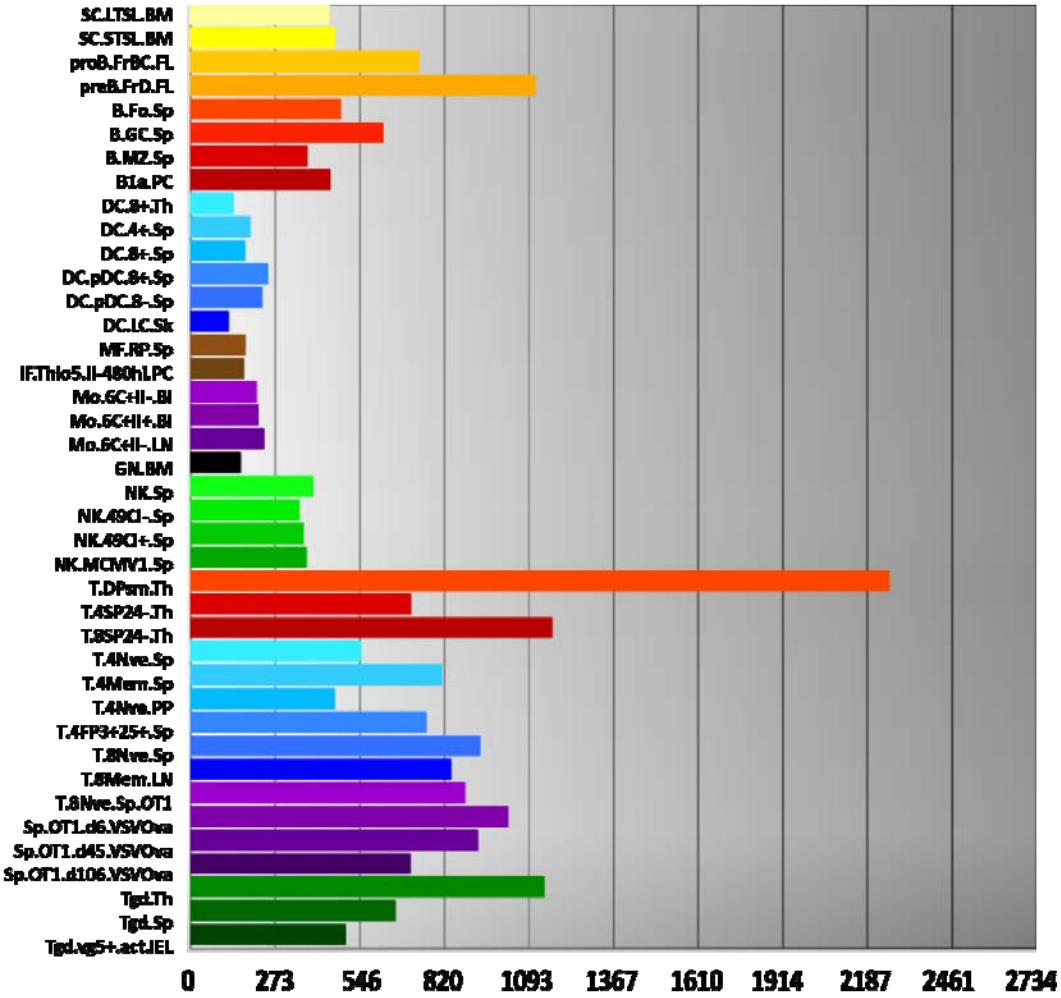

Figure 22R: HDAC7 is highly expressed in lymphoid cells but not in the myeloid lineage. Data obtained from the Immunological Research Project Database (Immgen) showing the expression of HDAC7 in all characterized immune cells in the mouse (http://www.immgen.org/).

2.2. Generation of HDAC7 conditional knockout mice in B cell progenitors

Definitive proof of the crucial role of HDAC7 in B lymphocytes requires its function to be evaluated *in vivo,* in whole organisms. Given the expression pattern of HDAC7 and our findings demonstrating that HDAC7 is a repressor of myeloid genes in pre-B cells, we decided to study the physiological role of HDAC7 in B lymphocyte development *in vivo,* using mutant mice. HDAC7 knockout mice show embryonic lethality at day 11 resulting from a failure to form tight junctions in the developing circulatory system (Chang, 2006). Therefore, to address the role of HDAC7 in adult B lymphopoiesis, we generated a conditional mouse model for HDAC7 deficiency in early B cell progenitors (pro-B cells). To this end, in collaboration with Dr. Eric N. Olson (University of Texas, Southwestern, USA), we obtained mice with exons 2-10, encoding for the entire N-terminal regulatory domain of the HDAC7 gene, flanked by loxP sites that are recognized by Cre recombinase. To delete HDAC7 specifically in pro-B cells we crossed HDAC7$^{loxP/ko}$ mice with mice engineered to express Cre recombinase under the control of Mb1 (Cd79a) gene promoter, which is expressed at the earliest pro-B cell stage (Hobeika, 2006) (Figure 23R).

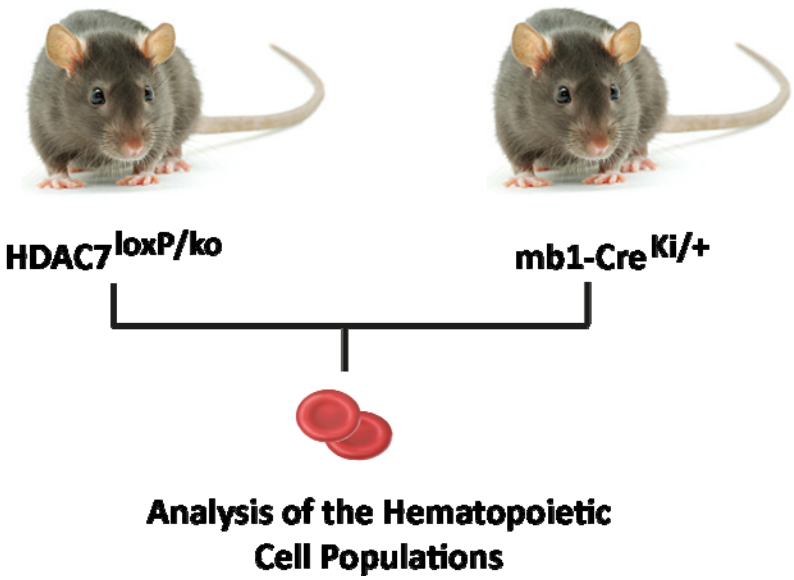

Figure 23R: Strategy for generating a conditional knockout mouse for the specific deletion of HDAC7 in the hematopoietic system. Mice bearing loxP flanked HDAC7 ($HDAC7^{loxP/ko}$) are crossed with mice engineered to express Cre recombinase under the control of the *Mb1* gene promoter, which is expressed at the earliest pro-B-cell stage.

2.3. Analysis of the hematopoietic cell population profile in the bone marrow of HDAC7 knockout mice

We first analyzed the effect of HDAC7 deficiency in different cell populations of the hematopoietic system by comparing the $HDAC7^{loxP/ko}$;$Mb1\text{-}Cre^{ki/+}$ mice with wild type littermates as the control, $HDAC7^{+/ko}$;$Mb1\text{-}Cre^{ki/+}$. Bone marrow (BM), spleen (SPL), thymus (TH) and peripheral blood (PB) from 5-6 week-old mice were analyzed by flow cytometry. The bone marrow of HDAC7 knockout mice has many fewer B cells ($B220^+$) than wild type mice (Figure 24R).

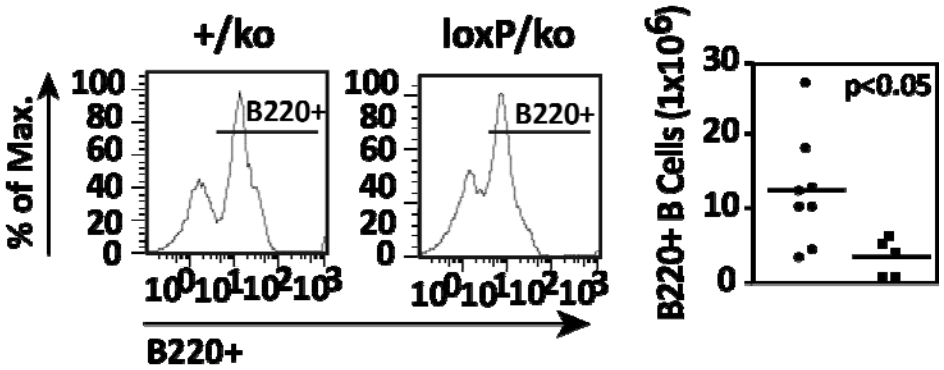

Figure 24R: B cell population is dramatically reduced in HDAC7 knockout mice. Flow cytometry analysis showing the percentage (left panel) and absolute number (right panel) of $B220^+$ B cells in the bone marrow of wt and HDAC7 knockout mice.

Next, we examined the effect of HDAC7 deficiency on the number of B cells at different developmental stages. We observed that the frequencies of pro-B ($B220^{+}CD43^{+}IgM^{-}$) and pre-B cells ($B220^{+}CD43^{-}IgM^{-}$) were also altered, resulting in a significant accumulation of pro-B cells and a reduction of pre-B cells in the bone marrow of HDAC7 knockout mice (Figure 25R-A). Consistent with this, absolute cell numbers of pro-B and pre-B cells were also significantly increased and decreased, respectively (Figure 25R-B).

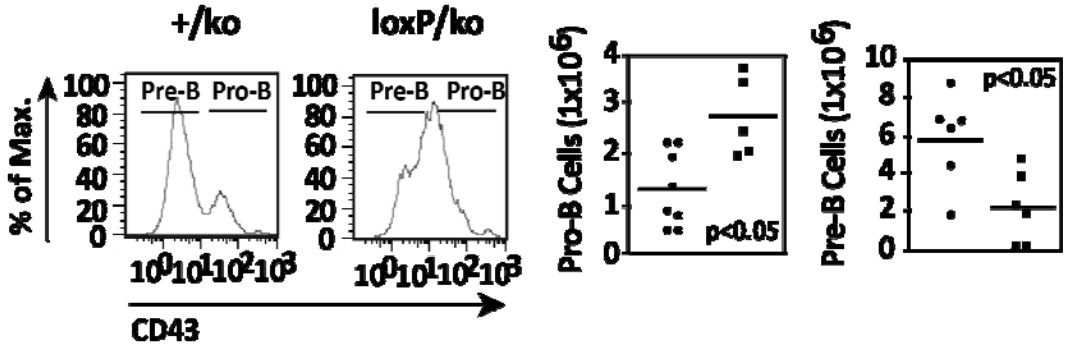

Figure 25R: Impairment of B cell development at the pro-B cell stage. Flow cytometry analysis showing the percentage (left panel) and absolute number (right panel) of pro-B and pre-B cells.

Remarkably, populations of immature ($B220^{+}CD43^{-}IgM^{+}IgD^{-}$) and mature recirculating ($B220^{+}CD43^{-}IgM^{+}IgD^{+}$) B cells almost entirely undetected in the bone marrow of $HDAC7^{loxP/ko}$;$Mb1\text{-}Cre^{ki/+}$ mice in comparison with $HDAC7^{+/ko}$;$Mb1\text{-}Cre^{ki/+}$ control mice (Figure 26R). Together, these results clearly demonstrate that HDAC7 is crucial to B lymphocyte development in bone marrow.

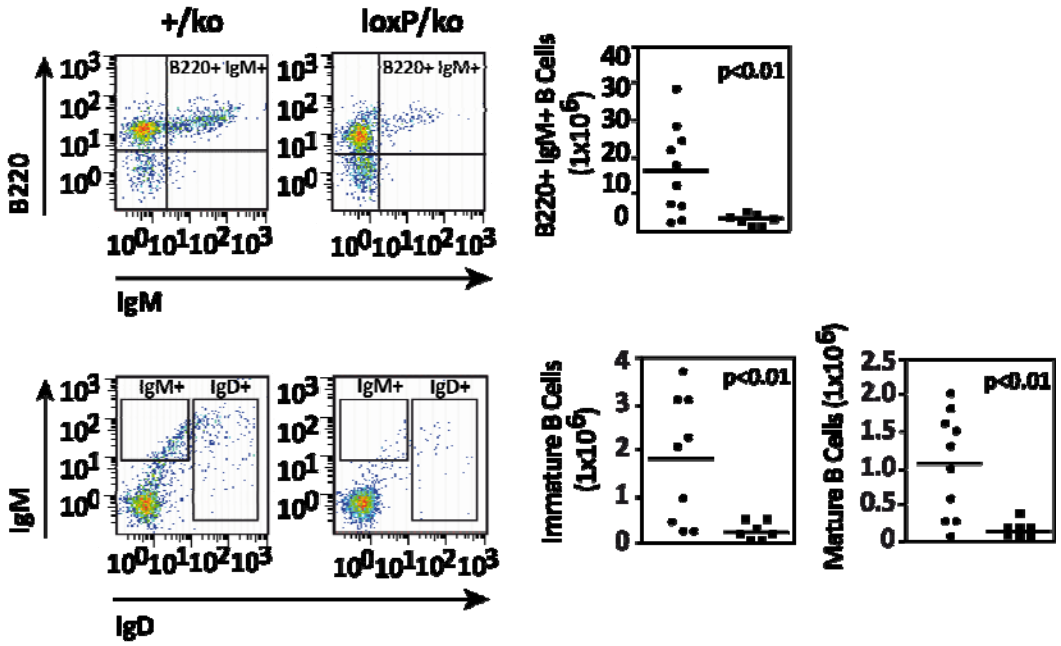

Figure 26R: Immature and mature B cell populations were absent in the bone marrow of HDAC7loxP/ko mice. Flow cytometry analysis showing the percentage (left panel) and absolute number (right panel) of immature and mature B cells.

Next, we wondered whether the absence of HDAC7 could affect the generation of cell types of other hematopoietic lineages. In contrast to the drastic reduction in B cells, granulocytes ($Gr1^+CD11b^+$), macrophages ($Gr1^-CD11b^+$) and erythrocytes ($Ter119^+$) were normal in the bone marrow of the HDAC7 knockout mice (Figure 27R). Collectively, these data demonstrate that HDAC7 deficiency leads to a significant block at the early stages of B cell development.

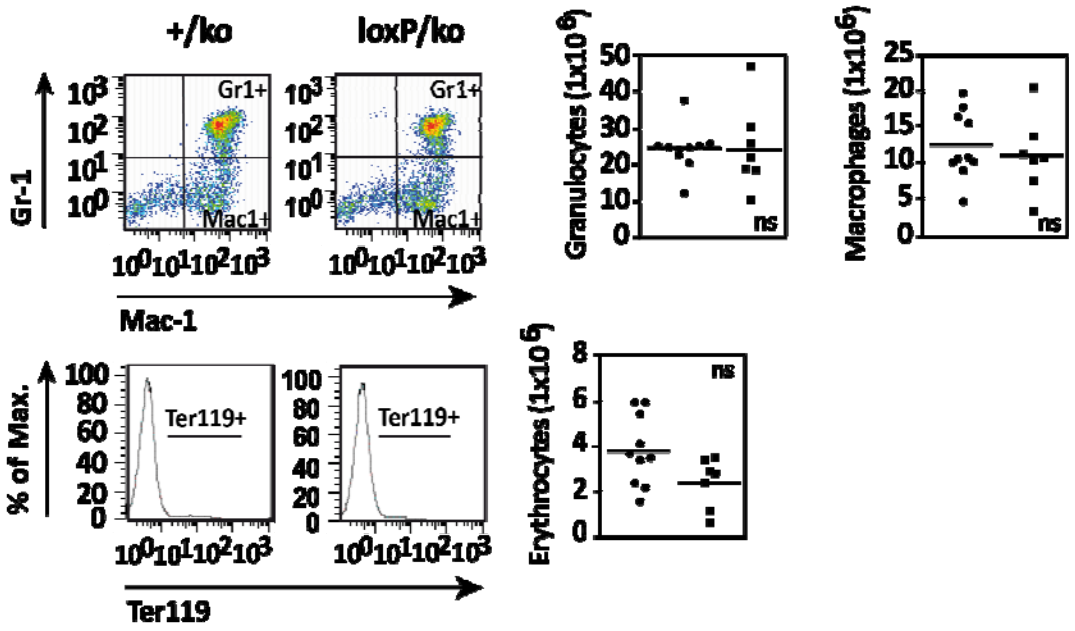

Figure 27R: HDAC7 has no effect on myeloid development. Flow cytometry analysis showing the percentage (left panel) and absolute number (right panel) of granulocytes, macrophages and erythrocytes in the bone marrow.

2.4. Analysis of the hematopoietic cell population profile in peripheral lymphoid organs of HDAC7 knockout mice

To assess whether HDAC7 is required at later B cell developmental stages, we analyzed B cell maturation in peripheral organs (spleen and blood) of HDAC7-deficient HDAC7$^{loxP/ko}$;Mb1-Cre$^{ki/+}$ mice and their littermate controls HDAC7$^{+/ko}$;Mb1-Cre$^{ki/+}$. We found that HDAC7 knockout mice had drastically and significantly fewer B cells (B220^{+}) in the spleen than did control mice (Figure 28R). Strikingly, the B220^{+} B cell population was almost nonexistent in peripheral blood in HDAC7-deficient mice (Figure 29R).

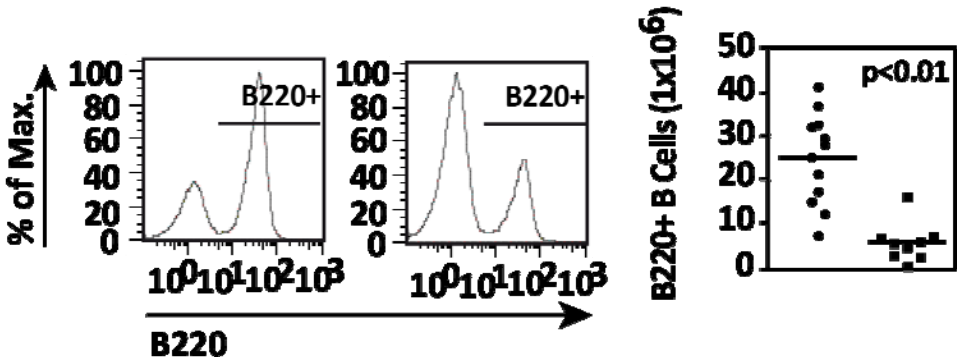

Figure 28R: B cell population is reduced in the spleen of HDAC7 knockout mice. Flow cytometry analysis showing the percentage (left panel) and absolute number (right panel) of B220+ B cells in the spleen of wt and HDAC7 knockout mice.

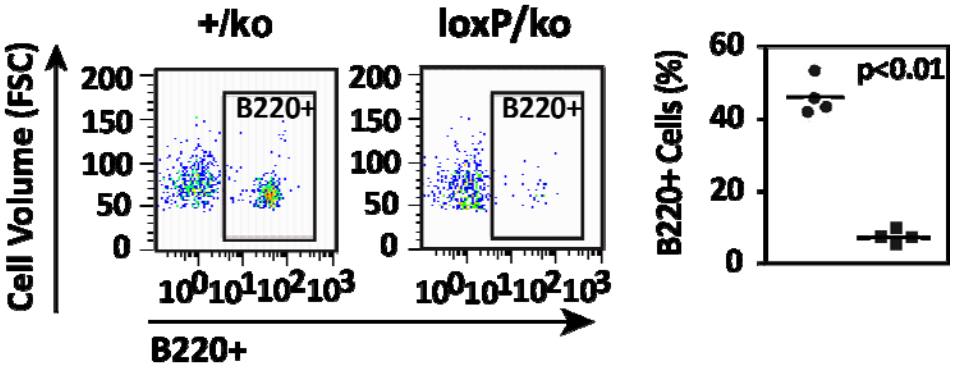

Figure 29R: B cell population is much smaller in the peripheral blood of HDAC7 knockout mice. Flow cytometry analysis showing the percentage (left panel) and absolute number (right panel) of B220+ B cells in the peripheral blood of wt and HDAC7 knockout mice.

Next, we analyzed specific B lymphocyte populations in the spleen of HDAC7$^{loxP/ko}$;Mb1-Cre$^{ki/+}$ knockout mice and their littermate controls. In particular, we

analyzed immature B cells ($B220^{+}CD43^{-}IgM^{+}IgD^{-}$), marginal zone (MZ) cells ($B220^{+}CD21^{+}CD23^{Low}CD93^{-}$), follicular (FO) cells ($B220^{+}CD21^{Low}CD23^{+}CD93^{-}$) and transitional (T) B cells ($B220^{+}CD21^{Low}CD23^{+}CD93^{+}$). We observed that the numbers of all the B cell subtypes were significantly lower in HDAC7-deficient mice, presumably due to a reduction in B cell production in the bone marrow of HDAC7 knockout mice rather than a potential functional dependency of splenic B cells on HDAC7 (Figure 30R).

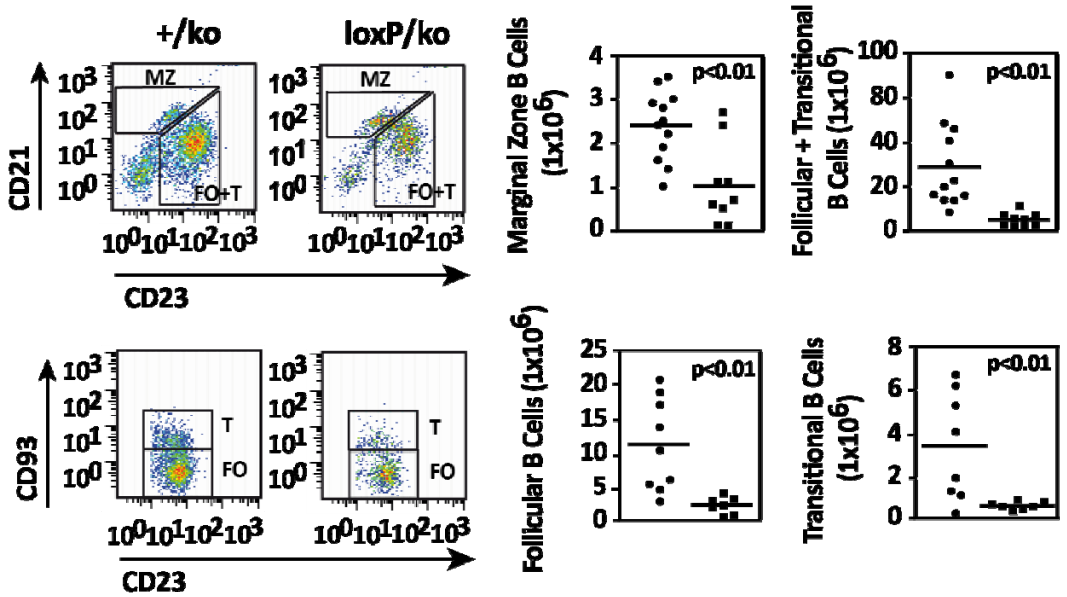

Figure 30R: Marginal zone, follicular and transitional B cell populations were dramatically reduced in the bone marrow of HDAC7loxP/ko mice. Flow cytometry analysis showing the percentage (left panel) and absolute number (right panel) of marginal zone, follicular and transitional B cells.

T cell developmental stages in the thymus were normal in HDAC7 knockout mice (Figure 31R), which indicates that T lymphocytes can undergo normal differentiation in the absence of HDAC7 from pro-B cells. Additionally, no changes were observed in macrophage and granulocyte numbers in the spleen (Figure 31R).

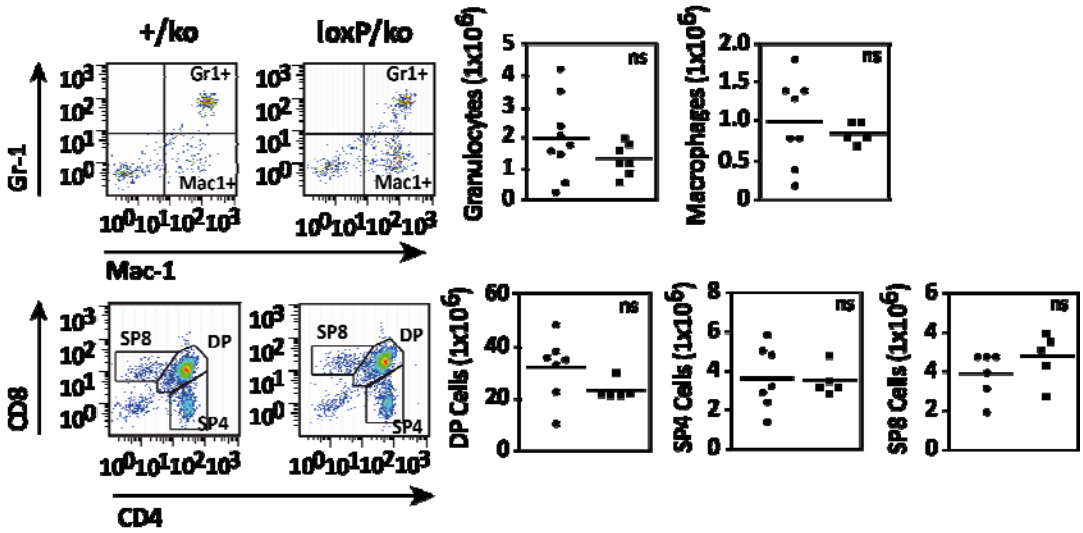

Figure 31R: HDAC7 has no effect in T and myeloid development in the periphery. Flow cytometry analysis showing the percentage (left panel) and absolute number (right panel) of granulocytes and macrophages in the spleen and T cells in the thymus.

2.5. HDAC7 knockout mice have small and unstructured spleens.

Given the dramatic block in B cell development in HDAC7 deficient mice we next analized the morfology of the spleens by histology assays. Remarkably, spleens of HDAC7$^{loxP/ko}$;Mb1-Cre$^{ki/+}$ knockout mice were quite smaller than those from their littermate controls (Figure 32R-A). Next, we sectioned the spleens from HDAC7$^{loxP/ko}$;Mb1-Cre$^{ki/+}$ mice and their littermate controls HDAC7$^{+/ko}$;Mb1-Cre$^{ki/+}$ and performed hematoxylin and eosin stainig. The spleens from control mice showed a normal structure with well defined follicles represented by the withe pulpe (hematoxylin staining in purple) surrounded by the red pulpe (eosin staining in pink). Strikingly, we observed that spleens from HDAC7 deficient mice showed an unstructred morphology, with smaller and not well defined follicles (Figure 32R-B). Immunohistochemistry analysis to detect the presence of T lymphocytes in the spleen showed that, whereas control mice contain T lymphocytes in

the inner surface of the follicles, HDAC7 knockout mice showed the presence of this cell type in all the the follicular area. Moreover, spleens from HDAC7 decicient mice showed increased density of macrophages in the red pulpe (Figure 32R-C and D).

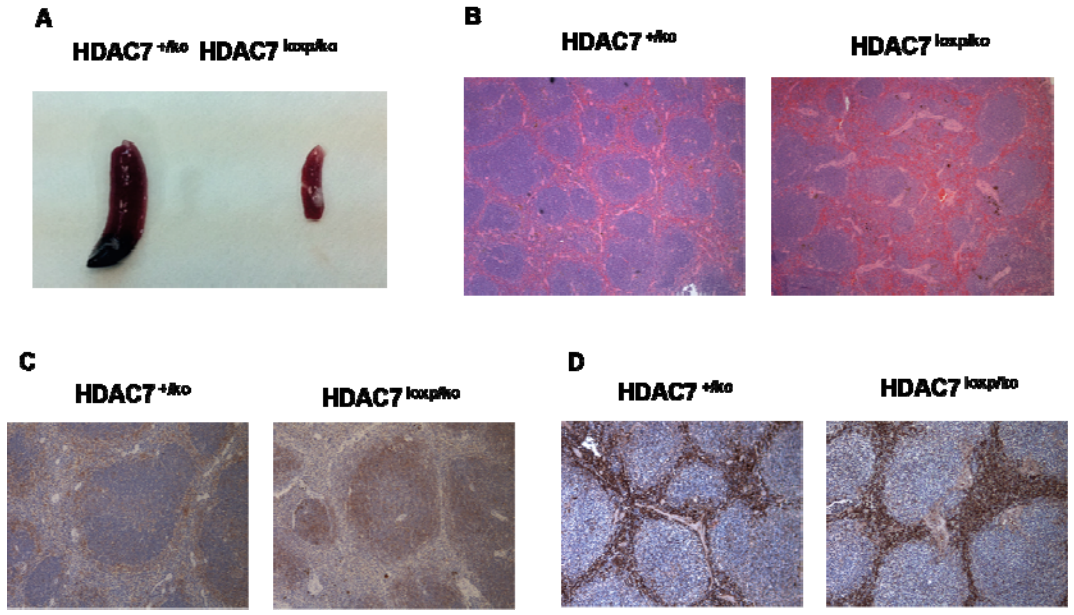

Figure 32R: HDAC7-deficiet mice have small and unstructured spleens. A. Spleens from HDAC7-knockout mice are quite smaller than those from their littermate controls. **B.** Hematoxylin (in purple) and eosin (in pink) staining reveals that spleens from HDAC7-deficient mice showed an unstructred morphology, with smaller and not well defined follicles. **C and D.** Immunohistochemistry analysis to detect the presence of T lymphocytes in the spleen shows the presence of this cell type in all the follicular area of the spleen of HDAC7 knockout mice and an increased density of macrophages in the red pulpe, in comparison with the wild type mice.

2.6. Gene expression profiling of HDAC7 deficient pro-B cells

Next, to explore the molecular mechanism underlying HDAC7-mediated B cell development, we performed global gene-expression profiling in pro-B cells purified from *Hdac7*$^{loxp/ko}$; mb1-Cre$^{ki/+}$ mice and their *Hdac7*$^{+/ko}$; mb1-Cre$^{ki/+}$ littermate control mice. Microarray analysis revealed that 3193 genes were differentially expressed in HDAC7-deficient pro-B cells, of which, 1750 were up-regulated and 1443 were down-regulated. Among the up-regulated genes, we found some genes that coded for transcription factors, chromatin-associated factors, myeloid-specific proteins, signal transducers, T-cell related proteins, and genes related to the cell cycle and ubiquitination processes (Table 1R). On the basis of our previous findings demonstrating that HDAC7 is involved in the repression of macrophage genes in pre-B cells, we examined the list of up-regulated genes and looked for the presence of myeloid genes and of other hematopoietic cell-lineage genes. Strikingly, we observed that the absence of HDAC7 from pro-B cells resulted in the up-regulation of key genes for macrophage functions, such as *Itgam, Itgax, Ifi204, Tet2, Ccl3, Ccl4* and *Ccr2*, among others (Table 1R). We also observed the up-regulation of many transcription factors, several of which are known to play a role in myeloid cell differentiation (*Fosb, Egr1, Crebzf, Cebpb, Cebpd* and *Cebpz*).

Transcription Factors

	Gene	Log2 Fold change
***Ap-1* Family**	*FosB*	4,6
	Fos	3,2
	Jun	2,7
	Jun B	1,8
	Jund	1,0
***C/ebp* Family**	*Cebpb*	0,7
	Cebpz	0,6
	Cebpd	0,7
***Runx* Family**	*Runx1*	0,5

	Runx2	1,2
	Runx3	0,7
***Notch* Family**	*Notch2*	0,7
	Notch3	0,7
***Klf* Family**	*Klf2*	1,6
	Klf4	2,0
	Klf6	2,18
	Klf7	0,84
***Zeb* Family**	*Zeb1*	1,7
	Zeb2	1,3
Others	*Foxp1*	1,4
	Egr1	3,5
	Nfat5	0,6
	Mef2a	0,7
	Crebzf	1,0
	Stat3	1,6
	Ikzf2	0,5

Chromatin-associated Factors	
Gene	**Log2 Fold change**
Brd1	0,65
Brd8	1,0
Cbx4	0,6
Chd2	1,0
Chd6	0,7
Chd8	1,0
Ezh1	0,6
Kdm2a	1,1
Kdm5a	0,6
Kdm6a	1,66
Kdm6b	1,8
Mll1	0,51
Mll2	1,0
Mll3	0,6
Mll5	0,72
Mllt10	0,6
Mllt3	0,55
Setd2	0,74
Setd3	1,0
Setd6	0,6
Setd8	1,0

	Smarca2	0,71
	Smarce1	1,22
	Suv39h2	0,56
	Suv420h1	0,8
	Tet2	1,0
	Jhdm1d	1,0
	Jmjd1c	1,52
	Jmjd6	0,8
	Hdac9	0,82
	Mbd1	0,92
	Mbd6	0,82
	Hdac9	0,82

Myeloid-related genes

	Gene	Log2 Fold change
	Ccl3	1,2
	Ccl4	1,2
	Ccr2	0,63
	Ccr6	0,52
	Ccr9	1,2
	Ccrl1	0,52
	Ccrl2	0,6

	Cd33	0,92
	Itgam	1,3
	Itgax	0,7
	Fcgr4	0,8
	Csf2ra	1,4
	Csf3r	0,8
	Cr1l	0,7

Inflamatory-response genes

	Gene	Log2 Fold change
	Il10ra	0,75
	Il12rb2	0,85
	Il17rb	0,56
	Il1f9	0,76
	Il1r2	1,1
	Il1rn	0,55
	Ifi203	0,55
	Ifi204	1,13
	Ifng	1,6

	Tnfaip2	1,0
	Tnfaip3	1,4

T cell-related genes

	Gene	Log2 Fold change
	Cd28	0,77
	Nfat5	0,85
	Lck	0,8
	Sla2	0,84

Intracellular signal cascade

	Gene	Log2 Fold change
***Mapk* pathway**	*Map3k2*	0,85
	Map3k8	1,34
	Map4k4	0,95
	Map4k5	1,0
	Mapk14	0,6
	Mapk1ip1	0,72
	Mapk1ip1l	1,6
	Mapk6	1,6
	Mapk8ip3	0,80
Nfkb Family	*Nfkbia*	0,92
	Nfkbiz	2,3
	Nfrkb	0,66
	Ikbkb	0,72
	Ikbkg	0,7
***Pkc* family**	*Prkca*	0,7
	Prkcb	0,7
	Prkcc	0,9
	Prkcd	0,8
	Prkd3	0,6

Protein Ubiquitination

	Gene	Log2 Fold change
	Cblb	1,2
	Birc3	1,2
	Birc6	1,8
	Ube2j2	1,3
	Ube2b	1,4
	Ubr2	0,6

	Ube2i	0,8
	Mdm2	1,4
	Malt1	1,1
	Cbl	0,8
	Cnot1	0,93
	Cnot2	0,8
	Cnot4	1,0
	Cnot7	0,83

Table 1R: Changes in gene expression of important myeloid genes in the absence of HDAC7. Microarray analysis revealed that 1750 genes were up-regulated in HDAC7-deficient pro-B cells. Among the up-regulated genes there were some genes that coded for transcription factors, chromatin-associated factors, myeloid-specific proteins, signal transducers, T-cell related proteins, and genes related to the cell cycle and ubiquitination processes.

Cell cycle related genes

Gene	Log2 Fold change
Bcl2	1,1
E2f5	1,0
Gadd45a	1,39
Gadd45b	0,51
Gadd45g	0,7
Pten	1,0
Trp53	1,1
Cdc40	0,5
Cdc42	1,23
Cdk13	0,7
Cdk16	0,73
Ccnc	0,62
Ccnd1	0,9
Ccni	0,7
Ccnl2	0,61
Ccnt2	0,74

Importantly, we also found that HDAC7 deficiency led to the up-regulation of genes characteristics of T lymphocytes, such as *Cd28, Cd69, Il*17 and *Lck*, and the T-cell transcription factors *Runx1, Runx3* and *Nfat5*.

To analyze further the type of genes that were upregulated in the absence of HDAC7 we performed a gene set enrichment analysis based on the gene ontology (GO) categories corresponding to Biological Processes, and on the KEGG pathways (Figure 33R).

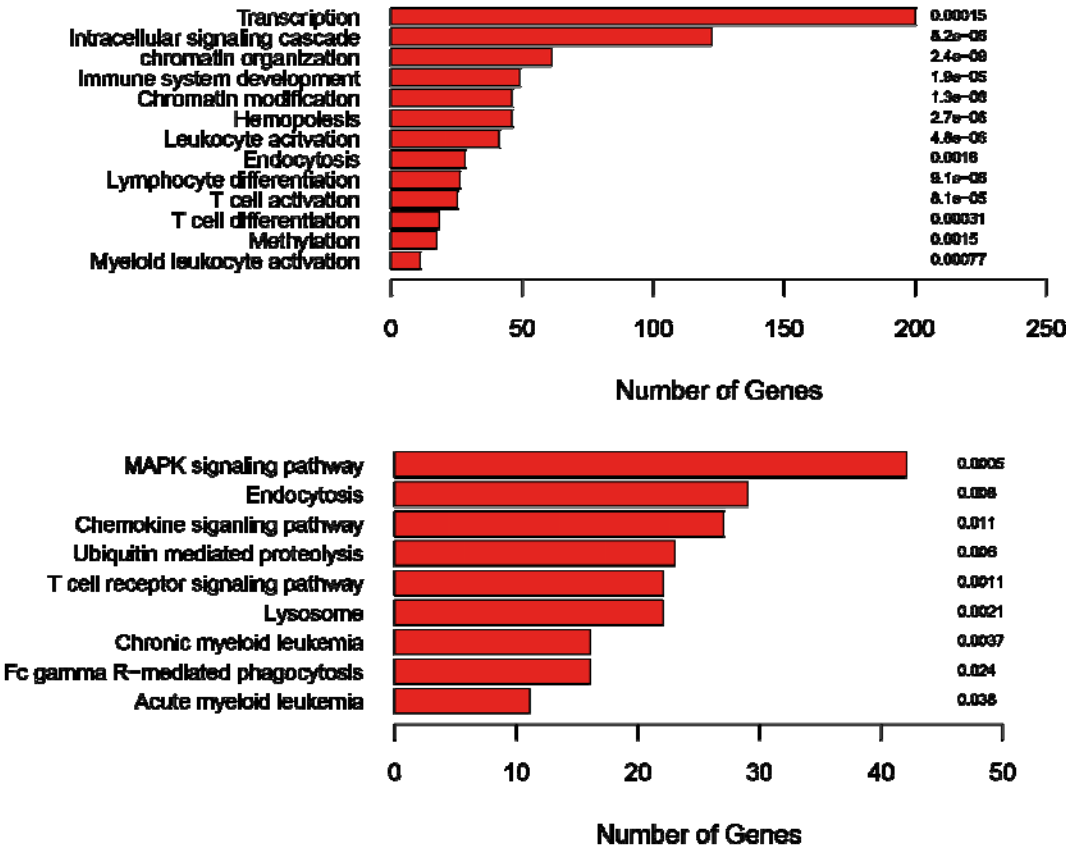

Figure 33R: Changes in the regulatory network of B cells and macrophages in the absence of HDAC7. Heat-maps showing significantly (corrected p-value < 0.05) enriched

GO Biological Process and Molecular Functions categories among the up-regulated genes altered in the absence of HDAC7.

The biological processes analysis revealed that the set of genes upregulated in the absence of HDAC7 belong to GO categories representing chromatin modification, hemopoiesis, leukocyte activation, intracellular signaling cascade, lymphocyte differentiation, immune sytem development and T cell activation and differentiation processes. The KEGG pathway enrichment analysis showed that the set of upregulated genes were enriched in MAPK, T cell receptor signaling, lysozyme, ubiquitin-mediated proteolysis, endocytosis, chemokine signaling and Fc gamma-mediated phagocytosis pathways. Changes in expression of selected genes (*Fosb*, *Jund*, *Junb*, *Crebzf, Klf4, Egr1, Cebpβ, Cebpz, Tet2, Itgam, Ccl3, Sykb, Map3k8, Nfkbid, Nfkbiz, Cd28, Usp7 and Mdm2*) were validated by qRT-PCR (Figure 34R). Together, these data demonstrate that HDAC7 is a crucial transcriptional repressor of lineage-inappropriate genes in B cell progenitors.

Comparison of the number of genes up-regulated in HDAC7-deficient pro-B cells and genes whose up-regulation was blocked by the presence of HDAC7 following 48 and 72 hours of β-estradiol treatment of C10 cells showed an overlap of 213 genes (representing 12% of the up-regulated genes in HDAC7-deficient pro-B cells), such as *Fos, Jun, Cebpd, Ccr2, Ccrl1, Ccrl2, Cd33, Fcgr4, Tlr7*, *Mapk3k8* and *Gadd45b* among others (Figure 35R). This finding corroborates the involvement of HDAC7 in the repression of genes from other hematopoietic lineages such as macrophages.

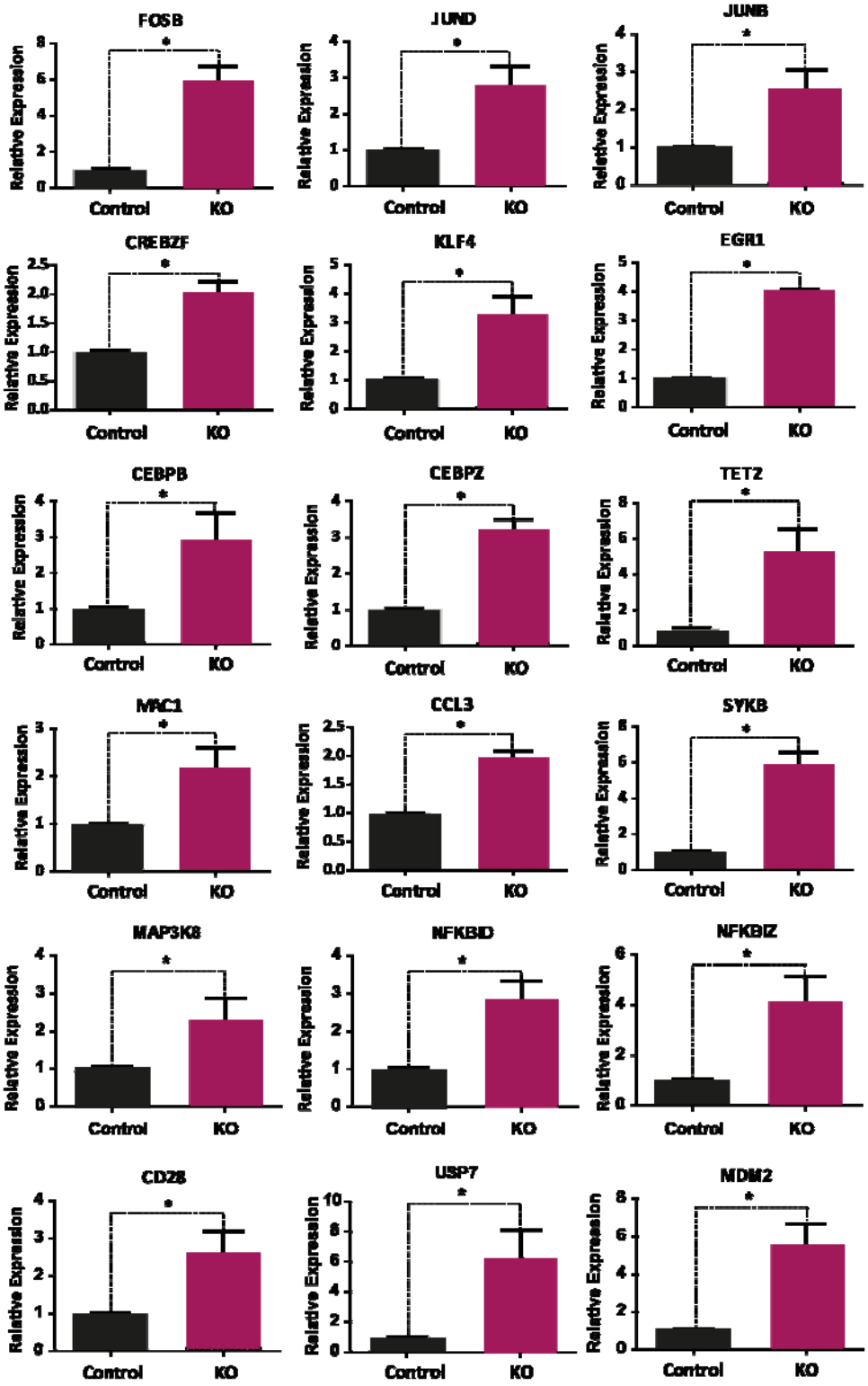
FOSB
JUND
JUNB
CREBZF
KLF4
EGR1
CEBPB
CEBPZ
TET2
MRC1
CCL3
SYKB
MAPSK8
NFKBID
NFKBIZ
CD28
USP7
MDM2
Relative Expression
Control
KO
*

Figure 34R: Validation by qPCR of selected genes whose expression were altered in the absence of HDAC7. Changes in expression of selected genes (*Crebzf, Klf4, Fosb, Egr1, Cebpβ, Tet2, Cd28 and Mdm2*) were validated by qRT-PCR.

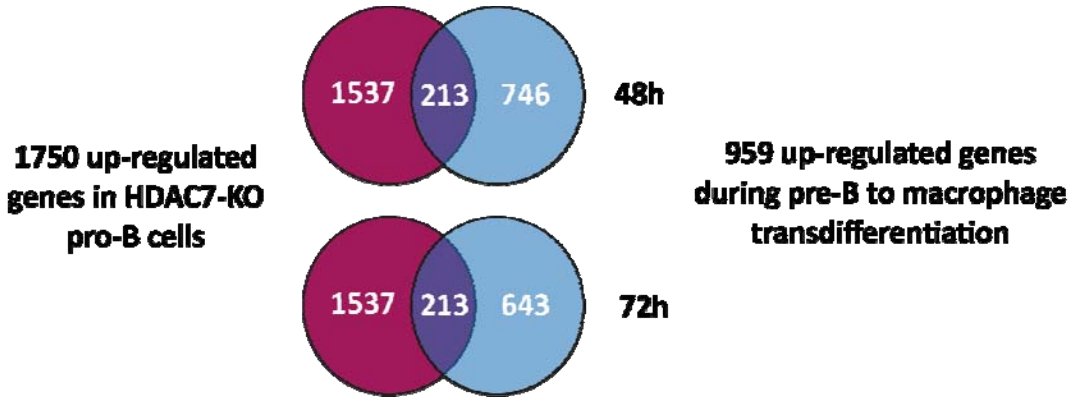

Figure 35R: HDAC7 deficiency leads to the up-regulation of macrophage-associated genes in pro-B cells. Venn diagrams showing the number of genes up-regulated in HDAC7 knockout pro-B cells and genes up-regulated after 48 and 72 hours of β-estradiol treatment during pre-B-to-macrophage transdifferentiation affected by HDAC7 and the overlap between the two models.

2.7. Molecular mechanisms underlying HDAC7 repression in pro-B lymphocytes

As mentioned above, class IIa HDACs exert their actions as transcriptional repressors by interacting with tissue-specific transcription factors, leading to them being recruited to the promoters of genes required for development and cell differentiation (Parra, 2010; Martin, 2009). We have shown that HDAC7 specifically interacts with the transcription factor MEF2C and not with other B cell-specific transcription factors in B cell precursors. This prompted us to consider whether HDAC7 interacts physiologically with MEF2C and is recruited to the promoter of non-lymphoid genes in pro-B cells, leading to their transcriptional silencing. To test this, we performed chromatin immunoprecipitation

(ChIP) experiments. First, using the TFconsite bioinformatic tool we found that promoters of *Itgam*, *Fosb, Cd69, Cd28, Ccl3, Mdm2* and *Egr1* contain putative MEF2 binding sites. We designed primers spanning the upstream regulatory regions containing the MEF2 putative binding sites of the different gene promoters. Chromatin prepared from wild type pro-B cells was subjected to ChIP assay with specific antibodies for MEF2C and HDAC7. qRT-PCR analysis of the immunoprecipitated material with specific primers for the *Itgam*, *Cd69, Cd28, Ccl3, Mdm2* and *Egr1* loci indicated that MEF2C and HDAC7 were both significantly enriched at the identified putative MEF2 binding sites in the gene promoters in pro-B cells (Figure 36R). Taken together, these results imply that HDAC7 is recruited to the promoters of lineage inappropriate genes in pro-B cells via interaction with MEF2C, resulting in their transcriptional silencing.

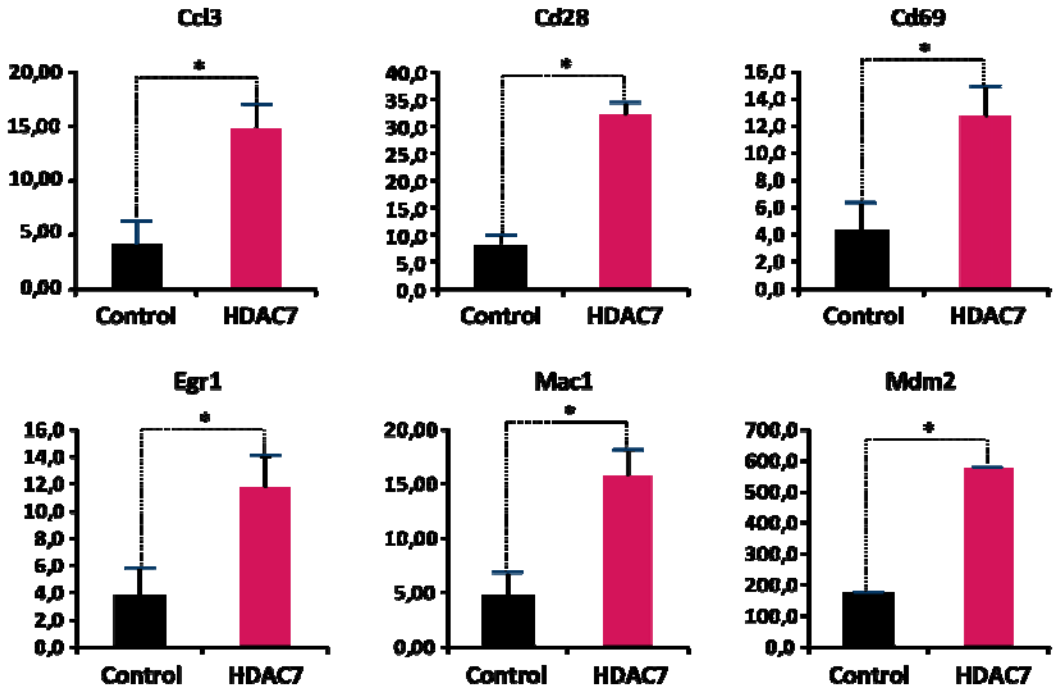

Figure 36: HDAC7 binds to the transcription factor MEF2C and is recruited to the promoter of non-lymphoid genes in B cells. Chromatin immunoprecipitation experiments

showing the enrichment of HDAC7 and MEF2C to putative MEF2 binding sites on the the *Itgam*, *Cd69, Cd28, Ccl3, Mdm2* and *Egr1* gene loci in B cell precursors. Results are presented as percentage immunoprecipitated over input and are representative of three independent experiments.

2.8. Analysis of the responsiveness of HDAC7-deficient pro-B cells *ex-vivo*

Having found that early pro-B cell development is blocked in the absence of HDAC7, we speculated that pro-B cells derived from HDAC7 knockout mice may be defective in IL-7R signaling, which is crucial for the proliferation of early B cell progenitors (Corcoran, 1996). In fact, we found that pro-B cells from HDAC7$^{loxP/ko}$; mb1-Cre$^{ki/+}$ mice failed to express the proper levels of IL-7Rα, whereas the control pro-B cells showed a higher level of expression of IL-7Ra (Figure 37R-Left panel). MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)) assays revealed that HDAC7-deficient pro-B cells failed to proliferate in *ex vivo* cultures, in comparison with wild type cells (Figure 37R-Right panel).

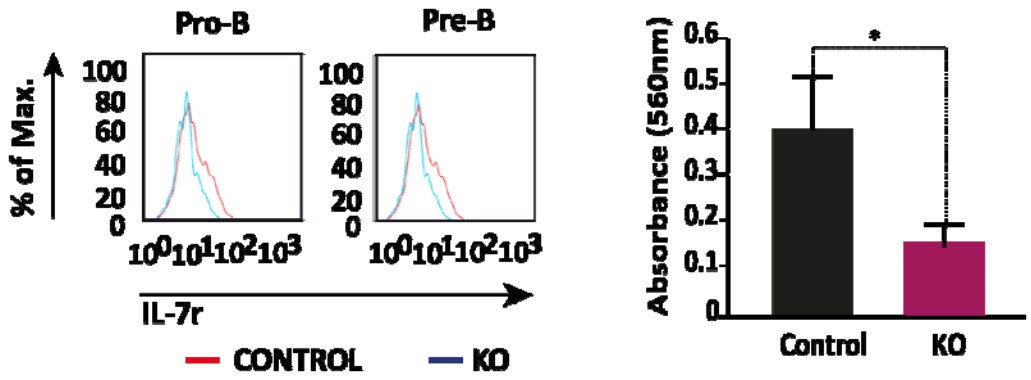

Figure 37R: Pro-B cells lacking HDAC7 fail to up-regulate IL-7Rα expression and, thus to proliferate in ex-vivo cultures. Il7r+ cells and MTT assays in both control and knock-out mice.

This set of results suggests that HDAC7-deficient pro-B cells were defective in proliferation as a result of the impairment in IL-7 signaling. Since the absence of HDAC7 from B cell progenitors results in the up-regulation of genes from other lineages, such as macrophages, we wondered whether the cells exhibit lineage promiscuity and express macrophage cell surface markers in *ex vivo* cultures. To test this possibility, pro-B cells were isolated from bone marrow from HDAC7$^{+/ko}$; mb1-Cre$^{ki/+}$ or HDAC7$^{loxP/ko}$; mb1-Cre$^{ki/+}$ mice. We cultured the purified cells on S17 stromal cells in the presence or absence of IL-7. Strinkingly, we observed that pro-B cells lacking HDAC7 expressed the macrophage marker Mac-1 after six days of culture (Figure 38R).

In summary, our findings indicate that, in the absence of HDAC7, early B cell development is blocked at the pro-B cell stage. Pro-B cells from HDAC7$^{loxP/ko}$; mb1-Cre$^{ki/+}$ mice failed to proliferate compared with the control pro-B cells and express surface markers from other hematopoietic lineages.

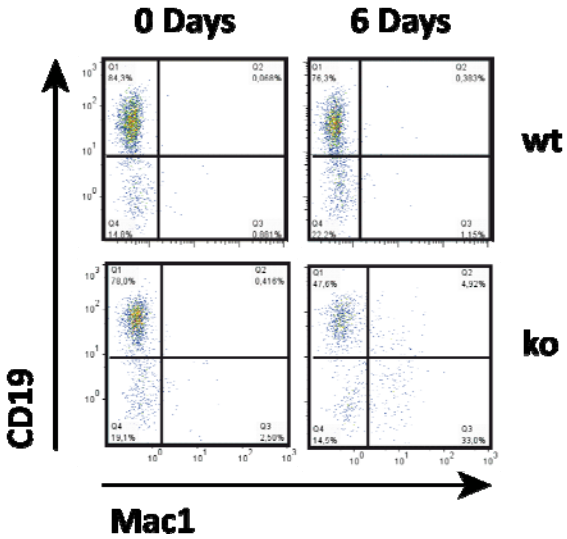

Figure 38R: Pro-B cells lacking HDAC7 express Mac-1. Mac1 and CD19 expression after three days of culture with myeloid cytokines.

PART IV

DISCUSSION, CONCLUSION AND REFERENCES

Discussion

Within the hematopoietic system, the generation of B lymphocytes is a sophisticated process involving cell lineage specification, commitment and differentiation. This is achieved by the action of B cell transcription factors responsible for activating specific genes characteristic of each particular cellular stage of B cell development. However, in order to acquire a particular "cellular identity" the cells also need to repress genes from other lineages. We know that these master regulators of B cell development also mediate the repression of lineage-inappropriate genes. However, the means by which B cell transcription factors mediate the process of gene silencing to acquire and maintain cellular identity is poorly understood. The main goal of this doctoral project was to elucidate the mechanisms of gene transcriptional repression occurring in B lymphocytes. We decided to study the potential contribution of the class IIa sub-family of HDACs to B cell biology. Class IIa HDACs might be suspected of being likely to participate in B cell development for several reasons: first, they are tissue-specific transcriptional repressors; second, they are involved in development and differentiation processes; and third, they exert their repressive actions by interacting with tissue-specific transcription factors that lead to the recruitment and silencing of target genes. To achieve our initial objective we adopted an *in vitro* experimental approach featuring a highly efficient immune reprogramming system and an *in vivo* experimental approach involving conditional knockout mouse models.

1. Role of HDAC7 during the reprogramming of pre-B cells into macrophages

The reprogramming of a particular differentiated cell into other cellular lineages is possible due to the action of regulatory networks of transcription factors. The means by which transcription factors modulate enhancers and promoters to establish a new gene transcription program has been widely studied and discussed in many reports and their action is recognized as being essential for correct cellular

reprogramming (Cantone, 2013; Papp, 2013; Van Oevelen, 2013). Using a previously described immune reprogramming system (Bussmann, 2009) we demonstrated that HDAC7 is highly expressed in B cell precursors but not in myeloid cells, such as macrophages. More importantly, depletion of HDAC7 in pre-B cells results in the derepression of key macrophage genes. Functionally, the presence of HDAC7 interferes with the acquisition of key functional features of the converted macrophages. These aberrant macrophage-converted cells do not express the Mac-1 protein levels normally present in macrophages, are not able to phagocytose bacteria properly and do not respond adequately to endotoxin by expressing major pro-inflammatory cytokines. At the mechanistic level, HDAC7 specifically interacts with the transcription factor MEF2C in pre-B cells and is recruited to MEF2 binding sites located at the promoters of genes critical to macrophage function. In addition to the interaction with MEF2C, the catalytic activity of HDAC7 is also necessary to repress macrophage genes. Our results demonstrate that HDAC7 is expressed in fetal and adult pre-B cells, suggesting that it might play a role in both types of B lymphopoiesis (Figure 2D).

From a mechanistic perspective, it is well established that class IIa HDACs, such as HDAC7, interact with tissue-specific transcription factors and this complex is recruited to important genes for cell development and differentiation (Martin, 2009; Parra, 2010). In the late 1990s, MEF2C was found, among the various MEF2 family members, to be specifically expressed in B cells within the lymphocyte lineage, suggesting that it could have a role in B cell development and function (Swanson, 1998). More recently, it has been reported that MEF2C regulates B cell proliferation and survival after BCR activation and p38 MAPK signaling (Khiem, 2008; Wilker, 2008). MEF2C is also expressed at earlier stages of lymphocyte development (Gekas, 2009; Stehling-Sun, 2009). Moreover, Stehling-Sun *et al.* recently reported that the transcription factor MEF2C is critical in lymphoid development, acting at the branching point where cells become committed to the lymphoid or myeloid lineages (LMPP stage). The absence of MEF2C in LMPPs resulted in the up-regulation of genes

related to immune and inflammatory response and cellular response to infections (Stehling-Sun, 2009). At the molecular level MEF2C is involved not only in the activation of lymphoid genes, but also in the repression of inappropriate ones, such as myeloid genes (Gekas, 2009). Using a Venn diagram to compare the genes whose up-regulation was impaired in the presence of HDAC7 during the conversion of pre-B cells into macrophages with the set of genes reported by Stehling-Sun (2009), we found a overlap, which was shown to be significant by a chi-square test, of 46 ($p < 10^{-6}$) and 30 ($p = 0.00044$) genes, respectively. Interestingly, several genes associated with phagocytosis (such as *Fcgr1*, *Fcgr2b* and *Fcgr3*) were targets for both HDAC7 and MEF2C. These results indicate that MEF2C and HDAC7 both silence inappropriate genes in hematopoietic precursors during B lymphocyte development. Lin *et al.* recently used ChIP-Seq analysis to describe the transcriptional regulatory targets of E2A, EBF1 and FOXO1 in pro-B cells and showed that HDAC7 contains binding sites for these transcription factors in putative regulatory elements, thereby demonstrating that HDAC7 is a direct target of these three transcription factors and suggesting that they may be involved in B lymphocyte development (Lin, 2010).

Our results show that, in pre-B cells, HDAC7 specifically interacts with MEF2C and is recruited to the MEF2 sites at the promoter of important macrophage genes, resulting in their transcriptional repression during B cell development. Moreover, we have demonstrated that the MEF2 interaction motif and the catalytic activity of HDAC7 are both essential for repressing inappropriate genes during the cellular reprogramming of pre-B cells into macrophages. We have therefore demonstrated that HDAC7 is an important regulator factor in B lymphocytes by acting as a transcriptional co-repressor that facilitates the silencing of lineage-inappropriate genes, such as myeloid genes.

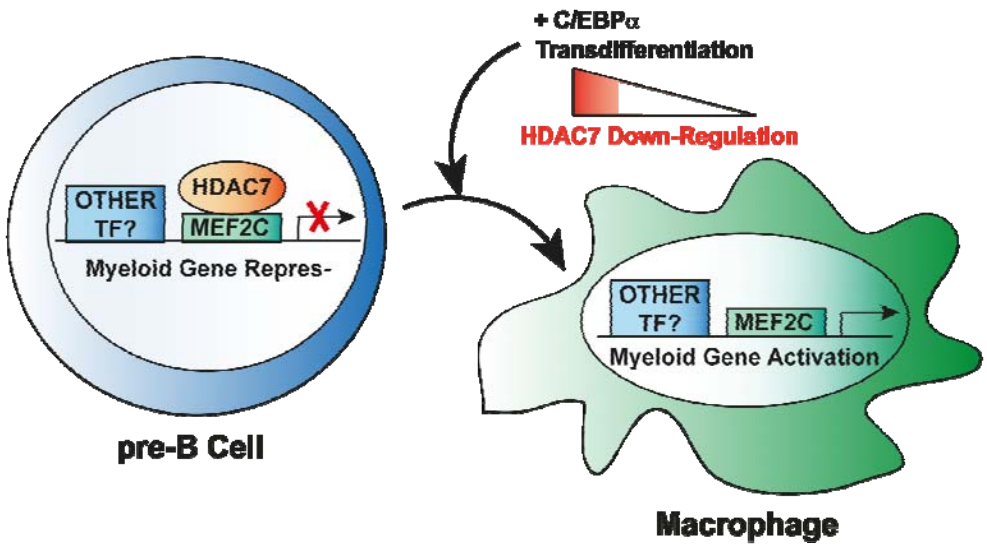

Figure 1D: Model for HDAC7-mediated transcriptional repression in pre-B cells. HDAC7 is expressed in pre-B cells and not in macrophages. In pre-B cells, HDAC7 specifically interacts with the transcription factor MEF2C and is recruited to promoters of myeloid genes. During the transdifferentiation of pre-B cells into macrophages HDAC7 is down-regulated allowing the expression of macrophage-specific genes.

Finally, in light of previous reports demonstrating the expression and functions of HDAC7 in T cells, our results have uncovered a more general role for HDAC7 in the lymphoid lineage compartment within the hematopoietic system. In addition, the finding that HDAC7 is dramatically down-regulated during the transdifferentiation of pre-B cells into macrophages not only reinforces this notion, but also reveals an additional level of complexity to the way its activity and function are regulated. During T cell development in the thymus and later in a differentiated and specialized T cell type, the cytotoxic T lymphocytes (CTLs), HDAC7 is regulated in a signal-dependent manner that is responsible for its phosphorylation and subsequent nucleo-cytoplasmic distribution (Dequiedt, 2003; Dequiedt, 2005; Parra,

2005; Kasler, 2007; Kasler, 2011; Navarro, 2011). In resting thymocytes, HDAC7 is localized in the nucleus, where it represses the expression of a large number of genes involved in the positive (survival) and negative (apoptosis) selection of the cells. However, in response to TCR signaling, HDAC7 becomes phosphorylated and translocates to the cytoplasm, where it can no longer repress its target genes (Dequiedt, 2003; Dequiedt, 2005; Parra, 2005; Kasler, 2007). Later, in the periphery, HDAC7 is constitutively phosphorylated and found in the cytoplasm of CTLs, enabling the expression of key genes for the function of this specialized T cell type (Navarro, 2011). We may postulate that, within a particular hematopoietic cellular lineage, the HDAC7 gene transcriptional repressive function is regulated in a signal-dependent way by controlling its phosphorylation status and altering its cellular distribution. In other words, HDAC7 is unphosphorylated or phosphorylated and localized either in the cell nucleus or in the cytoplasm depending on the requirement to silence or activate specific genes at different stages of T cell development. In contrast, at the branching points where cellular lineage has to be determined, e.g. lymphoid versus myeloid, HDAC7 may be regulated at the expression level. Our results showing that HDAC7 expression is down-regulated during the conversion of pre-B cells into macrophages further support this hypothesis. However, as is the case for T cells, we cannot rule out the possibility that HDAC7 is also regulated in a signal-dependent manner during B cell development and differentiation. In this regard, we found that HDAC7 is expressed in the nucleus of pre-B cells (data not shown), probably because there is no fully functional B cell receptor (BCR). However, it is highly probable that HDAC7 function is also regulated via BCR signaling at later cell differentiation stages when B cells develop into mature antibody-secreting cells. Therefore, we propose that, in the hematopoietic system, HDAC7 is not only a signal-dependent transcriptional repressor involved in different developmental steps of a particular lymphocyte type, but also a lineage-specific transcriptional repressor responsible for maintaining the identity of lymphocytes by silencing lineage-inappropriate genes.

2. Role of HDAC7 in B cell development

The development of B lymphocytes in the bone marrow occurs in a stepwise manner, with critical checkpoints tightly regulated at the transcriptional level. Proper expression of B lineage-specific genes and the repression of non-lymphoid genes are both essential for B cell commitment, survival, proliferation and differentiation (Cobaleda, 2007; Nutt, 2007; Stehling-Sun, 2009). Here, we showed that *in vivo* deletion of HDAC7 in early B cell progenitors results in a block in B cell development, demonstrating that HDAC7 plays an essential role in B lymphopoiesis. As mentioned above, HDAC7 specifically interacts with the transcription factor MEF2C, leading to the repression of non-lymphoid genes in a pre-B cell line. We found that, in primary pro-B cells, HDAC7 is recruited to MEF2 binding sites located at the promoters of important macrophage and T cell-related genes, such as Itgam, Cd69, Cd28, Ccl3 and Egr1. This group of genes was shown to be MEF2C targets in LMPPs (Stehling-Sun, 2009). Therefore, we may conclude that, in our knockout mouse model, MEF2C cannot recruit HDAC7 and so non-lymphoid genes (such as those that are related to myeloid and T cell transcription factors, or involved in chromatin modification, protein ubiquitination, intracellular signal transduction, and cell cycle regulation, and myeloid-related genes) become activated, resulting in a block of B cell development (Figure 2D). Moreover, our microarray experiments clearly confirmed that HDAC7 is an important and essential transcriptional repressor in pro-B cells. We found that in pro-B cells lacking HDAC7, 1750 genes became up-regulated relative to the wild-type pro-B cells. A gene-set enrichment analysis based on the GO categories demonstrated that, in the absence of HDAC7, most of the up-regulated genes corresponded to important biological processes and molecular functions related to other cellular lineages, such as macrophages and T lymphocytes. Moreover, not only genes associated with important macrophages functions, but also those involved in functions essential for cell survival and development were altered in the absence of HDAC7. In fact, we showed that genes related to transcription factors, chromatin

modification, protein ubiquitination, intracellular signal transduction and cell cycle regulation were all altered in the absence of HDAC7. We found a significant overlap between the set of genes up-regulated in HDAC7-deficient pro-B cells and the genes whose up-regulation was blocked by the presence of HDAC7 in our transdifferentiation assays. In addition, HDAC7-deficient pro-B cells show myeloid-lineage promiscuity and express the macrophage marker Mac-1 in *ex vivo* cultures. Together, our observations demonstrate that HDAC7 is a repressor of lineage-inappropriate genes in B lymphocytes.

We found that the absence of HDAC7 had no significant effect on the expression of B cell master regulators, such as PAX5, E2A, EB1 and IKAROS. The absence of HDAC7 leads to a dramatic block in B lymphopoiesis even though essential B cell transcription factors are present. Therefore, we can conclude that HDAC7 is a novel master regulator in B cell development. We also obtained strong evidence that the repression of lineage-inappropriate genes is also an essential mechanism for the correct development of a specific cellular type within the hematopoietic system. In this regard, one of the most important examples is PAX5, a master regulator involved in activating lymphoid genes and repressing specific genes from other cellular lineages, in order to ensure the fate and maturation of B cells. In fact, PAX5 is considered the 'guardian of B cell identity' (Cobaleda, 2007). In a recent ChIP-Seq analysis, Revilla-I-Domingo *et al.* reported that HDAC7 is a target of PAX5 in pro-B cells, and specifically that PAX5 induces HDAC7 expression (Revilla-I-Domingo, 2012). The mechanism by which PAX5 mediates the repression of lineage-undesirable genes in B lymphocytes is currently poorly understood. It is thought that PAX5 interacts and mediates the recruitment of transcriptional repressors to non-lymphoid genes. Our findings add to our knowledge of the molecular mechanisms involved in PAX5-mediated gene silencing. We propose that PAX5 induces the expression of a transcriptional repressor, HDAC7, which is in turn responsible for repressing genes from other cellular lineages, thereby ensuring the acquisition of a proper B cell genetic identity.

In summary, we have identified HDAC7 as a lymphoid-lineage specific transcriptional repressor that acts as a master regulator in B lymphocyte Biology.

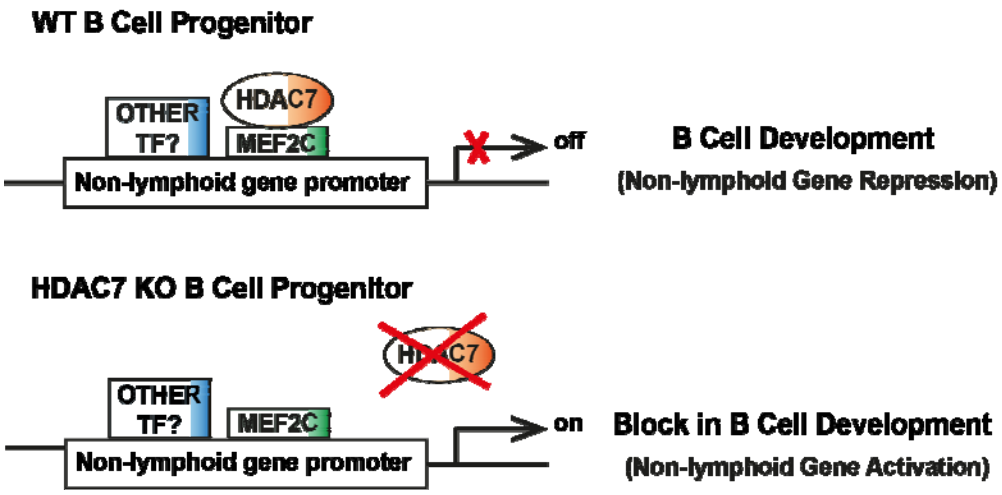

Figure 2D: Model for HDAC7-mediated transcriptional repression of non-lymphoid genes in pro-B cells. HDAC7 specifically interacts with MEF2C and this interaction leads to the activation of non-lymphoid genes, resulting in a block of B cell development.

Conclusion

1. Role of HDAC7 during the reprogramming of pre-B cells into macrophages.

1. HDAC7 is highly expressed in B cell precursors (pre-B cells) but not in myeloid cells, such as macrophages.

2. During the cellular conversion of pre-B cells into macrophages HDAC7 expression is down-regulated.

3. HDAC7 re-expression interferes with the up-regulation of key macrophage genes during the reprogramming of pre-B cells into macrophages

4. HDAC7 re-expression interferes with the functional properties of the reprogrammed macrophages.

5. HDAC7 knockdown in pre-B cells results in the de-repression of key myeloid genes, such as *Itgam, Fcgr1* and *Ccl3*.

6. In pre-B-cells, HDAC7 specifically interacts with MEF2C and is recruited to the MEF2 sites at the promoters of important macrophage genes resulting in their transcriptional repression.

7. Both MEF2 and the catalytic domain of HDAC7 are essential for the repression of inappropriate genes during the cellular reprogramming.

2. Role of HDAC7 in B cell development.

1. HDAC7-deficient mice show a block in B cell development at the pro-B cell stage.

2. From a mechanistic angle, HDAC7 is recruited to the MEF2 sites located at the promoters of lineage inappropriate genes in pro-B cells.

3. HDAC7 deficiency in pro-B cells results in the up-regulation of many genes from alternative lineages, such as macrophages and T lymphocytes.

4. The absence of HDAC7 has no significant effect on the expression of B cell-specific transcription factors, such as PAX5, E2A, EB1 and IKAROS.

5. HDAC7 deficiency in pro-B cells results in lower expression of IL-7R and reduced cell proliferation.

6. HDAC7 deficient pro-B cells show cellular lineage promiscuity and express Mac-1 macrophage marker in *ex vivo* cultures.

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ANNEX 1

LIST OF FIGURES AND ABBREVIATIONS

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Introduction

Results

Abbreviations

ABBREVIATIONS

ALL	Acute lymphoblastic leukemia
BCR	B-cell receptor
BM	Bone marrow
Bp	Base pair
BP	Biological processes
cDNA	Complementary deoxyribonucleic acid
ChIP	Chromatin immunoprecipitation
CLPs	Common myeloid progenitors
CMPs	Common myeloid progenitors
CRCs	Chromatin remodeling complexes
CSR	Class-switch recombination
DNA	Deoxyribonucleic acid
FO	Follicular B cell
GDH	Glutamate dehydrogenase
GMPs	Granulocyte/macrophage progenitors
GO	Gene ontology
HAT	Histone acetyltransferase
Hda1	Histone deacetylase 1
HDAC	Histone deacetylase
HDI	Histone deacetylase inhibitor
HSCs	Hematopoietic stem cells
Immgen	Immunological genome project database
KO	Knock-out
LMPPs	Multipotent lymphoid primed progenitors
LPS	Lipopolysaccharide
Mb	Megabase
MEF2	Myocyte enhancer factor 2

MEPs	Megakaryocytes/erythrocyte progenitors
MF	Molecular functions
MFI	Mean fluorescence intensity
MTT	(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MZ	Marginal zone B cell
NES	Nuclear export signal
NK	Natural killer
NLS	Nuclear localization signal
NuRD	Nucleosome remodeling and deacetylase
PB	Peripheral blood
PCR	Polymerase chain reaction
PKB	Protein Kinase B
PKC	Protein kinase C
PKD1	Protein kinase D1
PP	Protein phosphatase
PTM	Post-translational modification
qPCR	quantitative PCR
RNA	Ribonucleic acid
Rpd3	Reduced potassium dependency 3
RT-qPCR	Real time- quantitative PCR
siRNA	Small interference ribonucleic acid
SIRT	Sirtuin
SPL	Spleen
SWI/SNF	Switch/sucrose non-fermenter
T	Transitional B cell
TCA	Tricarboxylic acid
TCR	T-cell receptor
TH	Thymus
Treg	Regulatory T cells

VPA	Valproic acid
Wt	Wild type
YY1	Ying yang 1

ANNEX 2

PUBLICATIONS

LIST OF PUBLICATIONS

1. 'HDAC7 Is a Repressor of Myeloid Genes Whose Downregulation Is Required for Transdifferentiation of Pre-B Cells into Macrophages'.

   Barneda-Zahonero, B.,* **Roman-Gonzalez, L.,*** Collazo, O., Rafati, H., Islam, ABMMK., Bussmann, LH., di Tullio, A., De Andres, L., Graf, G., López-Bigas, N., Mahmoudi, T., Parra, M.

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   *Both authors have equally contributed to this work.

2. 'Epigenetic regulation of B lymphocyte differentiation, transdifferentiation, and reprogramming'.

   Barneda-Zahonero B, **Roman-Gonzalez L**, Collazo O, Mahmoudi T, Parra M.

   Comp Funct Genomics. 2012; 2012:564381.

3. 'Identification of HDAC7 as a novel therapeutic target in B cell acute lymphoblastic leukemia and B cell lymphoma.'

   Barneda-Zahonero, B., Collazo, O., Serra, J., Islam, ABMMK, Gomez, A., **Roman-Gonzalez, L**., Vidal, A., Lopez-Bigas, N., Villanueva, A., Esteller, M., Parra, M.

   Manuscript submitted to Blood.

4. 'HDAC7: A novel master regulator in B lymphocyte development'.

   This manuscript, which I will be the first author, reports the role of HDAC7 in B lymphocyte development by using an *in vivo* approach. I have generated a conditional knockout mouse for HDAC7 in B cell progenitors and have observed that the absence of HDAC7 results in a dramatic block of B cell development. The manuscript is in preparation and I intend to submit it to Science in May 2014.

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HDAC7 Is a Repressor of Myeloid Genes Whose Downregulation Is Required for Transdifferentiation of Pre-B Cells into Macrophages

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Abstract

B lymphopoiesis is the result of several cell-commitment, lineage-choice, and differentiation processes. Every differentiation step is characterized by the activation of a new, lineage-specific, genetic program and the extinction of the previous one. To date, the central role of specific transcription factors in positively regulating these distinct differentiation processes to acquire a B cell–specific genetic program is well established. However, the existence of specific transcriptional repressors responsible for the silencing of lineage inappropriate genes remains elusive. Here we addressed the molecular mechanism behind repression of non-lymphoid genes in B cells. We report that the histone deacetylase HDAC7 was highly expressed in pre-B cells but dramatically down-regulated during cellular lineage conversion to macrophages. Microarray analysis demonstrated that HDAC7 re-expression interfered with the acquisition of the gene transcriptional program characteristic of macrophages during cell transdifferentiation; the presence of HDAC7 blocked the induction of key genes for macrophage function, such as immune, inflammatory, and defense response, cellular response to infections, positive regulation of cytokines production, and phagocytosis. Moreover, re-introduction of HDAC7 suppressed crucial functions of macrophages, such as the ability to phagocytose bacteria and to respond to endotoxin by expressing major pro-inflammatory cytokines. To gain insight into the molecular mechanisms mediating HDAC7 repression in pre-B cells, we undertook co-immunoprecipitation and chromatin immunoprecipitation experimental approaches. We found that HDAC7 specifically interacted with the transcription factor MEF2C in pre-B cells and was recruited to MEF2 binding sites located at the promoters of genes critical for macrophage function. Thus, in B cells HDAC7 is a transcriptional repressor of undesirable genes. Our findings uncover a novel role for HDAC7 in maintaining the identity of a particular cell type by silencing lineage-inappropriate genes.

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Introduction

The generation of B cells is the result of several cellular transitions that take place in a stepwise manner and comprise cell lineage choices, cell commitment and differentiation. Every differentiation step leads to the activation of specific genes characteristic of the new cellular stage. This is achieved by the action of well defined networks of transcription factors specific to each particular cellular state [1,2]. In the bone marrow, lymphocyte development begins at the lymphoid-primed multipotent progenitor (LMPPs) stage. LMPPs become common lymphoid progenitors (CLPs), which have the potential to differentiate into B and T lymphocytes, as well as natural killer (NK) cells [3]. The transcription factors IKAROS, PU.1 and MEF2C are critical for the cellular commitment of LMPPs to the lymphoid lineage [3–5]. Later, the transcription factors E2A, EBF and FOXO-1 are required for the early specification of CLPs into pro-B cells, whereas PAX5 is required to maintain B cell identity along differentiation into mature B cells [6–11]. However, there is an increasing body of evidence indicating that the repression of lineage inappropriate genes is a pivotal mechanism to properly acquire a particular cellular state during B lymphopoiesis. For

Author Summary

Through the hematopoietic system, all the distinct mature blood cell types are generated, thereby constituting one of the best-studied paradigms for cell lineage commitment and differentiation in biology. B lymphocytes are generated through several cell-commitment, lineage-choice, and differentiation processes. To date, the central role of lineage-specific transcription factors in positively regulating these distinct developmental steps is well established. However, in the absence of proper transcriptional repression, an "adolescent cell" will never be able to reach its "adulthood identity," having a potential impact in the development of hematological malignancies. In this article, we examined the molecular mechanism responsible for the gene silencing of lineage undesirable genes in B cell precursors and uncovered the role played in this process by the histone deacetylase HDAC7. We show that HDAC7 is expressed in B cell precursors where it interacts with the transcription factor MEF2C and is recruited to the promoters of non-B cell genes. While HDAC7 is down-regulated during the lineage conversion of pre-B cells into macrophages, re-expression of HDAC7 interferes with both the acquisition of the myeloid gene transcriptional program and macrophage-specific cell functions. We therefore have identified a novel lineage-specific transcriptional repressor in the hematopoietic system.

example, PAX5 not only induces the expression of a B-cell specific genetic program, it also suppresses inappropriate genes of alternative lineages, thereby ensuring its role in maintaining B cell identity and differentiation [12–14]. Recently, it has been reported that the transcription factor MEF2C, by activating lymphoid specific genes and repressing myeloid genes, is involved in the cellular choice towards the lymphoid lineage [5]. These studies suggest that B cell transcription factors must also recruit transcriptional co-repressors to silence undesirable genes. To date, very little is known on the role of transcriptional repressors during B lymphopoiesis.

Histone deacetylases (HDACs) have emerged as crucial transcriptional co-repressors in highly diverse physiological systems. To date, 18 human HDACs have been identified and grouped into four classes. Class I HDACs (HDAC1, 2, 3, and 8), class II HDACs (HDAC4, 5, 6, 7, 9, and 10), class III HDACs, also called sirtuins, (SIRT1, 2, 3, 4, 5, 6, and 7) and class IV HDAC (HDAC11). Class II HDACs are further subdivided into class IIa (HDAC4, 5, 7, 9) and class IIb (HDAC6 and 10) [15,16]. Unlike other HDACs, Class IIa HDACs have three unique features. First, they are expressed in a tissue-specific manner and are involved in development and differentiation processes. They exert their transcriptional repressive function in skeletal, cardiac, and smooth muscle, the bone, the immune system, the vascular system, and the brain among others. Second, they are signal-dependent co-repressors which become phosphorylated at conserved serine residues in the regulatory N-terminal domain leading to their nuclear export. Third, they contain a regulatory N-terminal domain that mediates their interactions with tissue-specific transcription factors such as members of the MEF2 family [15,16]. This last feature prompted us to ask whether members of the class IIa HDACs subfamily could be lineage-specific transcriptional repressors crucial to maintain B cell identity and biology.

To address this question we have used a cellular transdifferentiation system that we reported recently [17]. This system consists of a pre-B cell line (HAFTL cells) transduced with a retroviral vector for stable expression of a β-estradiol-inducible form of C/EBPα (C10 and C11 cells). After addition of β-estradiol, C10 and C11 cells are converted into functional macrophage-like cells at 100% efficiency within 48–72 hours. The conversion of pre-B cells into macrophages is direct and does not involve overt retro-differentiation through hematopoietic stem and progenitor cells [18]. Unexpectedly, this cellular transdifferentiation process appears to occur in the absence of significant changes in the DNA methylation status of key lymphoid or myeloid genes, but involves changes in histone modification on both types of genes [19]. Since HAFTL cells are a fetal liver cell line immortalized by Ha-ras transformation we, in parallel, also investigated the involvement of class IIa HDACs in normal primary B cell precursors. Here, we report that during the conversion of pre-B cells into macrophages, HDAC7 expression is down-regulated. In pre-B cells HDAC7 specifically interacts with the transcription factor MEF2C and is recruited to putative MEF2 sites located at the promoters of key macrophage genes. Forced re-expression of HDAC7 interferes with the establishment of the gene transcriptional program and functional characteristics of macrophages. Importantly, HDAC7 depletion in pre-B cells results in the de-repression of macrophage genes.

Results

HDAC7 is down-regulated during the transdifferentiation of pre-B cells into macrophages

In order to study the potential role of class IIa HDACs in B lymphocyte biology we first analyzed their expression levels during the cellular transdifferentiation of pre-B cells into macrophages. RT-qPCR and Western blotting experimental approaches showed that HDAC7 expression was dramatically down-regulated during the conversion of C10 cells into macrophages at 72 hours after β-estradiol treatment (Figure 1A and 1B). Notably, no changes in *Hdac4*, *Hdac5* and *Hdac9* expression levels were observed during the cellular reprogramming process. As previously described [17], the B cell genes *Rag1* and *Pax5* became down-regulated, whereas the expression of the myeloid genes *C/Ebpb* and *Csf1r* were up-regulated during cellular reprogramming (Figure 1A). Consistent with our findings, HDAC7 is not present in RAW264.7 macrophages at neither the RNA nor protein levels (Figure 1A and 1B). As expected, the expression of the B cell transcription factors IKAROS, E2A, EBF and PAX5 were down-regulated during the cellular conversion. In contrast, protein levels of the transcription factors MEF2C and RUNX1 did not significantly change during transdifferentiation (Figure 1B). To further confirm our findings we analyzed the kinetics of HDAC7 expression during the cellular transdifferentiation in our previously reported microarray experiments [17]. Strikingly, similar to the B cell specific transcription factor PAX5, HDAC7 expression was down-regulated during cellular reprogramming (Figure S1). Since HAFTL cells are a fetal-derived pre-B cell line transformed by Ha-ras we wondered whether our findings could be extended to normal primary cells. We analyzed HDAC7 expression in both primary B cell precursors and primary macrophages in a recently reported microarray analysis [18]. Importantly, we found that, similar to PAX5, HDAC7 was highly expressed in B cell precursors compared to primary macrophages (Figure 1C and Figure S1). In agreement with our results with the C10 cell line, C/EBPα-mediated conversion of primary pre-B cells into macrophages also resulted in the down-regulation of HDAC7 (Figure 1C and Figure S1). Our data demonstrate that among the different class IIa HDACs, HDAC7 shows a lymphoid-specific expression pattern and suggest that by repressing lineage inappropriate genes

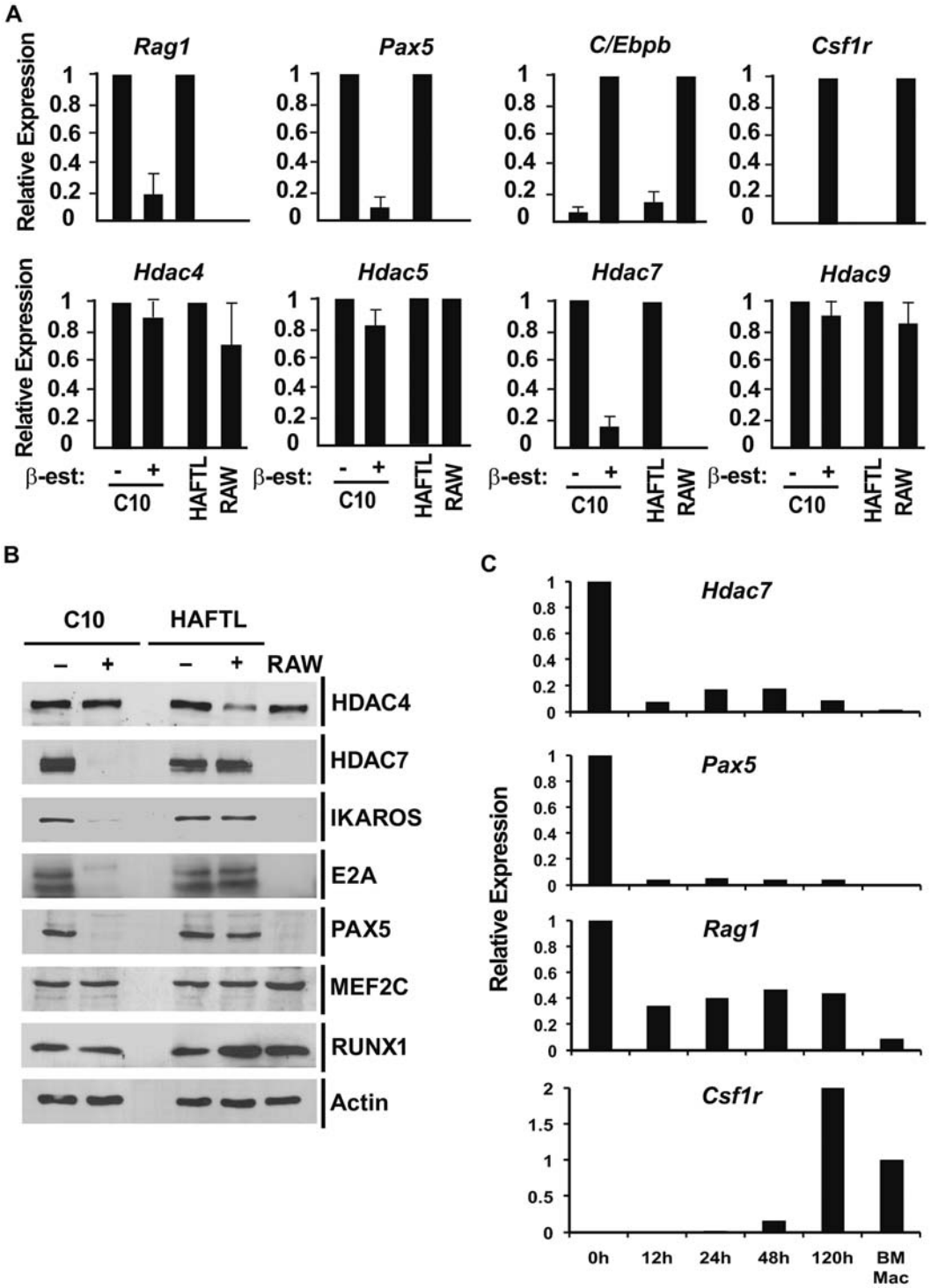

Figure 1. HDAC7 is down-regulated during the transifferentiation of pre-B cells into macrophages. A. RT-qPCR experiments for gene expression changes of class IIa HDACs, B cell and macrophages genes (uninduced cells (−) and β-estradiol induced C10 cells (+) for 72 hours). HAFTL

pre-B cells and RAW264.7 cells were used as control. Data are represented as the mean +/− standard error of the mean (SEM) of three independent experiments. B. Western blot of Class IIa HDACs and B cell transcription factors in the same experimental conditions shown in A. C. RT-qPCR experiments for gene expression changes of *Hdac7*, *Pax5*, *Rag1* and *Csf1r* genes (uninduced (0) and β-estradiol induced primary pre-B cells transduced with a retroviral vector for C/EBPα expression for the indicated times. Primary macrophages were used as control.
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in pre-B cells, such as genes characteristic of macrophages, HDAC7 might be crucial in maintaining B cell functions and identity.

HDAC7 re-expression interferes with the gene transcriptional program of the reprogrammed macrophages

Our observed dramatic down-regulation of HDAC7 upon transdifferentiation of pre-B cells into the macrophage lineage was consistent with its potential role as a transcriptional repressor of macrophage-specific genes in pre-B cells. To test whether the presence of HDAC7 could interfere with the acquisition of a macrophage-specific gene program during transdifferentiation of pre-B cells into macrophages, we performed a gain of function experimental approach followed by genome-wide microarray analysis. We transduced C10 cells with a retroviral vector carrying HDAC7-Flag (C10-HDAC7 cells). As a control, C10 cells were transduced with an empty retroviral vector (C10-MSCV cells). As expected, β-estradiol treatment of C10-MSCV cells resulted in the total down-regulation of endogenous HDAC7 protein levels, while C10-HDAC7 cells expressed the exogenous HDAC7 protein at similar levels as in untreated C10-MSCV cells even at 72 hours after β-estradiol treatment (Figure S2). We then examined the genome-wide effects of HDAC7 re-expression on the gene transcription program of C10 cells induced to transdifferentiate to macrophages. Microarray experiments were conducted in both C10-MSCV and C10-HDAC7 cells un-induced or induced to transdifferentiate for 48 and 72 hours. The addition of β-estradiol to C10-MSCV cells resulted in the up- and down-regulation of 1609 and 1798 genes at 48 hours and of 1531 and 1567 genes at 72 hours after treatment, in agreement with our previous report [17]. Importantly, the exogenous expression of HDAC7 in C10-HDAC7 cells treated with β-estradiol totally or partially abrogated the up-regulation of 988 and 866 genes, after 48 and 72 hours respectively. Earlier analyses showed that the vast majority of the up-regulated genes correspond to key genes for macrophage function, whereas the down-regulated genes are associated with cell cycle processes and with important functions for B cell development and biology [17]. To determine the type of genes whose up-regulation was affected by the presence of HDAC7 we performed a gene set enrichment analysis based on the gene ontology (GO) categories corresponding to Biological Processes, Cellular Components and Molecular Functions, as well as on KEGG pathways. To do so we took advantage of Gitools, a recently developed bioinformatics tool [20]. Gitools allows accessing many available biological databases, performing analysis and visualizing data using interactive heat-maps. Strikingly, the Biological Process enrichment revealed that the set of up-regulated genes affected by HDAC7 belong to GO categories representing key macrophage related functions, such as immune, inflammatory and defense response, cellular response to infections, positive regulation of cytokines production and phagocytosis (Figure 2A). Importantly, we found that the categories enriched in the KEGG pathways analysis correspond to similar biological processes (Figure S3). Gene ontology (GO) analysis corresponding to Cellular Components and Molecular Functions reinforce the above results (Figure 2B and 2C). Among the genes whose up-regulation is impaired by HDAC7 we found several that are involved in phagocytosis (such as *Fcgr1*, *Fcgr2b* and *Fcgr3*), genes related to the immune response (such as the chemokine genes *Cxcl10*, *Cxcl11* and *Ccl2*), Toll-like receptors (*Tlr3*, *Tlr4*, *Tlr7*, *Tlr8* and *Tlr9*), interleukins (*Il18* and *Il15*) and TNF pathway-related genes (*Tnfsf10*, *Tnfsf11* and *Tnfsf13b*) (Figure 3). A full list of the genes found in each category is presented in the interactive website http://bg.upf.edu/C10-HDAC7/ (for statistics see Dataset S1). To validate our microarray analysis, we selected several representative genes and tested their mRNA levels by RT-qPCR. We observed that the presence of HDAC7 significantly interfered with the increase in the mRNA levels of *Cxcl10*, *Fcgr1*, *Ifi35*, *Ifi47*, *Tlr3*, *Tlr9*, *Il18* and *C3* genes after β-estradiol treatment (Figure 4). Importantly, HDAC7 re-introduction did not interfere with the down-regulation of key genes for B cell differentiation and biology such as the transcription factor PAX5, reinforcing the notion that HDAC7 is a repressor of macrophage genes in B cell precursors (Figure S5). During the trasdifferentiation of C10 cells into macrophages the cells stop dividing and many genes related to the cell cycle, such as genes involved in mitosis, become down-regulated [17]. We have observed that the presence of HDAC7 also interferes with the down-regulation of these types of genes, corroborating that HDAC7 blocks, at least in part, the cellular transdifferentiation process (Figure S4, Dataset S2, and interactive website http://bg.upf.edu/C10-HDAC7/). Our data clearly demonstrate that HDAC7 represses the expression of central genes for macrophage function, and strongly suggest that HDAC7 acts as a specific transcriptional repressor of lineage inappropriate genes in B cell precursors.

HDAC7 knock down leads to the de-repression of macrophage genes

To determine the physiological function of HDAC7 in B cell precursors and test whether it is involved in the repression of macrophage genes, we performed a loss of function experimental approach. We knocked down HDAC7 by siRNA in both HAFTL cells and primary pre-B cells (Figure 5). Strikingly, we observed that the reduction in HDAC7 mRNA levels resulted in the de-repression of key macrophage genes such as *Itgam* (Mac-1), *Fcgr1* and *Ccl3* (Figure 5). These data demonstrate that HDAC7 is involved in the repression of lineage inappropriate genes in B cell precursors.

HDAC7 binds to the transcription factor MEF2C in pre-B cells and is recruited to the promoters of target genes critical for macrophage function

At the mechanistic level, class IIa HDACs exert their actions as transcriptional repressors by interacting with specific transcription factors recruited to the promoters of genes required for development and cell differentiation [15,16]. To address whether HDAC7 specifically interacts with particular sequence-specific transcription factors in pre-B cells, we undertook a candidate approach and performed co-immunoprecipitation experiments to test for potential interaction between endogenous HDAC7 and the B cell transcription factors IKAROS, E2A, PAX5, and MEF2C in the C10 parental line (HAFTL cells). HDAC7 specifically associated with MEF2C, but not with the other B cell transcription

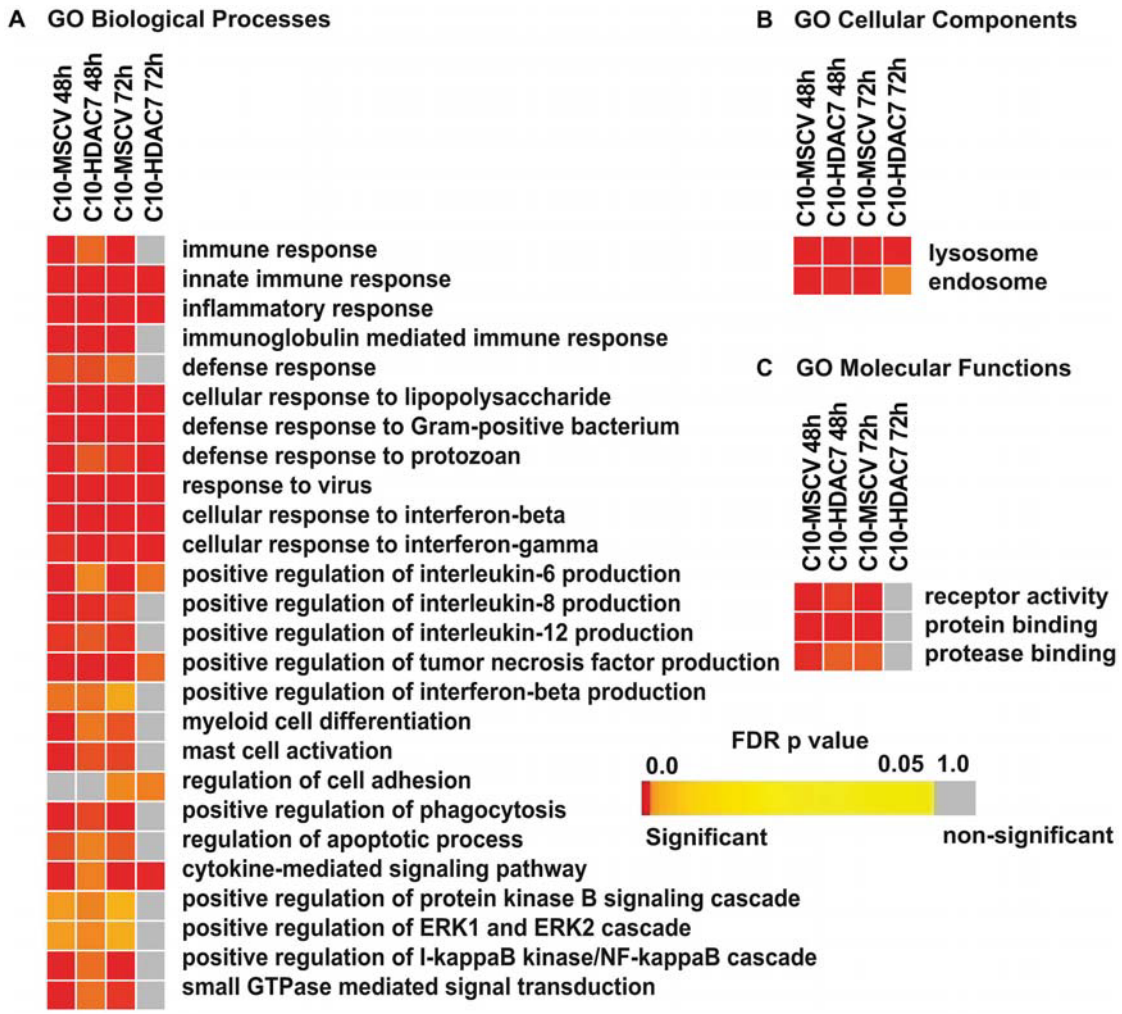

Figure 2. HDAC7 re-expression interferes with the gene transcriptional program of the converted macrophages. Heat-maps showing significantly (corrected p-value <0.05) enriched GO Biological Process (A), Cellular Components (B) and Molecular Functions (C) categories among the up-regulated genes affected by the re-expression of HDAC7 during transdifferentiation of pre-B cells into macrophages. Colours toward red indicate high statistic significance, yellow indicates low statistic significance, and gray indicates no statistical significance. The list of genes for the enrichment analysis in each column is as follows: C10-MSCV-48h includes genes up-regulated after β-estradiol treatment for 48 hours. C10-HDAC7-48h includes genes up-regulated after β-estradiol treatment for 48 hours which are down-regulated in the presence of HDAC7 (HDAC7 re-expression). C10-MSCV-72h includes genes up-regulated after β-estradiol treatment for 72 hours. C10-HDAC7-72h includes genes up-regulated after β-estradiol treatment for 72 hours which are down-regulated after re-expression of HDAC7.
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factors tested (Figure 6A). Recently, Camargo and colleagues have reported that MEF2C is crucial in the cellular choice towards the lymphoid versus the myeloid lineage in LMPPs [5]. Microarray analysis of control and MEF2C-deficient LMPPs revealed that the absence of MEF2C resulted in the up-regulation of genes enriched in GO categories related to the immune system, such as genes involved in the inflammatory and defense response of the cells [5]. Comparison of these data with our set of genes whose up-regulation was affected by HDAC7 re-expression during cellular transdifferentiation showed a significant overlap by Chi-square test of 46 ($p<10^{-6}$) and 30 ($p=0.00044$) genes, respectively (Figure 6B). Strikingly, the phagocytosis-related genes *Fcgr1*, *Fcgr2b* and *Fcgr3* were found to be targets of both HDAC7 and MEF2C. These data indicate that MEF2C represses genes characteristic of macrophages in hematopoietic progenitors and suggest that HDAC7 may also silence lineage inappropriate genes at earlier stages of lymphocyte development.

To test whether in pre-B cells HDAC7 is recruited to the promoters of macrophage-specific genes whose up-regulation is impaired in the presence of exogenously expressed HDAC7, we performed chromatin immunoprecipitation (ChIP) assays. Notably, using the TFconsite bioinformatic tool we found that

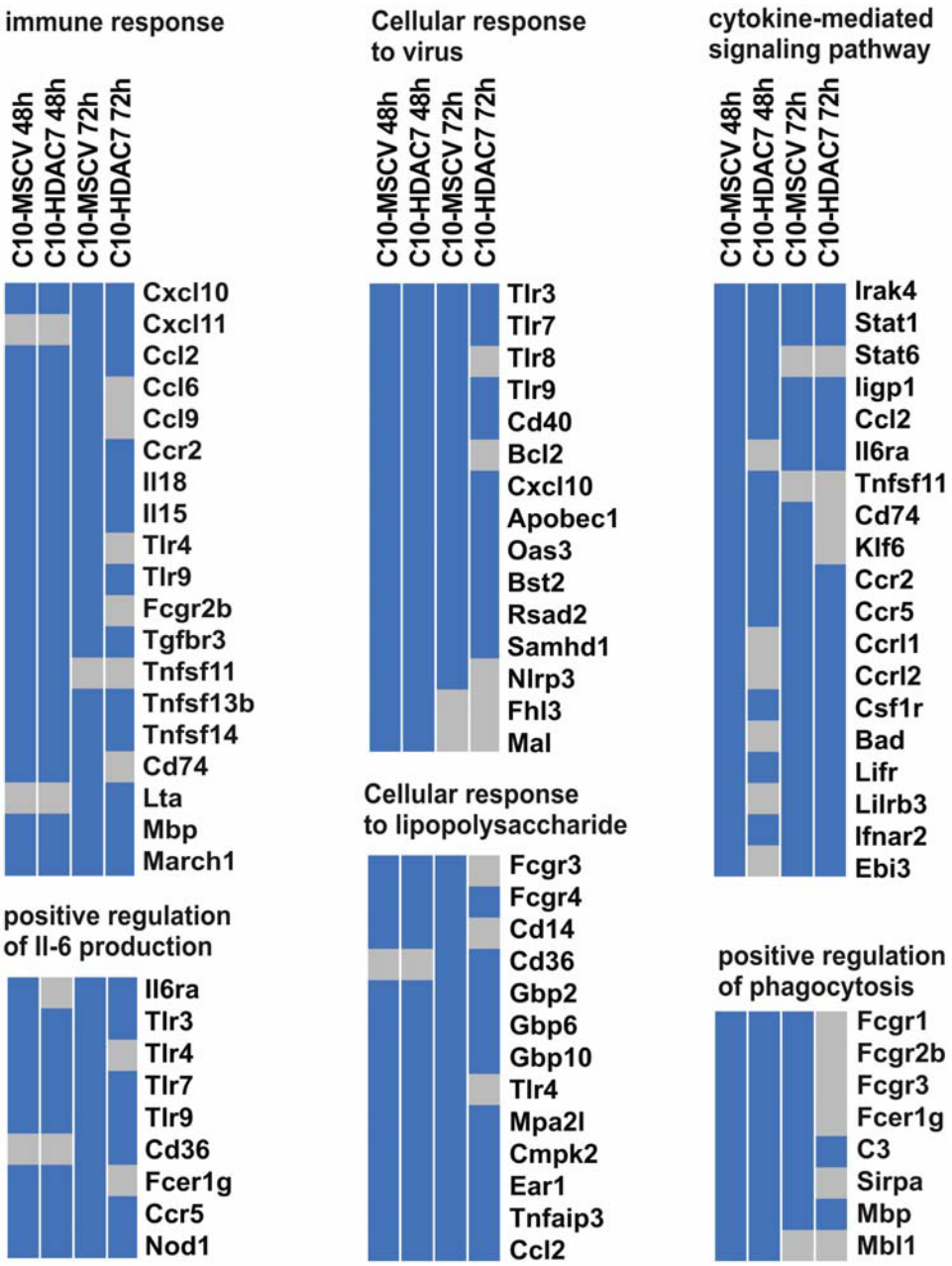

Figure 3. HDAC7 re-expression interferes with the gene transcriptional program of the converted macrophages. Heat-maps showing observed differentially expressed genes for selected enriched GO categories. Blue colour cell indicates positive events while gray colour indicates that the gene was not observed differentially expressed in that experimental condition.
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promoters of *Fcgr1*, *Cxcl10* and *Itgam* contain putative MEF2 binding sites. We designed primers spanning the upstream regulatory regions, gene body, and downstream regulatory regions of mouse *Fcgr1*, *Cxcl10* and *Itgam* (Figure 6C and 6F and Figure S6A). Chromatin prepared from C10 cells un-induced or induced with β-estradiol for 72 hours was subjected to ChIP with antibodies specific for MEF2C and HDAC7. qPCR analysis of the immunoprecipitated material with primers specific for the

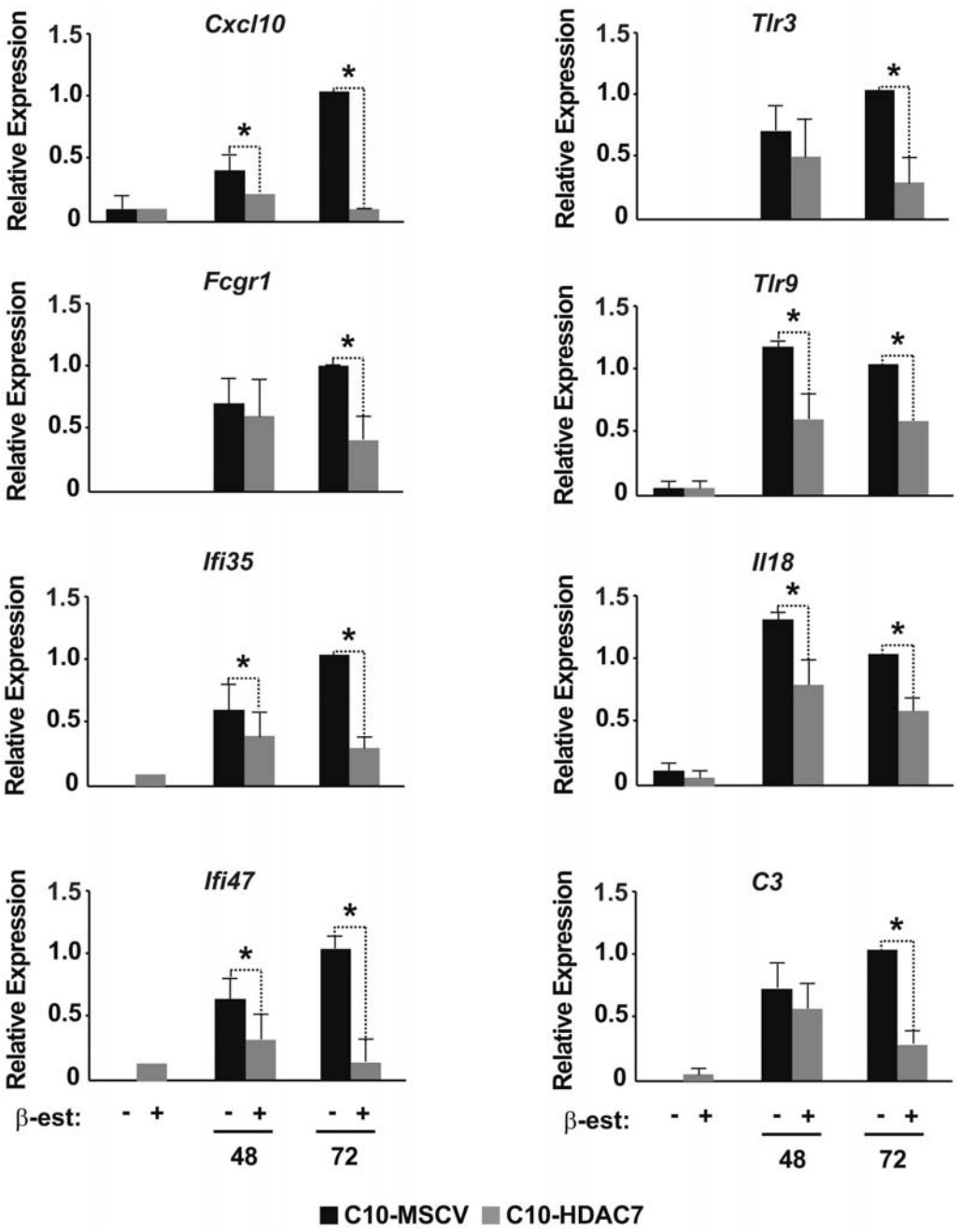

Figure 4. RT–qPCR for microarray validation of selected genes. RT-qPCR experiments for gene expression changes for 8 up-regulated genes, *Cxcl10*, *Fcgr1*, *Ifi35*, *Ifi47*, *Tlr3*, *Tlr9*, *Il18* and *C3* in the absence or in the presence of HDAC7. Data are represented as the mean +/− standard error of the mean (SEM) of three independent experiments. The two-way ANOVA test was used to calculate significant levels between the indicated groups. *$P<0.001$.

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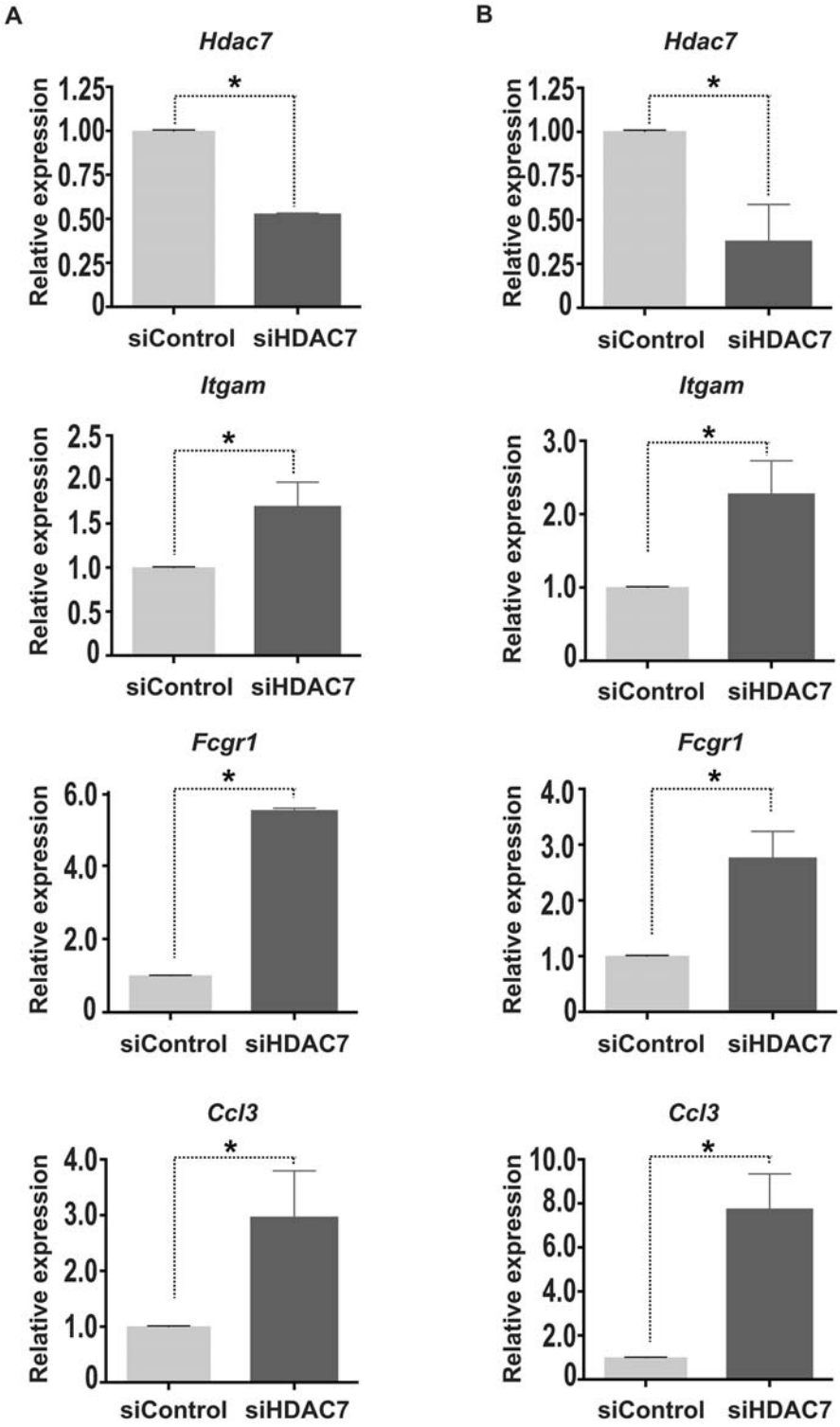
A
B
Hdac7
Relative expression
1.25
1.00
0.75
0.50
0.25
0
siControl
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*
Itgam
2.5
2.0
1.5
1.0
0.5
3.0
Fcgr1
6.0
4.0
Ccl3
10.0
8.0

Figure 5. HDAC7 knock down leads to the de-repression of macrophage genes. HAFTL cells (A) and primary B cell precursors (B) were transfected with control siRNA or siRNAs targeting HDAC7. 72 hours after transfection *Hdac7*, *Itgam*, *Fcgr1* and *Ccl3* mRNA levels were determined by RT-qPCR experiments. Data are represented as the mean +/− standard error of the mean (SEM) of three independent experiments. The two-way ANOVA test was used to calculate significant levels between the indicated groups. *$P<0.001$.
doi:10.1371/journal.pgen.1003503.g005

Fcgr1, *Cxcl10* and *Itgam* loci indicated that both MEF2C and HDAC7 were specifically and significantly enriched at the identified putative MEF2 binding sites in un-induced C10 cells (Figure 6D, 6E, 6G and 6H and Figure S6B). Importantly, while significant MEF2C enrichment around its putative binding sites was found in both un-induced and induced conditions, HDAC7 enrichment at the target genes was lost upon β-estradiol induction (Figure 6D, 6E, 6G and 6H and Figure S6B). Taken together these data indicate that via interaction with MEF2C, HDAC7 is recruited to the promoters of myeloid genes in B cell precursors, resulting in their transcriptional silencing.

HDAC7 re-expression interferes with the functional properties of the reprogrammed macrophages

We previously reported that during reprogramming of pre-B cells into macrophages there is an increase in the expression of Mac-1 and a down-regulation of CD19 levels, two cell surface markers characteristic for macrophages pre-B cells, respectively. The reprogrammed macrophages show high phagocytic activity and respond to LPS treatment with cytokine production [17]. To test whether the presence of HDAC7 could interfere with the functional characteristics of the reprogrammed macrophages we undertook three different experimental approaches. First, we followed the expression kinetics of CD19 and Mac-1 (CD11b), two cell surface markers characteristic for pre-B cells and macrophages, respectively by flow cytometry. In both C10-MSCV and C10-HDAC7 cells, 100% of the population became CD19 negative and Mac-1 positive 72 hours after addition of β-estradiol (Figure 7A). However, the presence of exogenous HDAC7 resulted in a significant block in the expression levels of Mac-1 (Figure 7A and 7B). In contrast, CD19 protein levels decreased to the same extent regardless of the presence of HDAC7 (Figure 7A and 7B). Exogenous expression of HDAC7 in primary B cell precursors also interfered with the up-regulation of Mac-1 in transdifferentiation experiments (Figure 7C). These results demonstrated that in the presence of HDAC7 the reprogrammed cells are not able to express the levels of Mac-1 protein normally present in macrophages. Second, we tested the phagocytic properties of the transdifferentiated C10 cells exogenously expressing HDAC7. Interestingly, C10-HDAC7 cells treated with β-estradiol showed a significantly reduced capacity to phagocytose red fluorescence protein-expressing bacteria compared to the control C10-MSCV cells (Figure 7D). Lastly, we analyzed the inflammatory response of both cell lines after treatment with LPS in the presence or absence of β-estradiol for 48 hours. Strikingly, expression of HDAC7 resulted in a significant inhibition in the expression of the pro-inflammatory cytokines, *Tnfα*, *Il-1α* and *Il-6*, by the macrophage-like converted cells in response to LPS (Figure 7E). We next tested whether HDAC7 re-expression results in the reduction of Mac-1 protein levels once the cells have been reprogrammed into macrophages. We first treated C10 cells with β-estradiol for 72 hours and the reprogrammed macrophages were transduced with a retroviral vector carrying HDAC7-Flag. We observed a decrease in the expression levels of Mac-1 24 hours after expression of exogenous HDAC7 (Figure S7A). However, we did not observe any effect on either the expression of macrophage genes or the phagocytic capacity of RAW cells expressing exogenous HDAC7 (Figure S7B, S7C and S7D). We have recently shown that C/EBPα-mediated reprogramming of pre-B cells into macrophages occurs in the absence of significant changes in the DNA methylation of crucial genes suggesting that the reprogrammed macrophages retained an epigenetic memory characteristic of the cell of origin [19]. Therefore, we speculate that the chromatin structure present in differentiated macrophages is not permissive for the recruitment of HDAC7 to its target genes and that such recruitment is only possible in a chromatin environment characteristic of B cells, in line the notion that HDAC7 is a lymphoid-specific transcriptional repressor. Taken together, these results demonstrate that HDAC7 significantly interferes with key functional characteristics of the transdifferentiated macrophages.

Interaction of HDAC7 with MEF2C and its catalytic activity are essential for repression of Mac-1

Class IIa HDACs interact with MEF2 proteins via a conserved motif of 17 amino acids located in the amino-terminal region of the proteins. To definitively prove that HDAC7 represses macrophage genes through its interaction with MEF2C, we generated retroviral vectors carrying mutants of HDAC7 with either a deletion of the entire 17 amino acids stretch (HDAC7-ΔMEF) or with substitutions of crucial lysine residues (HDAC7-K86AK88A). We tested the HDAC7 mutants for their ability to repress Mac-1 during the transdifferentiation of pre-B cells into macrophages. Expression of wild-type HDAC7 resulted in a significant decrease in Mac-1 positive cells and mRNA levels (Figure 8) whereas expression of the HDAC7 mutants defective for MEF2C binding had no significant effect. We next tested whether the enzymatic activity of HDAC7 is necessary for its repressive action on Mac-1 expression during the conversion of pre-B cells into macrophages. We generated a retroviral vector for HDAC7 mutated in its catalytic domain (HDAC7-H657A), a C-terminal truncated construct HDAC7(1–487) that completely lacks the HDAC catalytic domain but contains the MEF2 interacting motif and a N-terminal truncated construct HDAC7(438–915) bearing the enzymatic motif but lacking the MEF2 domain. As shown in Figure 8, forced expression of wild-type HDAC7 interfered with the up-regulation of Mac-1 protein levels 48 hours after induction of cellular reprogramming. In contrast, the HDAC7-H657A, the HDAC7(1–487) and the HDAC7(438–915) constructs did not block the up-regulation of Mac-1 levels. These experiments demonstrate that both the HDAC7-MEF2C interaction, as well as its catalytic activity, are necessary for HDAC7 to repress macrophage genes during cellular reprogramming.

Discussion

Our findings have revealed that HDAC7 is expressed in B cell precursors and not in macrophages (Figure 9). Using a cellular transdifferentiation system we have demonstrated that HDAC7 represses the expression of a large number of macrophage genes during the conversion of pre-B cells into this myeloid cell type. More importantly, depletion of HDAC7 in pre-B cells results in the de-repression of key macrophage genes. Functionally, the presence of HDAC7 interferes with the acquisition of key functional features of the converted macrophages. These aberrant macrophage-converted cells do not express the Mac-1 protein levels normally present in macrophages, are not able to properly

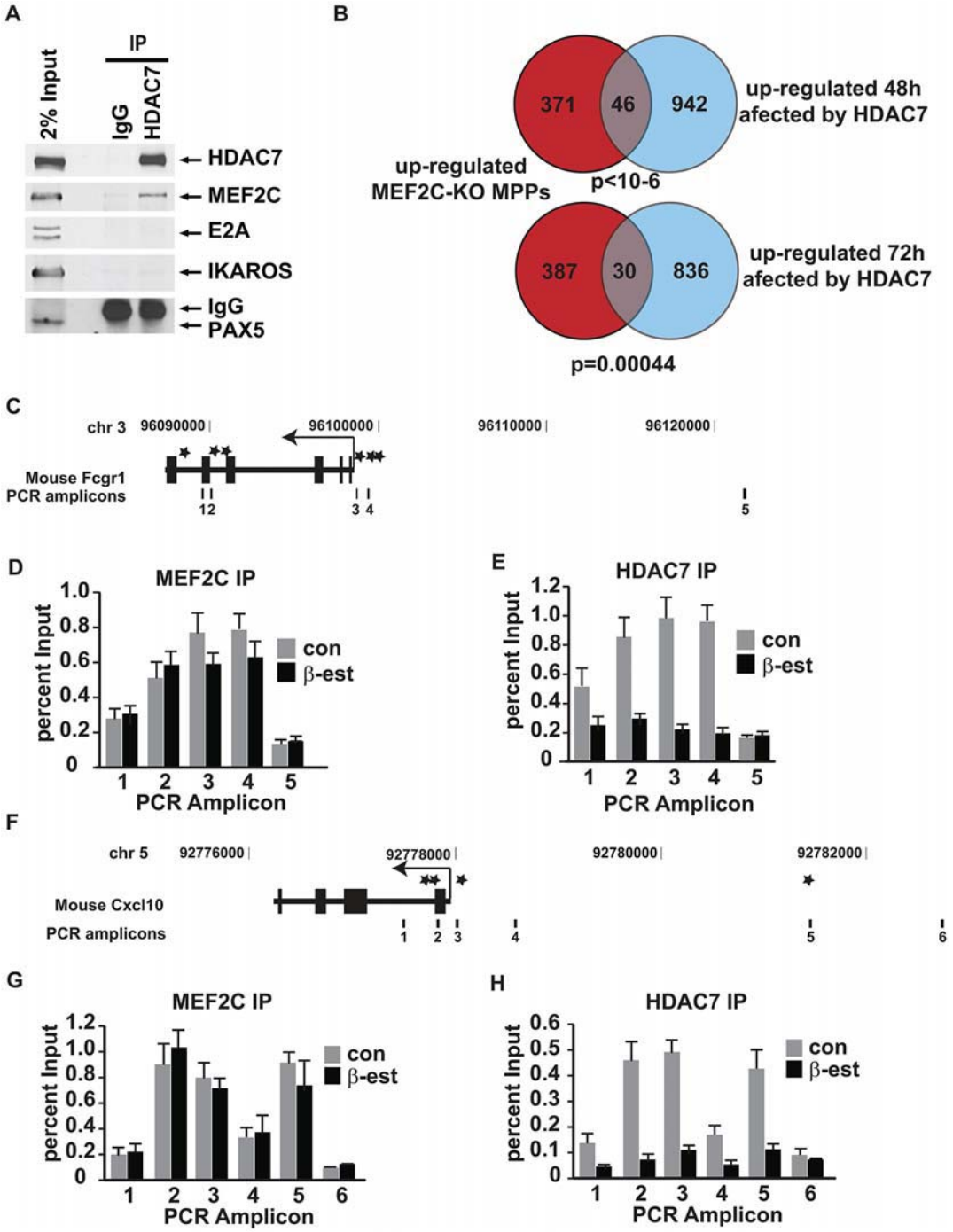

Figure 6. HDAC7 binds to the transcription factor MEF2C and is recruited to the promoter of key genes for macrophage function in pre-B cells. A. Co-immunoprecipitation experiments showing the specific binding of HDAC7 with MEF2C in pre-B cells. HDAC7 does not bind with IKAROS, PAX5 and E2A. B. Venn diagrams showing the total number of genes up-regulated in MEF2C deficient LMPPs, genes up-regulated at 48 and 72 hours of β-estradiol treatment affected by HDAC7 and the overlapping between both conditions. C and F. Schematic representation of the mouse

Cxcl10 and *Fcgr1* locus and amplicons scanned in Chromatin immunoprecipitation experiments by qPCR. Asterisks indicate MEF2 binding sites location. D, E, G and H. Chromatin immunoprecipitation experiments showing the enrichment of HDAC7 and MEF2C to putative MEF2 binding sites on the *Cxcl10* and *Fcgr1* gene loci in pre-B cells. Results are presented as percentage immunoprecipitated over input and are representative of three independent experiments.
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phagocytose bacteria and do not respond adequately to endotoxin by expressing major pro-inflammatory cytokines. At the mechanistic level, HDAC7 specifically interacts with the transcription factor MEF2C in pre-B cells and is recruited to MEF2 binding sites located at the promoters of genes critical for macrophage function (Figure 6 and Figure S6). In addition to the interaction with MEF2C, the catalytic activity of HDAC7 is also necessary to repress macrophage genes. Our results demonstrate that HDAC7 is expressed in fetal and adult pre-B cells suggesting that it might play a role in both types of B lymphopoiesis.

Why is HDAC7 specifically expressed in pre-B cells and not macrophages? Based on our results, we conclude that HDAC7 is a key transcriptional repressor of lineage inappropriate genes in pre-B cells. Using a ChIP-seq approach Murre and colleagues have identified HDAC7 as a target of the transcription factors E2A, EBF and Foxo1 in B cell precursors (pro-B cells) postulating that it could be an important regulator of B cell development and indicating that HDAC7 function is not restricted to T cells within the hematopoietic system [21]. In addition, our results showing that in pre-B cells HDAC7 interacts with the transcription factor MEF2C, allowing the recruitment to the MEF2 binding sites on promoters of genes characteristic of macrophages reinforces our conclusion. Indeed, in the late nineties, MEF2C was found, among the different MEF2 family members, to be specifically expressed in B cells within the lymphocyte lineage, suggesting that it could have a role in B cell development and function [22]. More recently, it has been reported that MEF2C regulates B cell proliferation and survival after BCR activation and p38 MAPK signaling [23,24]. MEF2C is also expressed at earlier stages of lymphocyte development [5,25]. It has been reported that MEF2C is involved in the cellular choice towards the lymphoid versus the myeloid lineage in lymphoid-primed multipotent progenitors (LMPPs) [25]. At the molecular level MEF2C activates the transcription of lymphoid specific genes and represses myeloid genes [25]. We have observed a significant overlap between MEF2C regulated genes in LMPPs and our identified HDAC7 target genes in pre-B cells. Moreover, HDAC7 mutants that do not interact with MEF2C are unable to suppress the up-regulation of macrophage genes during the reprogramming process. Given this scenario, we propose that HDAC7 is the MEF2C transcriptional co-repressor responsible for the silencing of myeloid genes in B cells and lymphoid precursors.

Finally, in light of previous reports demonstrating the expression and functions of HDAC7 in T cells, our results have uncovered a more general role for HDAC7 in the lymphoid lineage compartment within the hematopoietic system. In addition, the finding that HDAC7 is dramatically down-regulated during the transdifferentiation of pre-B cells into macrophages not only reinforces this notion, but also provides an additional level of complexity to the way its activity and function are regulated. During T cell development in the thymus and later in a differentiated and specialized T cell type, cytotoxic T lymphocytes (CTLs), HDAC7 is regulated in a signal dependent manner responsible for its phosphorylation and for its subsequent nucleo-cytoplasmic distribution [26–31]. In resting thymocytes, HDAC7 is localized in the nucleus, where it represses the expression of a large number of genes involved in both positive (survival) and negative (apoptosis) selection of the cells. However, in response to TCR signaling, HDAC7 becomes phopshorylated and translocates to the cytoplasm where it can no longer repress its target genes [26,27,29,31]. Later in the periphery, HDAC7 is constitutively phosphorylated and found in the cytoplasm of CTLs allowing the expression of key genes for the function of this specialized T cell type [30]. We could postulate that within a particular hematopoietic cellular lineage, the HDAC7 gene transcriptional repressive function is regulated in a signal-dependent way by controlling its phosphorylation status and altering its cellular distribution. That is, HDAC7 is unphosphorylated or phosphorylated and localized either in the cell nucleus or in the cytoplasm depending on the requirement to silence or activate specific genes at different stages of T cell development. In contrast, at the branching points where cellular lineage choice has to be made, e.g. lymphoid versus myeloid, HDAC7 might be regulated at the expression level. For instance, our results showing that HDAC7 expression is down-regulated during the conversion of pre-B cells into macrophages further support this hypothesis. However, as is the case for T cells, we cannot rule out the possibility that HDAC7 is additionally regulated in a signal dependent manner during B cell development and differentiation. In this regard, we have found that HDAC7 is expressed in the nucleus of pre-B cells (data not shown) probably due to the lack of a fully functional B cell receptor (BCR). However, it is highly probable that HDAC7 function is also regulated via BCR signaling at later cell differentiation stages when B cells develop into mature antibody-secreting cells.

Based on the findings of this study, we therefore propose that in the hematopoietic system, HDAC7 is not only a signal-dependent transcriptional repressor involved in different developmental steps of a particular lymphocyte type, but also a lineage-specific transcriptional repressor responsible for maintaining the identity of lymphocytes by silencing lineage inappropriate genes. We anticipate that other members of the class IIa HDACs subfamily might be specific repressors of undesirable genes in the cellular differentiation and developmental processes where they exert their actions (e.g. skeletal and cardiac muscle, bone formation and brain).

Materials and Methods

Plasmids

MSCV-puro-HDAC7, MSCV-GFP-HDAC7, MSCV-GFP-HDAC7(ΔMEF), MSCV-GFP-HDAC7(K86A/K88A), MSCV-GFP-HDAC7(H657A), MSCV-GFP-HDAC7(1–487) and MSCV-GFP-HDAC7(438–915) construct were generated by cloning the HDAC7-Flag cDNA PCR amplificated from the pCDNA3-HDAC7 wild-type and mutant plasmids previously described [20] into the MSCV-puro or MSCV-GFP vectors (Clontech).

Antibodies

Anti-HDAC7 (H-273), anti-HDAC7 (C-18), anti-HDAC4 (H-92), anti-E2A (V-18), anti-PAX5 (C-20) and anti-RUNX1 (DW71) were purchased from Santa Cruz Biotechnology. Anti-IKAROS (ab26083) was purchased from Abcam; anti-MEF2C (D80C1) XP from Cell Signaling Technology; and anti-α-Tubulin (T61999), from Sigma-Aldrich.

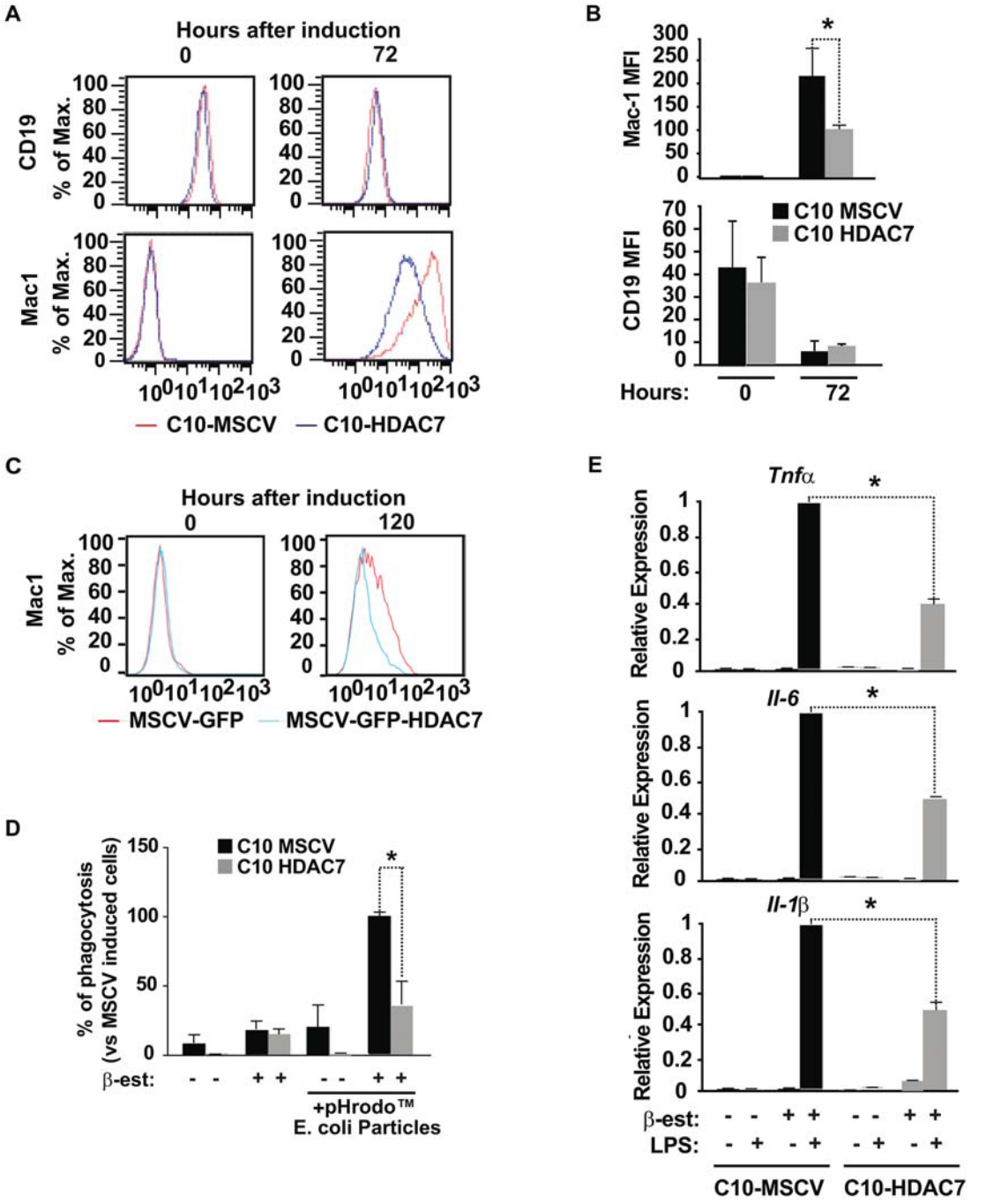

Figure 7. HDAC7 re-expression interferes with the functional properties of the converted macrophages. A. Histograms for Mac-1 and CD19 expression levels in C10-MSCV and C10-HDAC7 cells untreated or treated with β-estradiol for 72 hours. B. Mean fluorescence intensity (MFI) of Mac-1 and CD19 of C10-MSCV and C10-HDAC7 cells untreated or treated with β-estradiol for 72 hours. Data are represented as the mean +/− standard error of the mean (SEM) of three independent experiments. *$P<0.001$. C. Primary pre-B cells were transduced with the indicated retroviral vectors and 48 hours after induce to transdifferentiate. Histograms for Mac-1 proteins levels are shown. D. Capacity of C10-MSCV and C10-HDAC7 cells untreated or treated with β-estradiol for 48 hours to phagocytose red fluorescence bacteria. Data are given as mean ± SEM of values obtained

in three independent experiments. Statistical significance was determined by two-way ANOVA followed by Bonferrony multiple comparison test. *$P<0.001$. E. Effect of HDAC7 expression in LPS-mediated *Tnfα*, *Il-1α* and *Il-6* gene expression. C10-MSCV and C10-HDAC7 cells were treated or not with β-estradiol for 48 hours. Then, the cells where incuated or not with LPS for 6 hours and RNAs analyzed by RT-qPCR. Data are represented as the mean +/− standard error of the mean (SEM) of three independent experiments. P values were calculated by the two-way ANOVA test. *$P<0.001$.
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Cell culture and β-estradiol treatment

HAFTL cells were grown at 37°C in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM glutamine, and 50 U/ml streptomycin/penicillin. C10 cells (transduced with a MSCV-GFP-C/EBPα retroviral vector) and C11 cells (transduced with a MSCV-hCD4-C/EBPα retroviral vector) were cultured at 37°C in RPMI 1640 without phenol red supplemented with 10% of charcoal treated fetal bovine serum, 2 mM glutamine, and 50 U/ml streptomycin/penicillin. B-cell precursors and macrophages were obtained from mouse bone marrow as described [18]. For transdifferentiation induction, cells were treated with 100 nM β-estradiol in the presence of 10 nM of IL-3 and 10 nM of mCSF1 for the indicated periods of time.

Retroviral supernatant generation and cellular transduction

For retrovirus generation the MSCV-puro, MSCV-puro-HDAC7, MSCV-GFP, MSCV-GFP-HDAC7, MSCV-GFP-HDAC7(ΔMEF), MSCV-GFP-HDAC7(K86A/K88A), MSCV-GFP-HDAC7(H657A), MSCV-GFP-HDAC7(1–487) and MSCV-GFP-HDAC7(438–915) plasmids were transfected into the packaged cell line Platinum-E and supernatant were collected at 48–

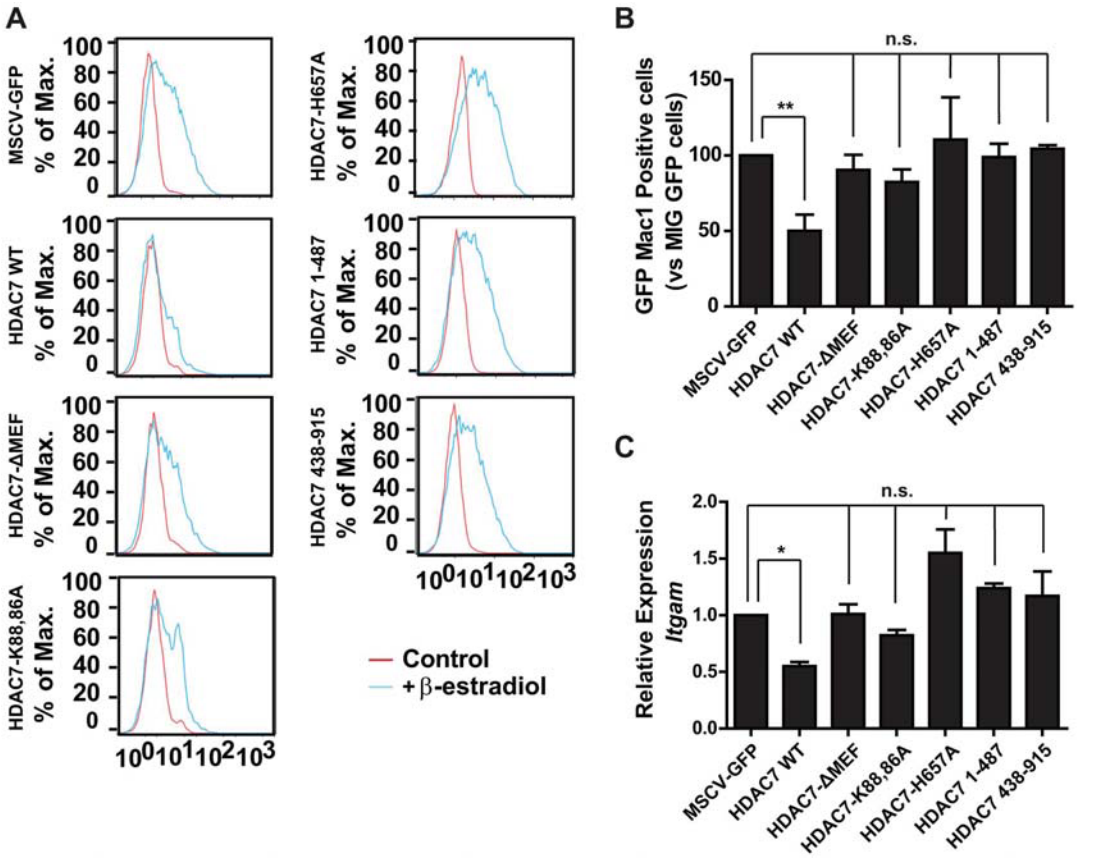

Figure 8. Interaction of HDAC7 with MEF2C and its catalytic activity are essential for repression of Mac-1. C11 cells were infected with MSCV-GFP, MSCV-GFP-HDAC7, MSCV-GFP-HDAC7(ΔMEF), MSCV-GFP-HDAC7(K86A/K88A), MSCV-GFP-HDAC7(H657A), MSCV-GFP-HDAC7(1–487) and MSCV-GFP-HDAC7(438–915) viruses and induced with β-estradiol for 48 hours. A. Cells were stained with a Mac-1 antibody and the GFP-positive fractions were gated and the results plotted. B. Percentage of Mac-1 positive cells treated with β-estradiol for 48 hours. Data are given as mean ± SEM of values obtained in three independent experiments. Statistical significance was determined by two-way ANOVA followed by Bonferrony multiple comparison test. *$P<0.0001$. C. RT-qPCR experiments for *Itgam* gene expression changes in cells treated with β-estradiol for 48 hours. Data are given as mean ± SEM of values obtained in three independent experiments. Statistical significance was determined by two-way ANOVA followed by Bonferrony multiple comparison test. *$P<0.001$.
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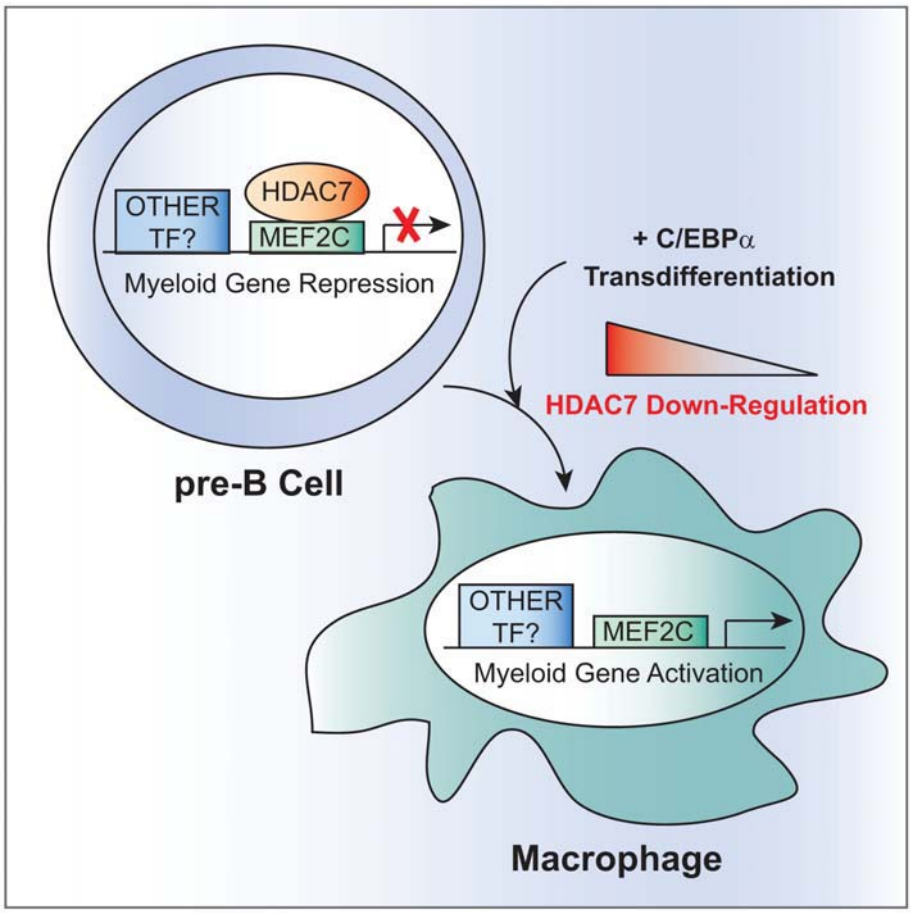

Figure 9. Model for HDAC7-mediated transcriptional repression in pre-B cells. HDAC7 is expressed in pre-B cells and not in macrophages. In pre-B cells, HDAC7 specifically interacts with the transcription factor MEF2C and is recruited to promoters of myeloid genes. During transdifferentiation of pre-B cells into macrophages HDAC7 is down-regulated allowing for expression of macrophages specific genes.
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72 hours post-transfection. For the generation of C10-MSCV and C10-HDAC7 cells, C10 cells were spin infected and 48 hours after were selected in the presence of 3 ug/ml of puromycin. C11 cells were spin infected with MSCV-GFP, MSCV-GFP-HDAC7, MSCV-GFP-HDAC7(ΔMEF), MSCV-GFP-HDAC7(K86A/K88A), MSCV-GFP-HDAC7(H657A), MSCV-GFP-HDAC7(1–487) and MSCV-GFP-HDAC7(438–915) and 48 hours after treated with β-estradiol.

siRNA depletion

Dharmacon siRNA control and on-target smartpools targeting transcript of the mouse HDAC7 gene were used to knockdown its expression HAFTL cells and primary B cell precursors. siRNA were transfected using Lipofectamine RNAiMAX Transfection Reagent (Invitrogen). mRNA levels were examined by RT-qPCR experiments 72 hours after siRNA transfection.

RT–qPCR experiments

RNA was extracted Trizol extraction (Qiagen) and cDNA synthesyzed using the High Capacity cDNA Reverse Trancription Kit (AB Applied Biosystems). RT-qPCR were performed in triplicate using SYBR Green I Master (Roche). PCR reactions were run and analyzed using the LightCycler 480 Detection System (Roche). Primers sequences upon request.

Co-immunoprecipitation and Western blot analysis

Total cellular extracts were prepared in PLB buffer (0.5% Triton X-100, 0.5 mM EDTA, 1 mM DTT in PBS) supplemented with protease inhibitors (Complete, Roche Molecular Biochemicals). Immunoprecipitation, SDS-PAGE and Western blot experiments were performed as previously described [32].

Flow cytometry

Cells were un-induced or induced to transdifferentiate. 48 hours later, cells were stained with fluorochrome conjugated antibodies against Mac-1 and CD19 (BD Pharmingen). Mac-1 and CD19 expression were monitored on a Gallios Flow Cytometer (Beckman Coulter) and analyzed by FlowJo software (Tree Star, Inc.).

Phagocytosis assays

C10-MSCV and C10-HDAC7 cells were induced or not for 48 h and subjected to the pHrodo *E.coli* phagocytosis KiT (Invitrogen) following the manufacturers protocol.

LPS induced inflammatory cytokines

C10-MSCV and C10-HDAC7 cells were induced to transdifferentiate. 48 h after induction they were incubated with LPS (1 μg/mL Sigma) for 6 h. RNA extraction, cDNA synthesis and RT-qPCR were performed as described above. Primers sequences upon request.

Microarray experiments

Biological duplicates of C10-MSCV and C10-HDAC7 cells were un-induced or induced to transdifferentiate for 48 and 72 hours (12 samples in total). Total RNA from cultured cells was extracted by Trizol and then purified. PCR amplified RNAs were hybridized against Affymetrix mouse arrays chip (Mouse Genome 430 PM strip) at the IRB Genomics Facility. Affymetrix raw CEL files and processed (normalized) data have been deposited in GEO database under accession number GSE36827.

Microarray data analysis

Affymetrix CEL files were background corrected, normalized using Bioconductor, package "affy" (version 1.28.1) using 'expresso' algorithm [33,34]. Since the Affymetrix chip version used in this study contains only perfect match (pm) probes, for normalization and acquiring raw probe intensities to expression values we used the following parameters: background correction method "rma"; normalization method "constant"; pm correct method "pmonly"; and summary method "avgdiff". Quality of microarray experiment (data not shown) was verified by Bioconductor package "arrayQualityMetrics" (version 3.2.4 under Bioconductor version 2.7; R version 2.12.1) [35]. To determine genes that are differentially expressed (DE) between two experimental conditions, Bioconductor package Limma was utilized to generate contrast matrices and fit the corresponding linear model [36]. Probe to gene annotation were performed using microarray vendor's annotation data. When more than one probe were annotated to same gene, highest absolute expression value was considered (maximizing). To consider a gene is differentially expressed, besides multiple test corrected, FDR p-value ≤ 0.05 as cut off, we also applied Log2 fold change (Log2FC) cut off 0.5 for β-estradiol treatment. We used Log2FC cut off 0.5 for genes that are affected by the expression of HDAC7 in β-estradiol treated cells. Expression data on Mef2c deficient multipotent progenitor cells were obtained from GEO database (accession No. GSE13686) [5]. Data were analyzed using the limma package from Bioconductor. Spots were not background corrected before within array loess normalization. After array normalization using the quantile method log2 ratios (mutant/control) was calculated. To define a gene up-regulated, we used Log2FC ≥ 1.0.

Functional and pathway enrichment analysis

Functional annotation of differentially expressed genes is based on Gene Ontology (GO) (http://www.geneontology.org) as extracted from EnsEMBL and KEGG pathway database. Accordingly, all genes are classified into three ontology categories (i) biological process (BP), (ii) cellular component, (CC) and molecular function (MF) and pathways when possible. We have taken only the GO/pathway categories that have at least 10 genes annotated. We used Gitools for enrichment analysis and heatmap generation (www.gitools.org). Resulting p-values were adjusted for multiple testing using the Benjamin and Hochberg's method of False Discovery Rate (FDR).

Chomatin immunoprecipitations assays

ChIP was performed essentially as previously described [32] on C10 cells un-induced or induced for 72 hours. To shear chromatin to an apparent length of ~500 bp, chromatin was sonicated using a BioRuptor sonicator (Cosmo Bio Co., Ltd) with either 40 45-s pulses (uninduced cells) or 30 45-s pulses (induced cells) at maximum setting. Input and immunoprecipitated DNA were subjected to Sybergreen Q PCR cycles with specific primers (provided upon request).

Supporting Information

Dataset S1 Microarray statistics analysis for genes up-regulated during the conversion of pre-B cells into macrophages that are affected by HDAC7.
(XLS)

Dataset S2 Microarray statistics analysis for genes down-regulated during the conversion of pre-B cells into macrophages that are affected by HDAC7.
(XLS)

Figure S1 HDAC7 is down-regulated during the transifferentiation of pre-B cells into macrophages. Kinetics of regulation (log2 Affymetrix expression values) of *Hdac7* and *Pax5* genes in C10 cells and primary pre-B cells transduced with a retroviral vector for inducible expression of C/EBPα. Cells were treated with β-estradiol for the times indicated. HAFTL cells, primary pre-B cells, primary bone marrow macrophages and RAW264.7 cells were used as control.
(EPS)

Figure S2 Western blot showing the protein levels of endogenous and exogenously expressed HDAC7 in C10 cells transduced with a untreated or treated with β-estradiol for 72 hours.
(EPS)

Figure S3 HDAC7 re-expression interferes with the gene transcriptional program of the converted macrophages. A. Heatmap statistics showing significantly (FDR p-value ≤ 0.05) enriched KEGG pathways among the up-regulated genes affected by the re-expression of HDAC7 during transdifferentiation of pre-B cells into macrophages. Colours toward red indicate high statistic significance, yellow indicates low statistic significance, and gray indicates no statistic significance. B. Heat-maps showing observed differentially expressed genes for selected KEGG pathways. Blue colour cell indicates positive events while gray colour indicates that the gene was not observed differentially expressed in that experimental condition.
(EPS)

Figure S4 HDAC7 re-expression interferes with the gene transcriptional program of the converted macrophages. Heatmap statistics showing significantly (FDR p-value ≤ 0.05) enriched A. GO Biological Processes categories, B. GO Cellular Components categories, C. GO Molecular Functions categories and D. KEGG pathways, among the down-regulated genes affected by the re-expression of HDAC7 during transdifferentiation of pre-B cells into macrophages. Colours toward red indicate high statistic significance, yellow indicates low statistic significance, and gray indicates no statistic significance.
(EPS)

Figure S5 HDAC7 re-expression does not interfere with the down-regulation of Pax5. A. Kinetics of down-regulation (log2 Affymetrix expression values) of *Pax5* in C10-MSCV and C10-HDAC7 cells un-treated or treated with β-estradiol for the times indicated. B. RT-qPCR validation of the results shown in A.
(EPS)

Figure S6 HDAC7 is recruited to the *Itgam* promoter in pre-B cells. A. Schematic representation of the mouse *Itgam* locus and amplicons scanned in Chromatin immunoprecipitation experiments by qPCR. Asterisks indicate MEF2 binding sites location. B. Chromatin immunoprecipitation experiments showing the enrichment of HDAC7 and MEF2C to putative MEF2 binding sites on the *Itgam* gene loci in pre-B cells. Results are presented as percentage immunoprecipitated over input and are representative of three independent experiments.
(EPS)

Figure S7 HDAC7 re-expression decreases Mac1 protein levels in the reprogrammed macrophages. A. Histograms for Mac-1 protein levels in reprogrammed machrophages transduced with either an empty vector or a retroviral vector for HDAC7 expression. B. Histograms for Mac-1 and CD19 protein levels in RAW-MSCV and RAW-HDAC7 cells. C. RT-qPCR experiments for gene expression changes for Hdac7, Itgam, Ccl3, and Fcgr1 genes in RAW-MSCV and RAW-HDAC7 cells. D. Capacity of RAW-MSCV and RAW-HDAC7 cells to phagocytose red fluorescence bacteria.
(EPS)

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Author Contributions

Conceived and designed the experiments: MP. Performed the experiments: BB-Z LR-G OC HR ABMMKI TM. Analyzed the data: BB-Z LR-G OC HR ABMMKI TM NL-B MP. Contributed reagents/materials/analysis tools: LHB AdT LDA TG NL-B. Wrote the paper: MP.

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Review Article

Epigenetic Regulation of B Lymphocyte Differentiation, Transdifferentiation, and Reprogramming

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B cell development is a multistep process that is tightly regulated at the transcriptional level. In recent years, investigators have shed light on the transcription factor networks involved in all the differentiation steps comprising B lymphopoiesis. The interplay between transcription factors and the epigenetic machinery involved in establishing the correct genomic landscape characteristic of each cellular state is beginning to be dissected. The participation of "epigenetic regulator-transcription factor" complexes is also crucial for directing cells during reprogramming into pluripotency or lineage conversion. In this context, greater knowledge of epigenetic regulation during B cell development, transdifferentiation, and reprogramming will enable us to understand better how epigenetics can control cell lineage commitment and identity. Herein, we review the current knowledge about the epigenetic events that contribute to B cell development and reprogramming.

1. Introduction

Hematopoietic stem cells (HSCs) give rise to mature B cells through the sequential differentiation of lymphoid progenitor cells. Long-term HSCs (LT-HSCs) have the ability to self-renew and reconstitute the entire immune system by differentiating into short-term HSCs (ST-HSCs). ST-HSCs differentiate into multipotent progenitors (MPPs) that then branch into common myeloid progenitors (CMPs) and lymphoid-primed multipotent progenitors (LMPPs). CMPs further differentiate into erythrocytes and megakaryocytes, whereas LMPPs retain the capability to give rise to myelomonocytic or lymphoid lineages [1, 2]. LMPPs become common lymphoid progenitors (CLPs) [3], which have the potential to differentiate into B and T lymphocytes as well as natural killer (NK) cells [4, 5]. Once committed to the lymphoid lineage, further differentiation steps lead to the formation of pro-B and pre-B cells, which are the early B cell precursors for immature B cells, the terminally differentiated plasma cells and germinal-center B cells (Figure 1).

Every step in B cell development is characterized by the activation of the specific genetic program characteristic of the new intermediate/progenitor generated and the repression/extinction of the genetic program of the previous cellular state. To achieve this, the different differentiation steps are tightly regulated at the transcriptional level. In recent years, the theory of the existence of networks of lineage-specific and identity-transcription factors responsible for establishing particular genomic landscapes has gained credence [6]. In the case of lymphocyte development, the transcription factors Ikaros and PU.1 are critical for the cellular commitment of LMPPs to the lymphoid lineage [2]. Subsequently, early B cell specification depends on the action of E2A, EBF, and FOXO1, whereas Pax5 is required for proper B cell development and for maintaining B cell identity [7–12]. Finally, during later developmental stages, the transcriptional repressors Bcl6 and Blimp-1 are crucial for the generation of germinal-center B cells and plasma cells, respectively [13–17] (Figure 1).

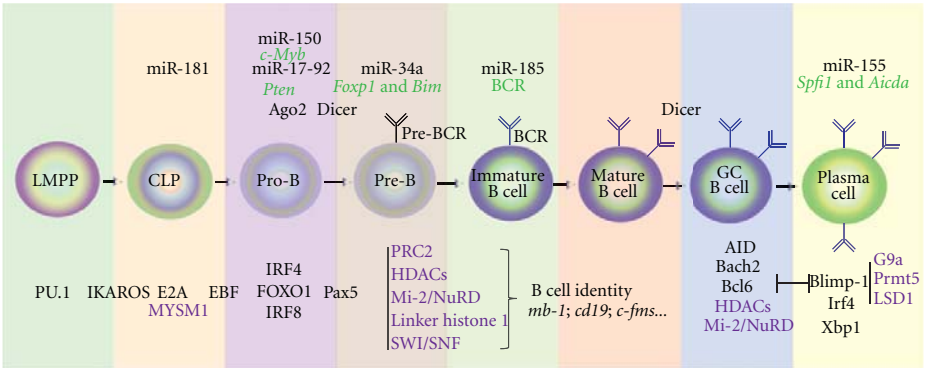

Figure 1: Scheme for B cell development. Successive stages of B cell differentiation and the key transcription factors and epigenetic regulators involved are shown. The epigenetic regulators that cooperate with specific transcription factors at every cell differentiation step are in purple. MicroRNA transcript targets are in green.

The picture of the hierarchical network of transcription factors that mediate the epigenetic signature needed to regulate the specific transcriptome of the fate of B-cells during their development has begun to emerge [18–20]. For example, Pax5, whose expression is induced by E2A and EBF, recruits chromatin-remodeling, histone-modifying and transcription-factor complexes to its target genes to activate the transcription of B cell-specific genes, and to silence lineage-inappropriate genes [19]. Extensive efforts have been made to elucidate the epigenetic mechanisms underlying the gene rearrangements of various components of the B cell receptor (BCR) [21–23]. Thus, epigenetic regulation is a critical event in B lymphocyte development. The relevance of transcription factors to the establishment and maintenance of cell-lineage identity has also been demonstrated in cellular reprogramming experiments [24–27]. The epigenetic mechanisms involved in the reprogramming and transdifferentiation of B cells have also been a focus of study in recent years.

Nucleosomes are the basic unit of the chromatin. They comprise 147 bp of DNA wrapped around a histone core, which contains two copies each of H2A, H2B, H3, and H4. This core is important for establishing interactions between nucleosomes and within the nucleosome itself [28]. Depending on the epigenetic modifications on the histone tails and in the DNA, chromatin can adopt different structural conformations that are correlated with its active, permissive (primed), or repressive status. The four main mechanisms by which epigenetic regulation occurs are DNA methylation, histone modification, chromatin remodeling, and regulation of gene expression by the action of noncoding RNAs. The methylation of cytosine residues at CpG dinucleotides (methyl-CpG), which is generally associated with transcriptional repression, is accomplished via the action of DNA methyltransferases (DNMTs) [29]. Methyl-CpG-mediated transcriptional repression can be explained by two nonmutually exclusive molecular mechanisms. First, methylation of DNA can interfere with the accessibility and recruitment of transcription factors to their DNA-binding sites. Second, DNA methylation results in the recruitment of methyl-CpG-binding proteins (MeCPs and MBDs) in association with corepressor complexes. Both mechanisms lead to the transcriptional silencing of the methylated genes [29]. The posttranslational modification of histones is another important epigenetic regulatory mechanism. Histones can be posttranslationally modified by a variety of enzymatic modifications, including acetylation, methylation, phosphorylation, sumoylation, and ubiquitination among others [30]. While acetylation is generally considered to be a mark of transcriptional activation, histone methylation can result in either transcriptional activation or repression, depending on the residue that is modified. In this regard, acetylation of histone H3 on lysine 9, 14, and or 18 (H3K9ac, H3K14ac, H3K18ac) is associated with transcriptional activation and considered "histone active marks." In the case of histone methylation, di- and tri-methylation of histone H3 at lysine 4 (H3K4me2, H3K4me3) are associated with transcriptional activation and therefore considered an active mark, whereas trimethylation of H3 at lysine 27 (H3K27me3) is found to be enriched at silenced genes and considered to be a repressive histone mark [28, 30]. Trimethylation of histone H3 at lysine 9 (H3K9me3) has also been characterized as a mark of transcriptional repression [30]. However, different reports suggest that it can also represent transcriptional activity [31, 32]. Another mechanism of epigenetic regulation involves the action of chromatin remodelers, which are multi-subunit complexes that use the energy from ATP hydrolysis to change the location or conformation of nucleosomes, resulting in increased or decreased DNA accessibility [28]. Chromatin-remodeling complexes can be divided into four groups, characterized by core ATPase subunits. Based on the defining ATPase, they are referred to as the SWI/SNF, ISWI, CHD, and INO80 families of remodelers [28]. Finally, microRNAs (miRNAs), a type of small noncoding RNAs, have been shown to anneal to 3′UTR of cognate mRNAs, leading to mRNA instability and/or the inhibition of translation, thereby making it possible to modulate the proteome of the cell [29].

In this papre we will summarize the recent advances in our understanding of the epigenetic mechanisms controlling B cell development and reprogramming.

2. B Cell Development: Early Specification towards the Lymphoid Lineage

When cells are at the LMPP stage, two transcription factors, Ikaros and PU.1, play critical roles in the early cellular specification towards the lymphoid lineage. Mice homozygous for a germline mutation in the Ikaros DNA-binding domain present a block at early lymphocyte development and therefore lack lymphocyte progenitors, T and B lymphocytes, as well as natural killer cells [33, 34]. More recently, Ikaros was shown to be a crucial transcription factor for the commitment of LMPPs into CLPs, clearly demonstrating its key role in the early cellular decision to undergo lymphocyte development [2]. LMPPs derived from Ikaros-null mice lack B cell potential and do not express *Flt3*, *Il-7r*, *Rag1* and *Rag2*, which are important genes for lymphoid commitment [2]. Mechanistically, Ikaros can either activate or repress transcription of target genes, depending on the recruitment of coactivators or corepressors. For example, in T cells, Ikaros has been shown to recruit corepressor or chromatin remodeling complexes in order to either repress or activate specific targets [35–37]. However, how Ikaros mediates the epigenetic regulation of its target genes during the differentiation of LMPPs into CLPs remains to be elucidated.

Likewise, the transcription factor PU.1 is crucial for the commitment of LMPPs to the lymphoid lineage. Strikingly, PU.1 is also required for the generation of GMPs and macrophages. In fact, mice deficient for PU.1 die around birth and lack B, T, NK and myelomonocytic cells [38, 39]. The promiscuity of PU.1 in regulating gene expression in different cell types raised the general question of what the mechanism of action is of a given transcription factor in different cell types. In this regard, Heinz et al. recently identified the genomewide binding sites of PU.1 in splenic B cells, macrophages and B cell progenitors [40]. They found that PU.1 cooperates with cell-type-specific transcription factors to activate the cisregulatory elements required for the development of a particular cell type. For example, in CLPs and pro-B cells, E2A induces PU.1 binding at B cell-specific genomic sites that contain closely located PU.1 and E2A binding motifs [40]. In addition, PU.1 binding initiates nucleosome remodeling, followed by H3K4me enrichment at many specific genomic regions [40]. These data could lead us to speculate that cooperation between PU.1 and Ikaros might be crucial for the activation of specific genes required to specify LMPP into CLPs. Also, the identity of Ikaros and PU.1 epigenetic partners remains unknown. This matter awaits investigation.

3. B Cell Development: Early B Cell Commitment

B cell development is characterized by the generation of the BCR, which consists of a heavy and a light immunoglobulin chain, IgH and IgL, respectively. The expression of the BCR subunits *VpreB*, *λ5*, and *mb-1* (*Cd79a*), and the initiation of D-J rearrangements at the IgH locus defines early B cell commitment [41]. The specification of CLPs in the B cell lineage requires two transcription factors, E2A and EBF1, which have been shown to activate the expression of genes essential for the formation of pro-B cells [42]. E2A and EBF knockout mouse models are phenotypically similar, and both transcription factors are considered to play key roles in initiating B lymphopoiesis. E2A-deficient mice show arrested B cell development at the pre-pro-B cell stage with compromised D-J rearrangements at the IgH locus and a lack of expression of *Rag1*, *mb-1*, *Iv*, *λ5*, *Cd19*, and *Pax5* genes [7–9]. More recently, it was shown that conditional deletion of E2A in pre-B cells did not result in a complete loss of expression of its target genes, indicating the involvement of E2A in the early steps of B cell commitment [43]. Similar to E2A, EBF is also known to play a crucial role in initiating B cell development. Mice lacking EBF do not express *Rag1*, *Rag2*, *mb-1*, *B29* (*Igβ*), *λ5*, *VpreB*, *cd19*, or *Pax5* genes [10]. Recent studies have also implicated the transcription factor FOXO-1 in early B lymphopoiesis. FOXO-1-deficient mice also show a developmental block at the pro-B cell stage [11]. Moreover, it has been reported that FOXO-1 regulates *Rag1* and *Rag2* expression [44].

Recent evidence indicates that the network of transcription factors Pax5, E2A and EBF also cooperate to regulate their target genes. For example, E2A, EBF, and Pax5 coordinate epigenetic events that lead to the expression of *mb-1*, which encodes the Igα subunit of the pre-BCR and BCR [45]. *mb-1* is methylated at CpG dinucleotides in HSCs and is gradually demethylated during B cell commitment correlating with its pattern of expression [21]. EBF and E2A contribute to the CpG demethylation and nucleosomal remodeling of the *mb-1* promoter, an event necessary for its transcriptional activation by Pax5. ATP-dependent chromatin remodeling complexes have also been implicated in EBF and Pax5-mediated regulation of the *mb-1* gene [21]. Knockdown of Brg1 and Brm, the catalytic subunits of the SWI/SNF chromatin-remodeling complex interfere with EBF and Pax5-mediated activation of *mb-1*. In contrast, knockdown of Mi-2, the catalytic subunit of the Mi-2/NuRD chromatin-remodeling complex, enhances chromatin accessibility and demethylation of the *mb-1* promoter and its transcription in response to both transcription factors [21]. These results are consistent with a model in which the SWI/SNF and Mi-2/NuRD chromatin remodeling complexes play antagonistic regulatory roles to enable or limit the reprogramming of target genes by EBF and Pax5 during B cell development [21]. The B-cell-specific gene *Cd19* is another example of a gene that is epigenetically regulated during early B cell development. *Cd19* encodes a cell surface protein that participates in signal transduction mechanisms via the BCR and pre-BCR. Chromatin remodeling at the upstream enhancer sequences of *Cd19* occurs in multipotent progenitors [22]. This chromatin remodeling has been shown to facilitate the recruitment of E2A to this locus followed by EBF and Pax5 recruitment [22]. Interestingly, the *Cd19* promoter is transcriptionally activated only after Pax5

binding. In this context, Mercer et al. recently reported that the monomethylation of H3K4 (H3K4me) at the enhancer regions of cell lineage-specifying genes is the main epigenetic mark, which is associated with their specific expression pattern throughout the lymphoid differentiation program [46]. Taken together, these reports provide clear examples of how B cell lineage-specific transcription factors cooperatively mediate the epigenetic regulation of target genes during B lymphopoiesis.

The recent advances in ultrasequencing technologies are helping to draw a global picture of how the networks of transcription factors modify the chromatin of their target genes. The laboratory of Cornelis Murre, using a ChIP-seq experimental approach, has elucidated how the network of transcription factors E2A, EBF and FOXO-1 orchestrates B cell commitment [18]. They found that during the transition of pre-pro-B cell to pro-B cells, E2A-associated genes become monomethylated at lysine 4 on H3 (H3K4me), a mark mainly found on gene enhancer elements. Subsequently, EBF and FOXO1 are involved in the enrichment of active histone modifications such as H3K4me3 on B-cell-specifying genes, such as *Pax5* [18]. Recently, Treiber and colleagues have shed light on the EBF-mediated epigenetic regulation of its target genes [47]. They classified EBF targets as activated, repressed, or primed genes. They observed that, in pro-B and pre-B cells, the "activated" genes are enriched in H3K4me3 and H3 acetylation active marks and show low levels of the repressive mark H3K27me3 [47]. In contrast, the "repressed" genes show the opposite pattern of histone modifications. The "primed" genes are enriched in the gene enhancer mark H3K4me in pre-B and pro-B cells and enriched in H3K4me3 and H3 acetylation in mature B cells [47]. The identification of the epigenetic regulators recruited by transcription factors to mediate gene expression changes during B lymphopoiesis remains to be addressed.

Other epigenetic marks, such as ubiquitination of Histone H2A, have proved to play a role in early B cell development. Jiang et al. pointed out that the histone H2A deubiquitinase MYSM1 is an important factor in B cell development [48]. *Mysm1* knockout mice show a drastic decrease in the number of B cells in the bone marrow, peripheral blood, and lymph nodes [48]. The authors concluded that MYSM1 antagonizes the action of the polycomb repressive complex 1 (PRC1) on the *Ebf1* promoter, enabling lineage-specific transcription factors, such as E2A, to be recruited to the *Ebf1* locus and to induce its transcription [48].

Early B cell development is also known to be regulated by microRNAs. Mice deficient in Ago2, which encodes a protein essential for microRNA biogenesis and function, display a block in B cell development at the pro-B cell stage [49]. Consistent with this, specific deletion of Dicer in pro-B cells, which abolishes the entire miRNA network in B cells, results in a complete block of B cell differentiation at the transition from pro-B to pre-B-cells [50]. Another study reported that miR-181, one of the approximately 100 microRNAs known to be expressed in mouse bone marrow cells, is more abundant in the B cell lineage than in other cell types [51]. Transplantation of multipotent hematopoietic progenitors overexpressing miR-181 into lethally irradiated mice resulted in an increase in the number of B cells [51]. Thus, miR-181 appears to target and repress the transcripts of critical genes involved in generating B cells. A similar experimental approach was used to show that another microRNA, miR-150, which is expressed in mature B and T cells, can block B cell differentiation at the pro-B cell stage when expressed prematurely [52]. Accordingly, the laboratory of Klaus Rajewsky reported that miR-150 plays a role during B cell differentiation through its action on *c-Myb* expression [53]. Other miRNAs have been associated with the early development of B cells. For instance, miR-34a ablation results in a developmental block at the pre-B cell stage, and miR-17-92 knockout mice exhibit a block in pro-B cells [54, 55]. They regulate the *Foxbp1* and *Bim* and *PTEN* genes, respectively, which are known to have a role in B cell differentiation [54, 55]. Recently, Kuchen et al. have elucidated the microRNAome during lymphopoiesis at the genome-wide scale, leading to the identification of miRNAs that are primed for expression at different stages of differentiation [56]. They reported that miRNA expression is tightly regulated by epigenetic modifications. In particular, they showed that the repressive mark H3K27me3 is associated with the gene silencing of lineage-inappropriate miRNA during lymphopoiesis [56]. However, they also observed that active epigenetic regulation by the presence of H3K4me also occurs in some of the microRNAs "primed" to be expressed. On the basis of the restrictive expression and abundance of miRNAs during B cell lineage specification, miR-320, miR-191, miR-139 and miR28 appear to be potential regulators of B cell differentiation [56]. The transcripts targeted by key miRNAs for the early differentiation of B cells remains to be identified.

4. B Cell Development: Pax5 in the Maintenance of B Cell Identity

The transcription factor Pax5 is essential for maintaining the fate of B cells and is therefore considered to be "the guardian of B cell identity" [57]. Its expression gradually increases in a stepwise manner during B cell development. Pax5 expression is first detected at the early pro-B cell stage and maintained up to the mature B cell stage. Pax5 knockout mice show a block in B cell development at the pro-B stage [12]. Pax5−/− pro-B cells express both E2A and EBF transcription factors, as well as their target genes. In contrast, E2A−/− and EBF−/− derived cells do not express Pax5. Collectively, these data indicate that Pax5 is a target for both transcription, factors. The laboratory of Meinrad Busslinger has shed light on the molecular mechanisms involved in the gradual expression of Pax5 during B cell development. In particular, they have identified an enhancer in the *Pax5* locus, which in combination with the promoter, recapitulates B lymphoid Pax5 expression [58]. Interestingly, the *Pax5* enhancer is silenced by DNA methylation in embryonic stem cells, while it becomes activated in multipotent hematopoietic progenitors. The presence of consensus binding sites for the transcription factors PU.1, IRF4, IRF8, and NF-*k* B within the *Pax5* enhancer suggests that these transcription factors play a role in sequential enhancer activation in hematopoietic

progenitors and during B cell development [58]. At the onset of pro-B cell development the transcription factor EBF1 induces chromatin remodeling at the *Pax5* promoter region. In non-B cells, Polycomb group proteins repress the *Pax5* promoter region [58].

In addition to the epigenetic regulation of its expression during B cell development, Pax5 induces the establishment of a B cell-specific transcription program that is associated with the suppression of inappropriate genes of alternative lineages, thereby ensuring its role in maintaining B cell identity and differentiation. Using gene expression microarrays and genome-wide ChIP-on-chip experimental approaches, the laboratories of Busslinger and Nutt have described the complex gene regulatory network regulated by Pax5 during B lymphopoiesis [59–61]. These studies have identified genes that are activated or repressed by Pax5 in wildtype pro-B cells. Pax5-activated genes appear to encode transcription factors and key proteins involved in B cell signaling, adhesion, migration, antigen presentation and germinal-center B cell formation [59, 61]. However, Pax5-repressed genes encode secrete proteins, cell adhesion molecules, signal transducers and nuclear proteins that are specific to erythroid, myeloid, and T cell lineages [59, 61]. Pax5-activated genes in pro-B cells were found to be enriched with epigenetically active marks, including H3K9ac, H3K4me2 and H3K4me3 [60]. Importantly, in Pax5-deficient pro-B cells, these active histone marks were dramatically reduced or lost, indicating that Pax5 is essential for guaranteeing the active chromatin structure at its target genes. These findings demonstrate that Pax5 is a master regulator of B cell identity, which, in conjunction with epigenetic regulators, coordinates a B-cell-specific target gene transcription program. Recently, McManus and colleagues have described the epigenetic mechanisms mediated by Pax5 during B lymphopoiesis [19]. By using a ChIP-on-chip analysis, they have identified Pax5 target genes in committed pro-B cells. The authors also apply a proteomic approach to identify Pax5 interacting partners. They found that Pax5 interacts with the members of the SWI/SNF chromatin remodeling complex Brg1. BAF57 and BAF170. They also reported that PAX5 recruits the NCoR1 repressor complex with its associated HDAC3 activity to repressed its target genes [19]. This study has provided novel important insight into the regulatory network and epigenetic regulation, by which Pax5 directly controls B-cell commitment at the onset of B lymphopoiesis.

The mechanism by which Pax5 mediates transcriptional repression of targets has also been informatively examined using a candidate gene approach. One of the important target genes repressed by Pax5 in B cells is the colony-stimulating factor receptor 1 gene (*csf1r* or *c-fms*), a gene essential for macrophage development. *Csf1r* is expressed at low levels in HSCs and downregulated in all nonmacrophage cell types. In HSCs, MPPs, CMPs, and CLPs the *Csf1r* promoter is bound by transcription factors and its chromatin structure in an active conformation [62]. However, the *Csf1r* gene is silenced during B cell differentiation. Interestingly, an intronic antisense transcription unit that is differentially regulated during lymphopoiesis overlaps with regions of de novo DNA methylation in B cells, highlighting DNA methylation as a mechanism for *Csf1r* silencing during B cell development. Despite being silenced, *Csf1r* chromatin remains in a poised or primed conformation even in mature B cell stages. Importantly, *Csf1r* expression can be reactivated by conditional deletion of the transcription factor Pax5 [62]. Pax5 was shown to bind the *Csf1r* gene directly, resulting in loss of RNA polymerase II recruitment and binding of myeloid transcription factors at cisregulatory elements [63]. Finally, Pax5 in conjunction with linker histone H1 also coordinates DNA methylation and histone modifications in the 3′ regulatory region of the immunoglobulin heavy chain locus and thus epigenetically regulates the IgH locus [64].

5. B Cell Development: Terminal Differentiation

The completion of V(D)J recombination and expression of the BCR on the surface of B cells marks the beginning of antigen-dependent B cell development. From this point, B cells undergo terminal differentiation dependent on signals emanating from the BCR after antigen triggering [65]. Peripheral B cells, without antigen-mediated signaling, are in a resting state [66]. Once activated, they either initiate the germinal center (GC) reaction or differentiate into antibody-secreting plasma cells. Entry into the GC reaction is regulated by Bcl6, whereas the generation of antibody-secreting plasma cells is controlled by Blimp-1. Bcl6 and Blimp-1 both act as transcriptional repressors and work in a mutually exclusive manner [67, 68].

After antigen triggering, Bcl6 is upregulated in some B cells that then enter the GC reaction [13–15]. In contrast, cells in which Bcl6 is not upregulated undergo differentiation into plasma cells [69, 70]. From a mechanistic angle, Bcl6 has been shown to interact with the chromatin remodeling complex Mi-2/NuRD in GC B cells, leading to the repression of specific genes that are characteristic of plasma cells [71, 72]. This Mi-2/NURD-mediated repression requires the recruitment of histone deacetylases HDAC1 and HDAC2 [71, 72].

After activation of GC B cells Bcl-6 expression is down-regulated in association with the expression of its target gene Blimp-1. Once expressed, Blimp-1 represses the gene expression program of mature B cells, thereby promoting plasma cell differentiation [16, 17]. Mechanistically, Blimp-1 exerts its repressive transcriptional activity by recruiting regulators and coordinating epigenetic modifications at its target genes. PRD1-BF1, the human orthologue of Blimp-1, silences the interferon beta gene in response to viral infection by recruiting the histone methyltransferase (HMTase) G9a to the interferon-beta promoter, resulting in H3K9me [73]. Blimp-1 has also been found in a complex with the arginine histone methyl transferase Prmt5, although the functional significance of this interaction in B cells is not clear [74]. The histone lysine demethylase LSD1 has also been shown to interact with Blimp-1 [75]. Chromatin immunoprecipitation (ChIP) experiments indicated that Blimp-1 and LSD1 share some target genes leading to a more accessible chromatin structure [75]. Importantly, disruption of the Blimp-1-LSD1 interaction resulted in attenuated antibody secretion of the

cells, highlighting the functional relevance of this interaction for B cell function.

In the last few years, additional transcription factors have emerged as being involved in B cell terminal differentiation. It has been reported that IRF4 and XBp1 control the maintenance of plasma cell identity. IRF4 is responsible for BLIMP-1 induction and, in conjunction with XBp1, determines the fate of the plasma cell. The network of transcription factors Pax5, Bach2, and Bcl6 direct B cell development into germinal center cells. It has been shown that Pax5 induces Bach2 expression after B cell activation, which in turn cooperates with Blc6 to repress Blimp-1 expression promoting activation-induced cytidine deaminase (AID) expression and antibody class switch [76, 77].

MicroRNAs are also involved in the terminal differentiation of B cells. Peripheral B cells in transit to their final maturation can give rise to two functionally distinct peripheral populations: follicular (FO) or marginal zone (MZ) B cells. FO versus MZ fate decision is functionally coupled to BCR signaling and it has been suggested that B cells bearing BCRs with autoreactive specificities are preferentially driven into a MZ fate [78]. In 2010, Belver and colleagues generated conditional Dicer-deficient mice at later stages of B cell development [79]. They observed that miRNA metabolism is important for such developmental stage since these mice presented an impairment in the generation of follicular B cells and an overrepresentation of marginal zone B cells. Accordingly, another phenotypic feature of these mice was the presence of high titers of autoreactive antibodies [79]. They identified miR185 as an important factor for the correct BCR-mediated development of B cells.

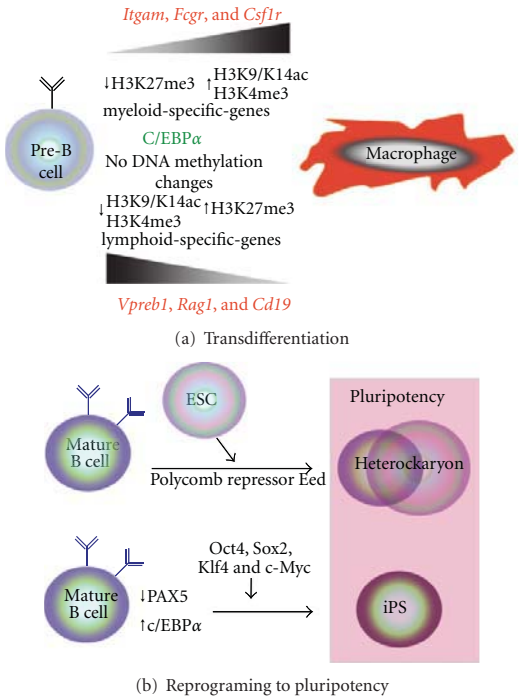

Figure 2: Transdifferentiation and reprogramming of B cells. (a) Ectopic expression of C/EBP in pre-B cells induces their transdifferentiation into macrophages. Epigenetic changes during the process are shown. (b) B cells can be reprogrammed to pluripotency by fusion with ESCs (heterokaryon) or by transgenic induction of Oct4, Sox2, Klf4 and c-Myc (iPS).

6. B Cell Reprogramming and Transdifferentiation

Since 1987, when the possibility of reprogramming specialized cells by the expression of a linage-specific transcription factor was first reported, many studies have tried to understand the molecular mechanisms that control all the processes involved. Due to the high developmental complexity that characterizes the hematopoietic system, it constitutes a model system with which study cell reprogramming and transdifferentiation in greater depth. In 1995, it was reported that overexpression of the erythroid lineage-specific transcription factor GATA-1 in myeloid leukemia cells induced their reprogramming into the megakaryocytic/erythroid lineage [24]. Subsequently, Nutt et al. reported that Pax5-defective pro-B cells differentiated into functional macrophages, granulocytes, natural killer cells, osteoclasts, and dendritic cells when specific cytokines were added to the culture medium [26]. Some years later, using knock-in and lineage-tracing technologies in mice, Xie and colleagues were able to demonstrate *in vivo* reprogramming of intrasplenic mature B cells into macrophages by the overexpression of the myeloid transcription factor C/EBPα [80]. More recently, the same laboratory generated a robust reprogramming system in which murine pre-B cells were converted into functional macrophages by the overexpression of C/EBPα [25] (Figure 2). This cellular conversion has been considered a transdifferentiation event since it is irreversible and does not require the retrodifferentiation of pre-B cells to previous progenitor stages [81]. Using the cellular system generated in Graf's laboratory, Radríguez-Ubreva and colleagues performed a high-throughput methylation analysis to study changes in DNA methylation during the transdifferentiation of pre-B cells into macrophages [82]. Surprisingly, they did not find any significant changes in DNA methylation during cellular conversion. However, they were able to identify the expected histone modifications in the genes that had previously been described to be upregulated or downregulated during the process. In particular, they reported an increase in the enrichment of the active histone marks H3K9/K14ac and H3K4me3, at the promoters of upregulated macrophage-specific genes, whereas a reduction of these modifications was observed in the B-cell-specific downregulated genes. In contrast, the repressive mark H3K27me3 was found to be enriched in the B cell downregulated genes and reduced in the upregulated macrophage-specific genes [82] (Figure 2). This study suggests that histone regulators are able to overcome the repressive effect of DNA methylation in

macrophage-specific genes in the converted cells. It also establishes an important difference from the process of reprogramming towards pluripotency in which promoter DNA demethylation plays a crucial role.

In this regard, Hanna and colleagues demonstrated that pro-B and pre-B cells can be reprogrammed into induced pluripotent stem (iPS) cells by the expression of the transcription factors Oct4, Sox2, Klf4, and c-Myc [27]. Interestingly, the expression of the four factors in mature B cells does not result in the reprogramming of mature B cells to pluripotency. They found that expression of c/EBPα in conjunction with the four "reprogramming" factors is necessary to generate iPS cells [27] (Figure 2). iPS cell lines derived from immature and mature B cells show promoter demethylation of the stem cell markers Oct4 and Nanog, whereas both promoters are heavily methylated in the original B cells. Finally, in mature B cells the promoter region of *Pax5* shows high and low levels of enrichment for the active mark H3K4me3 and the repressive mark H3K27me3, respectively. Conversely, equivalent enrichment of both histone modifications was observed in iPS lines derived from mature B cells [27]. This study raises the challenging question of how B lymphocytes at different developmental stages differ in their epigenetic landscape and how one factor can overcome this divergence to allow reprogramming into pluripotent cells.

A number of studies using experimental heterokaryons, in which a somatic cell is reprogrammed towards pluripotency by fusion with mouse embryonic stem (ES) cells, have also been used to reprogram B lymphocytes into pluripotent cells. The laboratory of Amanda Fisher has shown that when mouse ES cells are fused with human B lymphocytes the expression of human pluripotent-associated genes is rapidly induced [83]. Recently, the same group has elucidated some of the epigenetic mechanisms underlying this reprogramming process. They showed that deletion of Eed, Suz12, Ezh2, and Ring1A/B, which are members of either the polycomb repressor complex PRC1 or PRC2, in mouse ES cells abolishes their capacity to induce human B lymphocyte reprogramming towards pluripotency [84] (Figure 2).

7. Concluding Remarks

The impressive advances in genome-wide methods and the latest generation of ultrasequencing techniques are opening up new, and challenging lines of research focused on the elucidation of the epigenetic mechanisms underlying B cell differentiation and reprogramming. Many questions remain to be answered. Is there a specific "epigenetic signature" for the different cellular states comprising B cell development? How can lymphoid-specific transcription factors orchestrate the epigenetic machinery at different genes and genome regions to facilitate the choice to differentiate into a particular cellular lineage? Is the expression of epigenetic regulators lineage-specific? Epigenetic modification analyses, genome-wide RNA and ChIP-Seq studies, quantitative proteomics, and systematic functional studies offer us the opportunity to obtain high-quality measurements that will provide us with a draft of the "epigenetic-transcriptional" program that controls B cell development and reprogramming. Finally, conditional gene inactivation in mice will reveal the role of specific epigenetic regulators during B cell development. Thus, new regulatory networks connecting epigenetic and transcription factors seem likely to be revealed in the context of B lymphopoiesis in the future.

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