**Olive leaf components apigenin 7-glucoside and luteolin 7-glucoside
direct human hematopoietic stem cell differentiation towards
erythroid lineage**

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23 Abstract

24 The generation of blood cellular components from hematopoietic stem cells is important for the
25 therapy of a broad spectrum of hematological disorders. In recent years, several lines of evidence
26 suggested that certain nutrients, vitamins and flavonoids may have important roles in controlling
27 the stem cell fate decision by maintaining their self-renewal or stimulating the lineage-specific
28 differentiation. In this study, main olive leaf phytochemicals, oleuropein (Olp), apigenin 7-
29 glucoside (Api7G) and luteolin 7-glucoside (Lut7G) were investigated for their potential effects
30 on hematopoietic stem cell differentiation using both phenotypic and molecular analysis.
31 Oleuropein and the combination of the three compounds enhanced the differentiation of CD34+
32 cells into myelomonocytic cells and lymphocytes progenitors and inhibited the commitment to
33 megakaryocytic and erythroid lineages. Treatment with Lut7G stimulated both the erythroid and
34 the myeloid differentiation, while treatment with Api7G specifically induced the differentiation
35 of CD34+ cells towards the erythroid lineage and inhibited the myeloid differentiation. Erythroid
36 differentiation induced by Api7G and Lut7G treatments was confirmed by the increase in
37 hemoglobin genes expressions (*α-hemoglobin*, *β-hemoglobin* and *γ-hemoglobin*) and erythroid
38 transcription factor *GATA1* expression. As revealed by microarray analysis, the mechanisms
39 underlying the erythroid differentiation-inducing effect of Api7G on hematopoietic stem cells
40 involves the activation of JAK/STAT signaling pathway. These findings prove the
41 differentiation-inducing effects of olive leaf compounds on hematopoietic stem cells and
42 highlight their potential use in the *ex vivo* generation of blood cells.

Key words: Hematopoietic stem cells, differentiation, olive leaf components, erythroid differentiation, microarray analysis.

Introduction

Hematopoiesis is a tightly regulated process maintained by a small pool of hematopoietic stem cells (HSC) capable of undergoing self-renewal and generating mature progeny of all the hematopoietic cell lineages (Abdelhay et al., 2012). The two defining features of HSCs, self-renewal and multi-lineage differentiation, make these cells an attractive source for stem cell-based therapies. HSC transplantation and the infusion of the *ex vivo* expanded progenitors of a specific lineage can be a life-saving procedure in the treatment of a broad spectrum of disorders, including hematologic, immune, and genetic diseases (Walasek et al., 2012; Hino et al., 2000; Lippi et al., 2011; Siddiqui et al., 2011). Thus, strategies to expand either hematopoietic progenitors or the differentiated sub-populations are an area of active research. Characterizing and identifying regulators of stem cell fate decision may also help to design approaches that stimulate endogenous stem cells to promote healing and regenerative medicine as an alternative to stem cell transplantation (Bickford et al., 2006; Shytle et al., 2010). The decision of symmetric or asymmetric division of HSC depends on both intrinsic and extrinsic factors. The former include the specific hematopoietic lineage to which the cell belongs and its stage of maturation. In this regard, multiple proteins have been identified as regulators of HSC fate including transcription factors, epigenetic modifiers and cell cycle regulators (Walasek et al., 2012; Kubota and Kimura 2012). On the other hand, the extrinsic factors include multiple cytokines, adhesion molecules and other signals produced by stromal cells occupying HSC microenvironment and likely other cells in the body. Multiple cytokines, including SCF, TPO, EPO, Flt-3L, IL-11, IL-3, IL-6, and GM-CSF, and combinations of these, have been studied in *in vitro* HSC expansion and

differentiation protocols of mouse and human cells (Walasek et al., 2012; Sauvageau et al., 2004). In recent years, a growing body of evidence suggests that in addition to cytokines/factors, chemical and natural compounds can also have potent effects on hematopoietic cell expansion protocols. A number of compounds such as retinoic acids, valporic acid, 5-aza-2-deoxycytidine methyltransferase (5azaD), and the lipid mediator prostaglandin E₂ (PGE₂) have been identified for their ability to control stem cell fates in culture, where the effects ranged from the enhancement of stem cell expansion to the stimulation of lineage-specific differentiation (Walasek et al., 2012; Purton et al., 1999; De Felice et al., 2005; Hoggatt et al., 2009; Milhem et al., 2004; Bickford et al., 2006). Shytle et al. (2007) showed that a particular combination of blueberry extract, green tea extract, carnosine, and vitamin D3, a proprietary nutraceuticals formulation known as NT-020, demonstrated synergistic activity in promoting proliferation of hematopoietic stem cells. Therefore, it appears that it is possible to use certain natural products, either alone or in combination, in order to direct the stem cell culture to maintain the self-renewal or to stimulate the differentiation of the committed progenitors (Bickford et al., 2006; Shytle et al., 2010; Shytle et al., 2007).

Due to its established beneficial effects on health, olive leaf has gained the interest of the scientific and industrial community, and thus, has emerged as commercially valuable nutraceuticals. Olive leaves contain many bioactive compounds that have antioxidant, anti-microbial, antihypertensive, anti-viral, anti-inflammatory, hypoglycemic, neuroprotective, and anti-cancer properties (Kontogianni and Gerothanassis, 2012; Pereira et al., 2007; Somova et al., 2003; Micol et al., 2005; Wainstein et al., 2012; Seddik et 2011; Bouallegui et al., 2011). Olive leaf extract and compounds, such as apigenin 7-glucoside, showed anti-leukemia effects by inducing the differentiation of different leukemia cell lines (Abaza et al., 2007; Samet et al.,

2014a; Tzolmon et al., 2011). While myeloid leukemia cell lines are frequently used to study differentiation of myeloid cells, these cells have a highly abnormal karyotype and often display functional differences from their normal myeloid counterparts (Choi et al., 2014). We have previously demonstrated that olive leaf components have the potential to enhance the differentiation of hematopoietic stem cells rather than stimulating their self-renewal (Samet et al., 2014b). In this study, we identify the lineage-differentiation effects of each compound on the human hematopoietic stem cells and give insight into the underlying mechanism.

Materials and Methods

Sample preparation and cell culture

Stock solutions of 100 mM of oleuropein (Olp), apigenin 7-glucoside (Api7G) and luteolin 7-glucoside (Lut7G), (Sigma Aldrich), were prepared in dimethyl sulfoxide (DMSO) and stored at -20°C until use. Cryopreserved CD34+ hematopoietic stem/progenitor cells (HSPCs) were purchased from Life Technologies™ (GIBCO, Life Technologies™) and cultured in StemPro®-34 serum-free medium supplemented with StemPro®-34 Nutrient Supplement. Cytokines, SCF, IL-3 and GM-CSF (GIBCO, Life Technologies™) were used at final concentrations of 100 ng/mL, 50 ng/mL and 25 ng/mL respectively. For all experiments, cells were seeded at 2×10^4 cells/mL in 6-well plates and incubated for 24 h before the addition of olive leaf phytochemicals at the desired final concentrations.

The individual effect of each compound was assessed by treatment with Olp, Api7G or Lut7G at a final concentration of 50 μ M based on a preliminary study of the morphology and the viability of CD34+ cells cultured in presence of different concentrations of each compound (Samet et al., 2014b). DMSO (vehicle) at 0.05% was added to control cells. In order to evaluate the effect of

the combination of these three compounds, cells were also treated with combination (comb) of Olp, Api7G and Lut7G at 55 μ M, 5 μ M, and 5 μ M, respectively. The concentrations of the compounds in the mixture were determined based on the HPLC analysis of the ethanol extract of olive leaf and the compounds' effective concentration for the induction of differentiation of human chronic myelogenous leukemia K562 cells in our previous study (Samet et al., 2014a).

Cell viability and cell number

The viability and number of CD34+ cells were determined using flow cytometry on the 3rd, 6th and 9th days of culture with each compound (Olp, Api7G or Lut7G) or their combination (Comb). DMSO-treated CD34+ cells served as control cells. After incubation for the indicated time, treated cells were harvested, suspended in Guava ViaCount reagent (Guava Technologies) and allowed to be stained for at least 5 min in darkness. The Guava ViaCount reagent contains two DNA-binding dyes. The nuclear dye stains only nucleated cells, while the viability dye brightly stains dying cells. The differential permeability of these two dyes enables the Guava ViaCount assay to distinguish between viable and non-viable cells. The cell number and viability were automatically measured using the Guava ViaCount application in Guava PCA flow cytometry (Guava Technologies). Morphological changes were detected by observation under a phase contrast microscope (Leica Microsystem).

Cell cycle analysis

HSPCs were cultured for 3, 6 or 9 days, harvested, washed with 1x PBS, fixed with 70% ethanol, and incubated at 4°C for more than 12 h. The fixed cells were then centrifuged and washed with 1x PBS twice. Cell cycle reagent (Guava Technologies) was added, and the cells were incubated in the dark for 30 min at room temperature. The population of cells in each cell cycle phase was determined using the cell cycle software of Guava PCA.

Differentiation marker expression

Phenotypic analysis of the expanded cells were carried out on the 3rd, 6th and 9th days by analyzing the expression of surface markers such as CD34, CD38, CD33, CD10, CD14, CD41 and GlycophorinA (Abcam). The expanded control and treated cells were collected, the number adjusted to 1×10^5 cells and washed with 1x PBS. After the addition of antibodies, the cells were incubated for 40 min on ice. Excess antibody was removed by washing with 1x PBS before analysis using Guava PCA. The expression level of each surface antigen in treated cells was normalized to its expression level in control cells.

RNA isolation and real-time PCR analysis

Total RNA extraction of treated CD34+ cells, as well as control cells, was conducted on the 2nd, 5th and 8th day of culture using Isogen reagent (Nippon Gene Co., Ltd) following the manufacturer's instructions. The isolated RNA was quantified using Nanodrop 2000 (Thermo Scientific). First strand cDNA synthesis was carried out from about 1µg of total RNA using Superscript III (Invitrogen) and oligo(dT) primers according to manufacturer's protocol. Real-time PCR was performed using TaqMan master mix and specific gene primers from Applied Biosystems, specific for the 7500/7500 Fast RT-PCR system. Primers for *α-hemoglobin*, *β-hemoglobin*, *γ-hemoglobin*, *GATA1*, and the internal control *β-actin*, with the assay IDs listed in Supplementary table (Table S1), were all purchased from Applied Biosystems.

Microarray analysis

DNA microarray analysis was conducted on isolated RNA samples from cells treated with Api7G for 2 days and 5 days. Double-stranded cDNA was synthesized from 100 ng of total RNA using the GeneAtlas 3' IVT Express Kit (Affymetrix, Inc.). Biotin-labeled aRNA was

synthesized by *in vitro* transcription and purified. Purified aRNA (10 µg) was then fragmented using the GeneAtlas 3' IVT Express Kit and was hybridized to the Affymetrix HG-U219 (Affymetrix) for 16h at 45°C. The chips were washed and stained in the GeneAtlas Fluidics Station 400 (Affymetrix) and then the resulting image scanned using the GeneAtlas Imaging Station (Affymetrix). In order to identify the differentially expressed genes, data analysis was conducted using Affymetrix® Expression Console™ Software and Affymetrix® Transcriptome Analysis Console (TAC) 2.0 Software (Affymetrix). Gene ontology categories that are statistically overrepresented were assessed using BiNGO 2.4 software (Biological Network Gene Ontology) from Cytoscape (Maere et al., 2005). The differentially expressed genes were also subjected to hierarchical clustering using Euclidean distance and average linkage algorithm of the TIGR Mev version 4.9 software (Saeed et al., 2003).

Statistical analysis

Data are presented as the mean ± SD of three independent experiments. Statistical analyses were performed based on a paired two-tailed student's t-test (Microsoft Excel). A *p*-value < 0.05 was considered statistically significant.

Results

Major components of olive leaf and their combination were not cytotoxic on CD34+ hematopoietic cells

The cell viability and the cell number of CD34+ hematopoietic stem/progenitor cells were assessed every three days following treatment with the different samples. Results indicated that the number of viable cells was decreased by Comb on the 3rd day of culture compared to control cells, while no significant difference was observed on the 6th and 9th day of treatment. The

number of cells treated with Api7G was significantly lower than that of control cells throughout the treatment periods while treatment with Lut7G had no effect on the cell number (**Figure 1A**).

The results of cell viability analysis showed that the compounds, and their combination, did not affect the cell viability of CD34+ cells (**Figure 1B**). These findings suggest that treatment with 50 μ M of Olp, Api7G or Lut7G, as well as their combination, is not cytotoxic on hematopoietic stem/progenitor cells. Despite the decrease in the viable cell number observed during the culture with Api7G, the cell viability of Api7G-treated cells notably remained similar to the control cells. Consequently, the observed decrease in the cell number could be explained by a reduction in the growth rate of treated cells, rather than by cell death. Microscopic observations did not reveal significant morphological changes between the treated cells and the control cells (**Figure 2**).

Olive leaf components modulate the cell cycle distribution of CD34+ cells

Cell cycle analysis was conducted in order to understand the cause behind the inhibition of the growth rate of comb- and Api7G-treated cells. Data showed a significant increase of the cell population at G0/G1 phase on the 3rd and 6th day of culture in Api7G-treated cells associated with a slight decrease in the cell population of G2/M phase on the 6th day compared to control cells (**Table 1**). Culture of CD34+ cells in presence of the combination of the three compounds arrested the cell cycle at G0/G1 on the 3rd and 6th day of incubation (**Table 1**).

Olive leaf components induce the differentiation of hematopoietic stem/progenitor cells into various blood cell lineages

To assess the differentiation-inducing abilities of olive leaf compounds on hematopoietic stem cells, phenotypic analysis was carried out every three days of incubation using different antibodies for CD33, CD14, CD10, CD41, GlycophorinA (GPA), CD34 and CD38.

CD33 expression, a transmembrane receptor expressed on myelomonocytic cells, was significantly increased on the 3rd day of treatment with Comb and Olp. Lut7G-treated cells showed an increase in CD33 expression on the 9th day of treatment while its expression in cells incubated with Api7G was decreased compared to control cells (**Figure 3A and Figure S1A**).

The expression of CD14, a co-receptor for the detection of bacterial lipopolysaccharide used as specific marker for monocytes, was slightly increased after treatment with the combination of compounds on the 3rd and 6th day, but decreased in Olp-, Api7G- and Lut7G-treated cells. A significant decline in CD14 expression was observed on the 9th day of incubation for all the treatments in comparison with control cells (**Figure 3B and Figure S1B**).

Expression of CD10, a membrane metallo-endopeptidase commonly expressed on lymphoid progenitors, was significantly enhanced on the 3rd day in cells cultured with Olp and in cells treated with Comb (**Figure 3C and Figure S1C**).

As a marker for erythrocyte differentiation, the expression of GPA, a sialoglycoprotein of the membrane of red blood cells, was significantly increased on the 6th day and 9th day of incubation with Api7G. Similarly, treatment with Lut7G significantly enhanced the expression of GPA after incubation of CD34+ cells for 9 days. A reduction of GPA expression was observed for cells cultured in presence of Olp (**Figure 3D and Figure S1D**).

The expression of CD41, a membrane protein with coagulation properties used as a marker for megakaryocytes, was increased by the addition of Api7G to CD34+ cells after incubation for 6 days and 9 days. Meanwhile, treatment with Comb, Olp and Lut7G inhibited the expression of CD41 on the surface of hematopoietic stem/progenitor cells for all the treatment periods (**Figure 3E and Figure S1E**).

To investigate if olive leaf components maintain the primitive forms of hematopoietic cells, we analyzed the treated cells for the expression of CD34 and CD38. The expression of CD38 and CD34 was increased on the 3rd and 6th day, respectively and decreased after 9 days of treatment (**Figure S2**). This decrease could be indicative of loss of the primitive forms of hematopoietic cells by their differentiation.

Erythropoiesis-related genes expressions

Erythroid differentiation is accompanied by the synthesis of hemoglobin proteins, which are considered as specific markers of erythrocytes. We evaluated the expression of *α -hemoglobin*, *β -hemoglobin* and *γ -hemoglobin* genes by quantitative real-time PCR to confirm the erythroid differentiation induced by Api7G and Lut7G. Results presented in **Figure 4** showed a significant increase in the expression of these genes in Api7G-treated cells on the 5th day of incubation compared to control cells. The effect of Api7G treatment on the major erythroid transcription factor *GATA1* was investigated and results indicated an up-regulation of this gene. Treatment with Lut7G significantly increased the expression of *GATA1* on the 5th day followed by an up-regulation of hemoglobin genes expressions on the 8th day.

Microarray analysis results

240 In order to get an insight into the mechanisms involved in the erythroid differentiation effect,
241 gene expression profiles in cells treated with Api7G were assessed after treatment for 2 days and
242 5 days. Only genes with more than 2-fold change (treatment/control) in expression were
243 considered for the analysis. Following treatment with Api7G for 2 days, 769 genes were
244 modulated (764 genes were up-regulated and 5 genes were down-regulated). Treatment with
245 Api7G for 5 days up-regulated 494 genes and down-regulated 17 genes. Gene set enrichment
246 analysis according to molecular function (Supplementary data: Table S2) indicated that the up-
247 regulated genes in Api7G-treated cells for 2 days were mainly related to protein binding such as
248 the tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein (*YWHAQ*),
249 members of ATP-binding cassette subfamilies (*ABCD3*, *ABCE1*, and *ABCC4*), members of Rab
250 family (*RAB11A*, *RAB14*, *RAB18*, *RAB5A* and *RAB6A*), the heterogeneous nuclear
251 ribonucleoproteins (*HNRNPD*, *HNRNPF*, *HNRNPDL*), and the core binding factor beta (*CBFB*),
252 and genes related to transcriptional activity such as the pre-mRNA processing factor 4B
253 (*PRPF4B*), the CDC like kinase 1 (*CLK1*) and the serine/arginine-rich splicing factors (*SRSF1*,
254 *SRSF5*, *SRSF11*). After treatment for 5 days, genes related to protein binding formed the major
255 category of the up-regulated genes. An up-regulation of genes of molecules functioning in
256 cytoskeletal organization such as the nucleolar and spindle associated protein 1 (*NUSAP1*), the
257 tropomyosin 1 (*TPM1*), the coronin actin binding protein 2A (*CORO2A*) and the subunit of actin
258 related protein complex (*ARPC3*), has been also observed in Api7G-treated cells. Hierarchical
259 clustering of the differentially expressed genes from 2 days and 5 days treatment with Api7G
260 generated two groups that represent the up-regulated genes on 2nd and 5th day, respectively
261 (**Figure 5**). Particularly, the level of expression of genes encoding for clatherin, heavy chain
262 (*CLTC*), the *CCHC*-type zinc finger nucleic acid binding protein (*CNBP*), the eukaryotic

translation initiation factor 4A2 (*EIF4A2*), the matrin 3 (*MATR3*) and the cathepsin C (*CTSC*), was maintained for both 2 days and 5 days treatment (**Figure 5**).

A list of selected differentially expressed genes is presented in **Table 2**.

Discussion

Olive leaf components used alone, or in combination, did not affect the cell viability of CD34+ cells even though Comb-treatment and Api7G-treatment decreased the cell number, indicating a slight inhibition in the growth rate (**Figure 1**). This decrease could be explained by the arrest of the cell cycle at G0/G1 phase observed in the cells treated with Comb and Api7G (**Table 1**). It is a well-established fact that cell cycle arrest at G0/G1 phase is closely associated with differentiation. Methods aimed at inhibiting the cell cycle at G0/G1 phase drive a rapid, condensed differentiation to terminally differentiated cells, demonstrating that the cell cycle is a principle rate-limiting step of differentiation throughout the early and late stages (Li and Kirschner, 2014). Accordingly, it is suggested that the G0/G1 arrest in CD34+ cells by Api7G or Comb treatment enabled the cells to undergo the differentiation process. The molecular pathways that couple cell cycle to differentiation involve molecules of the G1/S transition which includes growth factors, downstream signaling pathways, Myc, the Rb/E2F pathway, and CDK inhibitors (e.g. p21) (Li and Kirschner, 2014; Muñoz-Alonso and León, 2003). Interestingly, microarray analysis revealed an increase in the expression of genes that regulate the G1/S transition in Api7G-treated cells after 2 days of treatment, particularly the cyclin-dependent kinase inhibitor 3 (*CDKN3*) whose encoded protein specifically dephosphorylate and inactivate CDK2, thereby inhibiting G1/S cell cycle progression (Chen et al., 2014).

Treatment with Comb, as well as with Olp, significantly increased the myelomonocytic population and enhanced the commitment to lymphocyte progenitors on the 3rd day. The simultaneous increase in CD14 expression indicates that the myelomonocytic population is consisting in part of monocyte/macrophage cells. No further enhancement of CD33 and CD10 expressions has been observed on the 6th and 9th day of treatment. This could be explained by the arrest of the proliferative activity in the differentiated cells which decrease their proportion compared to other cells that maintain the proliferative activity. The decrease in CD14 and CD41 expressions in Comb- and Olp-treated cells, and the reduction in GPA expression in Olp-treated cells on the 9th day suggests an inhibition of monocytes, megakaryocytes and erythrocytes differentiation. These findings suggest that treatment with Comb, as well as with its major compound Olp at 50 μ M, favors the differentiation of HSPCs towards the myelomonocytic lineages and their commitment to lymphocyte progenitors. Previous studies also reported the enhancement of myelomonocytic differentiation when compounds such as Vitamin D3 (1 α ,25-dihydroxyvitamin D3) and ATRA (all trans retinoic acids) were added to the culture medium supplemented with growth factors such as GM-CSF, SCF and IL-3 (Choi et al., 2009; Douer et al., 2000). The *ex vivo* expansion of cells of the immune system is needed in cellular therapies during infusion in order to substitute the decrease in the immune activity after intensive administration of chemotherapeutic agents which causes a high vulnerability to infections (Haylock et al., 1992; Choi et al., 2009).

HSPCs Incubated with Api7G expressed significant levels of GPA and CD41 on the 6th day of treatment. On the other hand, CD14 and CD33 expression were significantly decreased in all the incubation periods, particularly on the 9th day. These findings suggest that Api7G treatment enhance the differentiation of HSPCs towards erythroid and megakaryocytic lineages and

inhibits their myelomonocytic commitment. The simultaneous increase in both erythroid and megakaryocytic lineages could be explained by their derivation from a common bipotent progenitor, called the megakaryocyte/erythrocyte progenitor (Chang et al., 2007). The megakaryocyte differentiation-inducing effect was not as strong as the erythroid differentiation-inducing effect (around 1.2-fold increase in CD41 expression and 1.4-fold increase in GPA expression). The up-regulation of the expression of hemoglobin genes (*α -hemoglobin*, *β -hemoglobin* and *γ -hemoglobin*) as well as gene of the erythroid transcription factor, *GATA1* in Api7G-treated cells' total mRNA collected on the 5th day of treatment confirmed the occurrence of erythroid differentiation. These findings are in correlation with our previous study wherein an increase in the number of burst-forming unit-erythrocytes and mixed colonies of granulocytes-erythrocytes-macrophages-megakaryocytes in cells treated with Api7G was observed (Samet et al., 2014b).

Phenotypic analysis of HSPCs treated with Lut7G revealed an increase in the GPA expression after 9 days of incubation associated with a decrease of CD41 expression indicating a commitment to the erythroid lineage and an inhibition of megakaryopoiesis. In addition, Lut7G treatment significantly increased the expression of *GATA1* mRNA on the 2nd day and the 5th day of incubation followed by an increase in hemoglobin gene expression on the 8th day. A slight increase in CD33 expression was also detected in Lut7G-treated cells for 9 days associated with a significant reduction of CD14 expression suggesting that the increase in the myelomonocytic population is due to the expansion of the granulocytic lineages rather than the monocytic lineages. These results validate our previous report in which treatment with Lut7G increased the number of colony-forming unit-granulocytes/macrophages and burst-forming unit-erythrocytes (Samet et al., 2014b).

A variety of factors, such as insulin-like growth factor 1 (IGF-1), insulin, glucocorticoids, and cyclosporine A, have been proposed to support human red blood cells development when used as supplement to a liquid culture containing cytokines, mainly the glycoproteins erythropoietin (EPO) and SCF (Hattangadi et al., 2011; Miagawa et al., 2000; von Lindern et al., 1999; Ronzoni et al., 2008). These factors are reported to cooperate with EPO, the major factor governing erythropoiesis, in increasing erythrocytes production from CD34+ cells. In this study, despite the absence of EPO in the culture conditions, hematopoietic stem/progenitor cells were induced to differentiate towards the erythroid lineage by the addition of either Api7G or Lut7G highlighting the role of these flavonoids in stimulating the molecular mechanisms involved in erythroid development. These findings also suggest that in this culture condition (medium supplemented with SCF, IL-3 and GM-CSF), addition of Api7G or Lut7G could substitute erythropoietin in inducing erythroid differentiation. With the limitations of the current system based on voluntary blood donations, there is an increase in the demand for the generation of red blood cells for blood transfusion in several situations such as massive bleeding or as prolonged quality-of-life therapies in patients with chronic anemic disorders (Lippi et al., 2011). Thus, more interest is given to the improvement and optimization of the production of erythrocytes from different cell sources including somatic stem cells, human embryonic stem cells and induced pluripotent stem cells (Larochelle, 2013). To the best of our knowledge, this is the first report on inducing the differentiation of hematopoietic stem cells towards the erythroid lineage using plant-derived molecules.

The observed difference in the treatment time needed for the induction of erythroid differentiation between Api7G and Lut7G (6 days and 9 days respectively) could be partly explained by their different levels of potencies. It has been previously demonstrated, in a study

regarding insulin-resistance and type 2 diabetes, that apigenin is clearly more potent than luteolin in the inhibition of gluconeogenic and lipogenic genes expression (Bumke-Vogt et al., 2014). This difference in potency is probably related to the difference in their structures and thus to their affinity to interact with cellular components. The human cellular targets of apigenin have been identified by Arango et al. (2013) who concluded that the heterogeneous nuclear ribonucleoprotein A2 (hnRNPA2) is the most important of apigenin targets among molecules functioning mainly in GTPase activation, membrane transport and mRNA metabolism/alternative splicing, and that luteolin also interacts with this small-molecule, but with lower affinity than that of apigenin (Arango et al., 2013). Interestingly, the gene *HNRNPA2B1* encoding hnRNPA2 protein was up-regulated in Api7G-treated cells after 5 days of incubation (Table 2) suggesting that Api7G exerts its effects in CD34+ cells, partly by binding to hnRNPA2 and modulating the activity of alternative splicing of several human hnRNPA2 targets, which in turn modulates the activity of a large number of downstream genes. Other heterogeneous nuclear ribonucleoprotein could also be cellular targets for Api7G in CD34+ cells, as suggested by the up-regulation of *HNRNPD*, *HNRNPDL* and *HNRNPF* genes.

In order to explore the mechanisms involved in the erythroid differentiation-inducing effect of Api7G, microarray analysis was conducted on cells treated with Api7G for 2 days- and 5 days. Interestingly, among the differentially expressed genes were those that have significant role in erythroid differentiation, and the obtained results, therefore, are in correlation with the observed effect. Api7G up-regulated the genes involved in heme biosynthesis such as the *SLC25A37* gene coding for the solute carrier family 25 member 37, a mitochondrial iron transporter that specifically mediates iron uptake in developing erythroid cells, and the *CPOX* gene encoding the coproporphyrinogen oxidase, a key enzyme in heme biosynthesis (Chen et al., 2009; Taketani et

al., 2001). Moreover, *GYP A* and *GYP B* genes encoding the major sialoglycoproteins of the human erythrocyte membrane were respectively up-regulated after treatment for 2 days and 5 days (Table 2).

Results of the differentially increased genes indicated an up-regulation of janus kinase 2 (*JAK2*) and signal transducer and activator of transcription 1 (*STAT1*) genes in Api7G-treated cells after 2 days of incubation (Table 2). Several studies have reported the modulation of JAK/STAT pathways by the flavonoid apigenin (Shukla and Gupta, 2007). It has been reported that JAK2 is crucial for erythrocytes development and that mice which do not express an active protein for this gene exhibit embryonic lethality associated with the absence of definitive erythropoiesis (Neubauer et al., 1998). STATs play important roles in numerous cellular processes including immune responses, cell growth and differentiation, cell survival and apoptosis, and oncogenesis (Shuai, 2000). The drop in the expression levels of *JAK2* and *STAT1* genes on the 5th day could be explained by the transient expression of these genes as what have been reported in several studies in the development of different tissues such as the neointima formation and the neuron regeneration (Seki et al., 2000; Yao et al., 1997). After being phosphorylated by JAK, STATs translocate into the nucleus where they function as transcription factors to initiate gene activation. STATs interact with a number of proteins that modulate STAT signaling at various steps. Interestingly, genes encoding for some STAT-interacting proteins were up-regulated in Api7G-treated cells on the 5th day such as *KPNA1*, *NMI*, *EP300* and *CREBBP* encoding respectively for the nuclear protein importer NPI-1, the coactivator proteins N-myc-interactor Nmi-1, the histone acetyltransferase p300 and the CREB binding protein CRB. Taken together these findings indicate an activation of the JAK/STAT signaling pathway suggesting that this pathway plays an important role in the initiation of erythroid differentiation by Api7G-treatment.

Conclusion

This study provides the first report on the differentiation-inducing effects of olive leaf phytochemicals on human hematopoietic stem cells. The described effects highlight the potential use of olive leaf components in optimizing the *ex vivo* generation of the selected hematopoietic sub-populations. Addition of Api7G in particular as a supplement to the culture medium, specifically induces the differentiation of CD34+ cells towards the erythroid lineage despite the absence of EPO. Our findings suggest the involvement of the JAK/STAT pathway. A more in depth analysis of the different components of JAK/STAT pathway will be carried out in the future at both gene and protein levels. Furthermore, the potential synergistic effects of flavonoid Api7G and the lineages specific-cytokine EPO on erythroid differentiation will be investigated. It will be also interesting to evaluate the effect of this flavonoid on the engraftment potential of the *ex vivo* generated hematopoietic cells in *NOD/SCID mice*.

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558

Figure legends

Figure 1. Cell number (**A**) and cell viability (**B**) of CD34⁺ cells cultured in presence of olive leaf components. Cells were treated with oleuropein (Olp), apigenin 7-glucoside (Api7G) and luteolin 7-glucoside (Lut7G) used alone (at 50 μ M) or in combination (Comb). Cell number and cell viability were measured by flow cytometry after incubation for 3, 6 and 9 days. Control represents cells treated with 0.05% DMSO. Results are presented as the mean $\pm$ SD of three independent experiments. * and ** significantly different from the control ($p < 0.05$ and $p < 0.01$ respectively).

Figure 2. Morphological observations on CD34⁺ cells cultured in the presence of olive leaf components for 3, 6 and 9 days. Cells were treated with oleuropein (Olp), apigenin 7-glucoside (Api7G) and luteolin 7-glucoside (Lut7G) used alone (at 50 μ M) or in combination (Comb). Control represents cells treated with 0.05% DMSO. Cells were observed under a phase contrast microscope at 100x magnification. Scale bars represent 200 μ m.

Figure 3. Expressions of cell differentiation markers on hematopoietic stem cells cultured with olive leaf components. (**A**), (**B**), (**C**), (**D**) and (**E**) represent results of expression of CD33, CD10, CD14, GPA and CD41, respectively. Cells were treated with oleuropein (Olp), apigenin 7-glucoside (Api7G) and luteolin 7-glucoside (Lut7G) used alone (at 50 μ M) or in combination (Comb). Surface marker expression was measured by flow cytometry after incubation for 3, 6 and 9 days. Control represents cells treated with 0.05% DMSO. Results are presented as the mean $\pm$ SD of three independent experiments. * and ** significantly different from the control ($p < 0.05$ and $p < 0.01$ respectively).

580 **Figure 4.** Erythropoiesis-related genes expressions in CD34+ cells cultured with olive leaf
581 components for 2 days (**A**), 5 days (**B**) and 8 days (**C**). Cells were treated with apigenin 7-
582 glucoside (Api7G) and luteolin 7-glucoside (Lut7G) at 50 μ M each. Total RNA extraction was
583 carried out on the 2nd, 5th and the 8th day of incubation. Control represents cells treated with
584 0.05% DMSO. Results are presented as the mean $\pm$ SD of three independent experiments. * and
585 ** significantly different from the control ($p < 0.05$ and $p < 0.01$ respectively).

586 **Figure 5.** Gene expression map of the differentially expressed genes in CD34+ cells treated with
587 apigenin 7-glucoside (Api7G) for 2 days and 5 days. Genes with more than 2-fold change were
588 subjected to hierarchical clustering. Horizontal stripes represent genes and columns show
589 experimental samples. Clustering was performed on genes using the Euclidian distance method.
590 Red and green color codes for up- and down-regulation, respectively.

Table 1: Cell cycle distribution of CD34+ cells cultured in presence of olive leaf components.

		Day 3	Day 6	Day 9
G0/G1	Control	36.07 ± 3.11	38.19 ± 2.62	43.24 ± 5.38
	Comb	42.02 ± 3.62 **	43.07 ± 2.15 *	42.67 ± 3.95
	Olp	35.37 ± 4.43	41.99 ± 2.98	41.54 ± 4.62
	Api7G	41.08 ± 4.04 *	44.17 ± 1.61 **	39.38 ± 3.95
	Lut7G	37.67 ± 2.21	39.06 ± 2.86	38.25 ± 4.42
S	Control	14.39 ± 1.62	16.30 ± 1.17	10.35 ± 2.41
	Comb	15.38 ± 1.63	15.72 ± 1.66	10.49 ± 3.12
	Olp	11.18 ± 2.3	15.73 ± 1.52	10.49 ± 1.84
	Api7G	12.79 ± 2.22	15.07 ± 0.85	10.49 ± 2.13
	Lut7G	16.04 ± 2.5	16.28 ± 1.05	10.49 ± 1.55
G2/M	Control	26.32 ± 1.82	28.02 ± 1.58	26.5 ± 4.8
	Comb	26.91 ± 1.81	27.33 ± 1.27	24.84 ± 4.5
	Olp	28.46 ± 3.01	27.44 ± 0.94	26.27 ± 2.65
	Api7G	24.63 ± 3.31	25.79 ± 1.72 *	25.08 ± 3.17
	Lut7G	24.43 ± 1.31	27.92 ± 0.88	28.65 ± 4.22

Cells were treated by oleuropein (Olp), apigenin 7-glucoside (Api7G) and luteolin 7-glucoside (Lut7G) used alone (at 50 µM) or in combination (Comb). Cell cycle analysis was conducted using flow cytometry after incubation for 3, 6 and 9 days. Control represents cells treated with 0.05% DMSO. Results are represented as the mean ± SD of three independent experiments. * and ** significantly different from the control ($p < 0.05$ and $p < 0.01$ respectively).

Table 2. List of selected differentially expressed genes in CD34+ cells treated with apigenin 7-glucoside (Api7G) for 2 days and 5 days.

Gene Symbol	Transcript ID	Gene name	Molecular function	Gene expression Fold change (Treated/Control)	
				2 days	5 days
<i>YWHAQ</i>	Hs.74405.1	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, theta polypeptide	binding; protein binding	2.71	1.51
<i>ABCD3</i>	Hs.700576.3	ATP-binding cassette, sub-family D (ALD), member 3	protein binding; catalytic activity; nucleotide binding; purine nucleotide binding; adenyly nucleotide binding; hydrolase activity; ATP binding	2.66	-1.03
<i>RAB11A</i>	Hs.321541.2	RAB11A, member RAS oncogene family	protein binding; catalytic activity; nucleotide binding, GTP binding; phosphatase activity	2.53	-1.03
<i>PRPF4B</i>	Hs.159014.1	PRP4 pre-mRNA processing factor 4 homolog B (yeast)	protein binding; catalytic activity; nucleotide binding; purine nucleotide binding; hydrolase activity; ATP binding	2.55	-1.12
<i>CBFB</i>	Hs.460988.1	core-binding factor, beta subunit	binding; protein binding; transcription factor binding	2.41	1.32
<i>CLK1</i>	Hs.433732.2	CDC-like kinase 1	binding; catalytic activity; nucleotide binding; purine nucleotide binding; adenyly nucleotide binding; ATP binding	2.56	1.23

<i>SRSF1</i>	Hs.68714.1	serine/arginine-rich splicing factor 1	protein binding; nucleotide binding	3.3	1.08
<i>HNRNPD</i>	Hs.480073.5	heterogeneous nuclear ribonucleoprotein D (AU-rich element RNA binding protein 1, 37kDa)	binding; protein binding; nucleotide binding	4.7	1.07
<i>HNRNPF</i>	Hs.808.1	heterogeneous nuclear ribonucleoprotein F	binding; protein binding; nucleotide binding	2.32	1.99
<i>HNRNPA2B1</i>	Hs.487774.1	heterogeneous nuclear ribonucleoprotein A2/B1	binding; protein binding	1.14	2.09
<i>NUSAP1</i>	Hs.615092.6	nucleolar and spindle associated protein 1	binding; protein binding; cytoskeletal protein binding; tubulin binding; microtubule binding	2.64	1.03
<i>TPM1</i>	Hs.133892.8	tropomyosin 1 (alpha)	binding; protein binding; cytoskeletal protein binding	1.32	2.21
<i>ARPC3</i>	Hs.524741.5	actin related protein 2/3 complex, subunit 3, 21kDa	binding; protein binding; cytoskeletal protein binding	1.9	2.82
<i>CORO2A</i>	Hs.113094.1	coronin, actin binding protein, 2A	binding; protein binding; cytoskeletal protein binding	-1.08	2.19
<i>CLTC</i>	Hs.491351.1	clathrin, heavy chain (Hc)	binding; protein binding	2.75	3.14
<i>CNBP</i>	Hs.518249.4	CCHC-type zinc finger, nucleic acid binding protein	binding; protein binding	2.33	2.45
<i>EIF4A2</i>	Hs.518475.2	eukaryotic translation initiation factor 4A2	nucleic acid binding; translation initiation factor activity	2.31	2.39
<i>CDKN3</i>	Hs.84113.13	cyclin-dependent kinase inhibitor 3	protein binding, hydrolase activity, phosphatase activity	2.4	-1.04

<i>SLC25A37</i>	Hs.716436.2	solute carrier family 25 (mitochondrial iron transporter), member 37	binding	1.53	2.04
<i>CPOX</i>	Hs.476982.2	coproporphyrinogen oxidase	protein binding, catalytic activity	2.73	1.08
<i>GYPA</i>	Hs.434973.2	glycophorin A (MNS blood group)	binding; protein binding	2.26	1.18
<i>GYPB</i>	Hs.654368.8	glycophorin B (MNS blood group)	Binding	1.14	2.25
<i>JAK2</i>	Hs.656213.1	janus kinase 2	protein binding; catalytic activity; nucleotide binding; purine nucleotide binding; adenylyl nucleotide binding; ATP binding	2.18	1.3
<i>STAT1</i>	Hs.715518.1	signal transducer and activator of transcription 1, 91kDa	binding; protein binding	2.01	-1.02
<i>KPNA1</i>	Hs.161008.1	karyopherin alpha 1 (importin alpha 5)	binding; protein binding	1.12	2.12
<i>NMI</i>	Hs.54483.2	N-myc (and STAT) interactor	binding; protein binding; cytoskeletal protein binding	1.58	2.24
<i>EP300</i>	Hs.517517.1	E1A binding protein p300	protein binding; catalytic activity; transcription factor binding	2.05	-1.2
<i>CREBBP</i>	Hs.708378.1	CREB binding protein	binding; protein binding	1.02	2.01

Figure 1A.

Figure 1B

Figure 2

Figure 3A

Figure 3B

Figure 3C

Figure 3D

Figure 3E

Figure 4A

Figure 4B

Figure 4C

Figure 5