Mechanism of human Hb switching: a possible role of the kit receptor/miR 221-222 complex

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Acknowledgments: we thank Dr M Sargiacomo for helpful discussion on the imatinib experiments. We are grateful to AM Cerio and G Agostino and for their technical support, and to M Blasi, A Zito and G Loreto for the editorial assistance and graphics. CP is supported by the Italy-USA Oncology Program, Istituto Superiore Sanità.

Manuscript received on October 9, 2010. Revised version arrived on January 21, 2010. Manuscript accepted on March 8, 2010.

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ABSTRACT

Background
The human hemoglobin switch (HbF→HbA) takes place in the peri/post-natal period. In adult life, however, the residual HbF (<1%) may be partially reactivated by chemical inducers and/or cytokines such as the kit ligand (KL). MicroRNAs (miRs) play a pivotal role in normal hematopoiesis: downmodulation of miR-221/222 stimulates human erythropoietic proliferation through upmodulation of the kit receptor.

Design and Methods
We have explored the possible role of kit/KL in perinatal Hb switching by evaluating: i) the expression levels of both kit and kit ligand on $CD34^+$ cells and in plasma isolated from pre-, mid- and full-term cord blood samples; ii) the reactivation of HbF synthesis in KL-treated unilineage erythroid cell cultures; iii) the functional role of miR-221/222 in HbF production.

Results
In perinatal life, kit expression showed a gradual decline directly correlated to the decrease of HbF (from 80-90% to <30%). Moreover, in full-term cord blood erythroid cultures, kit ligand induced a marked increase of HbF (up to 80%) specifically abrogated by addition of the kit inhibitor imatinib, thus reversing the Hb switch. MiR-221/222 expression exhibited rising levels during peri/post-natal development. In functional studies, overexpression of these miRs in cord blood progenitors caused a remarkable decrease in kit expression, erythroblast proliferation and HbF content, whereas their suppression induced opposite effects.

Conclusions
Our studies indicate that human perinatal Hb switching is under control of the kit receptor/miR 221-222 complex. We do not exclude, however, that other mechanisms (i.e. glucocorticoids and the HbF inhibitor BCL11A) may also contribute to the peri/post-natal Hb switch.

Key words: hemoglobin switch, cord blood, cytokines, kit ligand, microRNAs.

Citation: Gabbianelli M, Testa U, Morsilli O, Pelosi E, Saulle E, Petrucci E, Castelli G, Giovinazzi S, Mariani G, Fiori ME, Bonanno G, Massa A, Croce CM, Fontana L, and Peschle C. Mechanism of human Hb switching: a possible role of the kit receptor/miR 221-222 complex. Haematologica 2010;95(8):1253-1260. doi:10.3324/haematol.2009.018259

Introduction

In human fetal life, red blood cells (RBCs) are characterized by a predominant content of fetal hemoblobin (HbF, $\alpha 2\ \gamma 2$), representing 85-90% of total Hb. Starting from late gestation, Hb production shows a gradual switch from HbF to adult Hb (HbA, $\alpha 2\ \beta 2$), i.e. HbF synthesis declines from 85-90% to less than 30% and further drops to less than 2% by one year of age and less than 1% in adults.[1,2] Numerous studies have attempted to identify the cellular and molecular mechanisms controlling the perinatal Hb switch.[3] *In vitro* and *in vivo* studies on fetal liver and cord blood (CB) hematopoietic progenitor cells (HPCs) indicate that the level of HbF synthesis in erythroid cells is regulated by a developmental clock,[4-6] i.e. the HbF synthesis program in HPCs is inversely related to the gestational age.[4-6] However, it has not been possible so far to clarify the extrinsic (i.e. environmental) and/or intrinsic mechanisms underlying this developmental clock.

In normal adults, the residual HbF, restricted to the F cell compartment,[7] may be reactivated in "stress erythropoiesis" to up to 10-20% of total Hb,[8,9] thereby providing an interesting model of partial reverse of the HbF to HbA switch. *In vitro* and *in vivo* inducers of HbF reactivation include histone deacetylase inhibitors,[10] particularly butyrate analogs,[11] and hydroxyurea,[12] as well as hematopoietic growth factors (HGFs), particularly the kit receptor ligand (KL).[13] In fact KL reactivates HbF production in normal erythropoiesis,[13,14] as well as in sickle cell anemia[15] and β-thalassemia.[16] Furthermore butyrate, hydroxyurea and dexamethasone potentiate the reactivating effect of KL.[17-19] Notably, HbF reactivation is also induced by downmodulation of the HbF repressor BCL11A.[20]

MicroRNAs (miRNAs or miRs) are a class of ~22-nt long non-coding RNAs which suppress protein expression at posttranscriptional level, mainly by annealing with the 3' UTR of the target mRNA.[21] MiRNAs play a pivotal role in the regulation of basic cell functions, including proliferation, differentiation and apoptosis in normal and abnormal conditions.[21-23] Importantly, several miR-based pathways underlying hematopoiesis have been identified.[24-27] In particular, miR-221 and -222 have been shown to control human erythropoiesis through the kit receptor.[24] Specifically, miR-221/222 are highly expressed in CD34$^+$ HPCs but gradually and sharply down-modulated during erythroid differentiation: this decline unblocks kit protein production at mRNA level, thus leading to expansion of early erythroblasts linked to prevalent HbF synthesis. On this basis, we have explored the possible role of the kit/miR-221/222 complex in the mechanism of perinatal Hb switching.

Design and Methods

Plasma, reticulocytes and, particularly, CD34$^+$ HPCs were isolated from cord blood (CB) and adult peripheral blood (APB) in order to evaluate kit/KL expression levels, the HbF reactivation as well as the functional role of miR- 221/222. Technical procedures for cellular and molecular studies are described in detail in the *Online Supplementary Appendix*.

Results

Gradual decline of both kit expression on CD34$^+$ cells and KL plasma levels during the perinatal period

In a first set of experiments, CD34$^+$ cells from 61 CB samples (from 33 to 41 weeks of gestation) were isolated and kit expression was evaluated by flow cytometry, as compared to that observed on CD34$^+$ cells purified from APB. The analysis of kit fluorescence profile always showed a unimodal peak, with a normal distribution of fluorescence values (representative cases in *Online Supplementary Figure S1*).

A large majority of the CD34$^+$ cells (ranging from 85 to 98% for CB and from 75 to 91% for APB) were distinctly kit+, but the labeling intensity greatly varied among the various samples analyzed, showing a clear and progressive decline during the perinatal period (from 67 to 18 MFI, mean values, Figure 1A). The results in 20 APB samples (8.58±0.5, mean value ± SEM) were even lower than in full-term CB (Figure 1A).

We then analyzed the relationship between kit expression and HbF content in 50 CB reticulocyte samples (since RBCs have a long life-span, the HbF content in reticulocytes provides a better estimate of the HbF level of each sample at birth).[28] This analysis showed a highly significant direct correlation between HbF content (from approximately 30% to 80-90%) and kit expression level (Figure 1B). In reticulocytes of 20 control APB samples, very low kit expression values were associated with very low HbF content levels (<1%; Figure 1B).

In a second set of experiments, we evaluated plasma KL concentration in 64 CB and 20 APB samples. In CB samples, the KL levels (1.374±0.04 ng/mL) were significantly higher than those observed in APB samples (0.85±0.02 ng/mL). Interestingly, when KL values were plotted against the CB gestational age, a significant inverse correlation was observed (Figure 1C); specifically, KL plasma levels in 35 week CB (1.95±0.2) were more elevated than in 41 week CB (0.89±0.04). We then analyzed the relationship between the plasma KL concentration and HbF content in CB reticulocytes. Here again, this analysis showed a significant direct correlation between these parameters (Figure 1D).

Reactivation of HbF synthesis in cord blood HPCs treated with kit ligand

In an attempt to evaluate the kit ligand effect on the cord blood HbF synthesis program, CD34$^+$ cells purified from 8 CB samples (including pre-term and mid-full term samples) were grown in unilineage erythroid culture supplemented with graded amounts of kit ligand (1, 10 or 100 ng/mL). The cytokine addition induced a dose-related enhancement of cell proliferation (i.e. the cell output showed an approximately 2-log rise over control cultures) while delaying terminal erythroid maturation (from 15-20 up to 25-30 days of culture) (Figure 2A and B).

More important, analysis of the HbF content in these samples indicated that: i) the level of HbF in CB reticulocytes (61%, mean value) was lower than in RBCs (82%), but significantly higher than in control cultures (33%); ii) addition of kit ligand to the erythroid cultures induced a dose-related increase in HbF up to 73%, i.e. approximately up to the level observed in CB reticulocytes and RBCs (Figure 2C). HbF analysis was carried out at optimal ery-

throid maturation. These results indicate that the perinatal HbF to HbA switch may be reversed by *in vitro* kit ligand addition in a dose-dependent fashion.

Imatinib abrogates the stimulatory effect of kit ligand on HbF production

STI571 (Imatinib or Gleevec) is a specific inhibitor of kit and PDGF receptor tyrosine kinase activity.[29] In our studies we have evaluated whether this compound is able to abrogate the KL effects on cord blood BFU-E culture. Addition of imatinib (2×10^{-6}M) to 5 cord blood erythroid cultures abrogated the stimulatory effect of KL treatment (100 ng/mL) on erythroid proliferation and maturation (Figure 3A and B) and down-modulated the HbF content (Figure 3C) to the level observed in control cultures. Imatinib treatment did not modify kit protein expression (*data not shown*).

HbF synthesis in sibling BFU-E colonies.

In these experiments, we analyzed the effect of KL, alone (1, 10 or 100 ng/mL) or in combination with imatinib, in sibling BFU-E colonies. The results confirm the data obtained in minibulk cultures (Figure 3D). In 5 different sibling clones, KL induced a dose-related HbF reactivation (from 40% to 65%, mean values), while addition of imatinib abrogated this stimulatory effect (from 65% to 30%) (Figure 3D, left panel). Similarly, KL induced an increase of the sibling colony size (from 5×10^4 to 1.6×10^5 cells/colony) which was fully inhibited by imatinib (4×10^4 cells/colony) (Figure 3D, right panel).

Role of miR-221/222 in the regulation of perinatal erythropoiesis and HbF production

In a first series of experiments, the endogenous expression of miR-221 and -222 was evaluated by quantitative real-time RT–PCR in CD34$^+$ cells isolated from 21 cord blood (including 9 pre-term and 12 mid-full term) and 20 adult peripheral blood samples. The results clearly showed decreased levels of both miR-221 and 222 in cord blood HPCs, as compared to adult peripheral blood progenitors (Figure 4A). Interestingly, miR-221/222 levels in pre-term cord blood were significantly lower than in mid-full term cord blood (Figure 4A).

In order to evaluate the functional role of these microRNAs in perinatal erythropoiesis and HbF synthesis, CD34$^+$ HPCs, isolated from 3 separate cord blood samples were transfected with the mature miR-221 and 222 or a control scrambled double-stranded RNA oligonucleotide and grown in unilineage erythroid culture up to terminal differentiation. Kit expression, evaluated by FACS and Western blot analysis in day 2 and 4 cultures, respectively, was reduced by approximately 50% in miR-221/222 transduced cells, as compared to control cultures, whereas kit mRNA was only slightly modified (Figure 5A and D; *data not shown*).

The erythroid cell progeny over-expressing miR-221 and -222 showed a significant decrease in its proliferative rate (up to 70%), cell cycling and HbF synthesis (50%), as compared to untreated or control miR-treated cells (Figure 4B; *data not shown*). Exogenous KL (100 ng/mL) was added to unilineage erythroid cultures and utilized as a positive control of cell proliferation and HbF modulation (Figure 4B, lower panels). Importantly, addition of KL to erythroid progenitors over-expressing miR-221 and 222 failed to induce a significant stimulation of both HbF synthesis and erythroid proliferation (Figure 4B, lower panels). This result clearly suggests that the inhibitory effect induced by miR-221 and 222 overexpression is specifically and mainly exerted through kit targeting.

Furthermore, the suppression of endogenous miR-221 and -222 was investigated by using antagomirs, a new class of anti-miR oligonucleotides that have been shown to efficiently and specifically inhibit miR function *in vitro* and *in vivo*.[30,31] *In vitro* treatment of CD34$^+$ HPCs with antagomir 221 and 222 increased erythroid proliferation rate (Figure 4C, left panel), reactivating HbF synthesis of approximately 50% as compared to cells either untreated or treated with a control antagomir (Figure 4C, right panel). The antagomir-induced downmodulation of miR-221 and 222 (Figure 5B) was associated to upmodulation of kit protein as shown by quantitative real-time RT-PCR and Western blot analysis, respectively. (Figure 5C and D)

To further show that the effect of miR-221/222 is mediated through kit targeting, experiments of knock-down of kit receptor expression using siRNA have been performed. These confirmed the results obtained in experiments of miR-221 and miR-222 enforced expression. Particularly, treatment with c-kit siRNA inhibited the response to exogenous KL in terms of erythroid cell proliferation and HbF induction, down to the levels observed in imatinib-supplemented or untreated control cultures (Figure 4D).

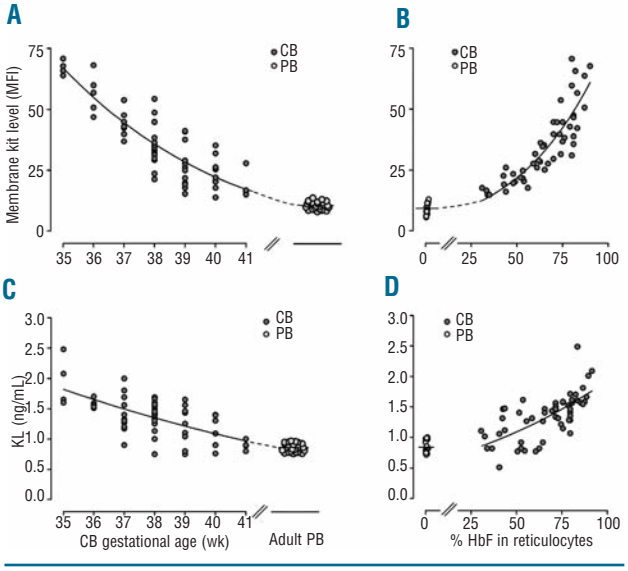

Figure 1. Kit/KL expression in CB CD34$^+$ cells in perinatal life. (Top). Kit expression levels in 61 CB and 20 control APB samples, as related to gestational age (A) and reticulocyte HbF content (B). The best fitting curves for CB values are presented. The curves are extrapolated to the mean adult value. The inverse correlation between kit level and gestational age and the direct one between kit level and HbF content are highly significant ($P<0.001$ and $P<0.001$, respectively). (Bottom). Plasma KL levels in CD34$^+$ cells from 64 CB samples and 20 control APB samples, as related to gestational age (C) and reticulocyte HbF content (D). The best fitting curves for CB values are presented. The left panel curve is extrapolated to the mean adult value. The inverse correlation between KL level and gestational age, and the direct one between KL level and reticulocyte HbF content are highly significant ($P<0.001$ and $P<0.01$, respectively).

Discussion

We previously reported that KL reactivates HbF synthesis in normal adults, as indicated by experiments carried out by KL addition in unilineage erythroid culture of purified BFU-Es.[13,19] These studies prompted us to hypothesize a possible role for the kit/KL system in Hb switching.

In an attempt to verify this hypothesis, we evaluated the kit expression level and the reticulocyte HbF content, as compared to APB samples, in a large series of pre- and mid-full term cord blood.

The results showed that kit expression on $CD34^+$ cells gradually and markedly declines during the perinatal period. Notably, this decline is directly related to the reticulocyte HbF content, which drops from 80-90% to less than 30%. Extension of the perinatal kit/HbF correlation curve to the post-natal period, specifically to the kit/HbF level in adult life, suggests that the residual Hb switch taking place after birth directly relates to a further decrease of kit expression level.

A second series of studies was focused on cord blood kit ligand levels. We confirmed that plasma KL concentration is more elevated in cord blood than adult peripheral blood, as reported.[32,33] Furthermore, we observed that during the perinatal phase KL levels show a moderate but significant decrease, directly related to the decline of HbF synthesis. In the first year after birth[32] and adult life (our results in Figure 1C) KL production shows a further moderate decrease, which correlates with the final drop of HbF content from less than 30% to less than 2% and less than 1%, respectively. A previous study[34] showed a small non-significant decrease of KL levels in cord blood samples at 33-36 versus 37-40 weeks of gestation (3.9 *vs.* 3.4 ng/mL of serum, respectively, mean values). Two technical aspects of this study may reconcile the apparent discrepancy with our results: i) the KL levels are more elevated than reported by us and others,[32,33] i.e. 1-2 ng/mL of plasma; ii) the spread of KL levels is more pronounced than in our studies. We, therefore, suggest that in Han *et al.*[34] the perinatal KL decline was attenuated by the elevated level and the wide spread of KL values.

The decrease of kit and KL expression are seemingly interlinked at functional level. The kit level on HPCs is the major determinant of their response to KL:[35] the gradual drop of kit expression on perinatal HPCs therefore causes a progressive reduction of their KL response. On the other hand, KL treatment of cord blood cultures stimulates and sustains kit expression in erythroid cells,[36] as confirmed by our studies.[24] Therefore, in the perinatal period, the KL decline seemingly contributes to the marked decrease of kit expression on HPCs.

A major source of KL is represented by the placenta:[37] in the perinatal period, the gradual loss of placenta function and its removal at birth may contribute to the decline of KL production. Furthermore, newborn endothelial cells (ECs) release KL at a level markedly higher than adult ECs:[38] the decreasing production of KL by ECs during development may contribute to the KL drop in the perinatal phase, and possibly also in the post-natal period.

Altogether, these observations led us to hypothesize that in the perinatal period the declining kit activity may represent a mechanism underlying the HbF to HbA switch.

To validate this hypothesis, further experiments were performed to evaluate the *in vitro* effect of exogenous KL on the HbF synthesis program in CB BFU-Es. The studies were carried out in two unilineage erythroid culture systems: i) minibulk erythroid culture of purified BFU-Es.[24] In this system, the BFU-Es undergo a gradual and homogeneous wave of differentiation/maturation along the erythroid lineage up to greater than 95% mature erythroblasts; ii) a two-step unicellular culture system.[19,39] In the first step, a unicellular BFU-E culture generates sibling progenitors. In the second step, the sibling BFU-Es are grown in unicellular erythroid-specific culture and comparatively analyzed at the level of their progeny. Importantly, the sibling BFU-Es generate pure erythroblast colonies and show an equivalent proliferative/differentiating capacity.

In the minibulk BFU-E culture, KL induces a dose-dependent increase of HbF. This parameter was always evaluated in mature erythroblasts: this excludes the possi-

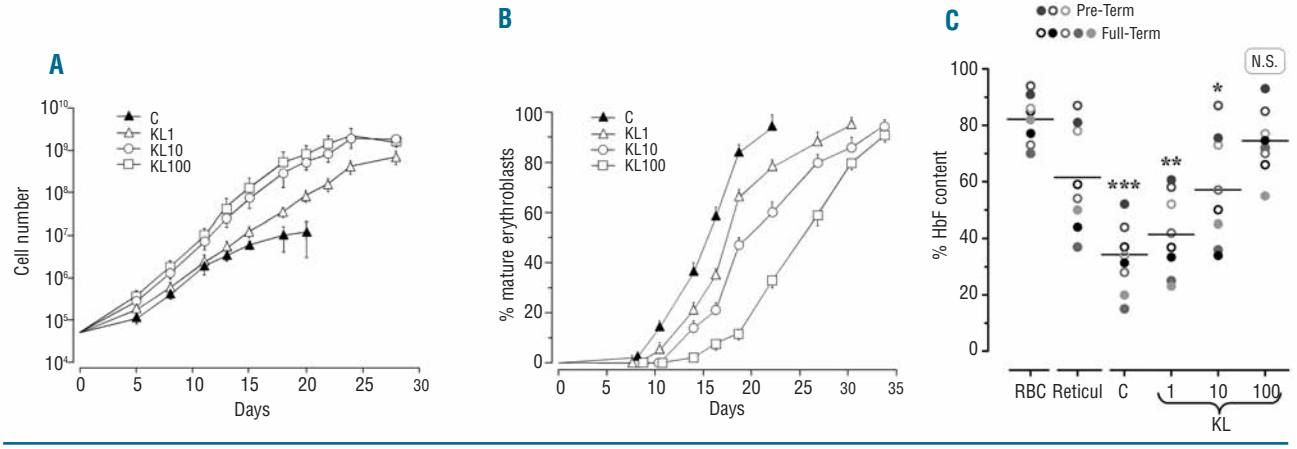

Figure 2. Reactivation of HbF synthesis in CB erythroid culture. Growth curve (A) and percentage of mature (polychromatophilic + orthochromatic) erythroblasts (B) of minibulk HPC erythroid cultures supplemented or not with KL (1, 10 and 100 ng/mL). C indicates control cultures. Mean ± SEM values of 8 separate experiments. (C) Percentage of HbF content in whole blood RBCs, reticulocytes and mature erythroblasts from minibulk HPC erythroid cultures supplemented or not with KL (1, 10 and 100 ng/mL). Results from 8 pre-term and full-term CB samples are shown. Each experiment is represented by a different symbol; mean values are indicated. ***P<0.001 and 0.01 when compared to RBC and reticulocyte group respectively. **P<0.01 when compared to RBC group but not to reticulocyte group. N.S. (not significant) when compared to RBC and reticulocyte groups.

bility that the HbF rise relates to differences in the stage of differentiation-maturation of the KL-treated *versus* control erythroblasts. Furthermore, equivalent results were obtained in unicellular sibling BFU-E cultures, thus excluding the possibility that KL addition recruits BFU-Es endowed with elevated HbF synthetic potential. We also evaluated the effect of the tyrosine kinase inhibitor STI571 (Imatinib or Gleevec), which inhibits kit signaling originating from stimulation of kit receptor on normal HPCs.[40] Imatinib fully blocked the KL action on both cell proliferation and HbF synthesis, down to the levels observed in control cultures; this ensures the specificity of the KL action on BFU-Es.

Importantly, exogenous KL induces a rise in HbF level almost up to that observed in cord blood RBCs, which is essentially the pre-switch level. These key experiments indicate that the HbF to HbA switch is largely reversed by KL addition. Specifically, exogenous KL counterbalances the decline of kit expression on HPCs,[24] thereby restoring the pre-switch kit activity and reversing the biological clock of HbF synthesis to the pre-switch period.

The rise of HbF content induced by KL is directly related to an increase of early erythroid cell proliferation and a delay of erythroblast maturation, as observed in KL-treated adult BFU-E culture.[13,17] This suggests that the KL-induced rise of HbF content may, at least in part, relate to a marked amplification of the early phase of erythropoiesis, which is characterized by peak γ-chain synthesis and minimal β-chain production.[41]

Notably, KL treatment of adult HPCs induces a relatively mild increase of HbF up to 10-20%,[13-17] i.e. it reverses only partially Hb switching. These findings suggest that mechanisms other than kit activity, e.g. glucorticoids[19] and the BCL11A HbF repressor,[20] may contribute to the postnatal component of the Hb switch; in fact, combined addition of KL+dexamethasone to adult erythroid culture potentiates HbF reactivation up to 40-50%.[19] Altogether, this first series of studies indicates that the perinatal drop of kit/KL activity is at least in part responsible for the perinatal Hb switch.

A second series of studies attempted to unveil the molecular mechanism underlying the decline of kit expression and HbF production in the perinatal period. As previously mentioned, miR-221/222 inhibit normal erythropoiesis via kit downmodulation:[24] the expression of these miRNAs decreases in erythroid culture of cord blood CD34$^+$ unblocking kit mRNA translation and promoting early erythropoietic proliferation. Other studies reported the analysis of miR-221 and miR-222 expression in normal erythroid cells; both studies basically confirmed the downmodulation of miR-221 expression during normal erythropoietic differentiation, although the kinetics of expression varied in these studies.[42,43] In order to ascertain a possible role of miR-221/222 in human perinatal erythropoiesis and Hb switching, we evaluated: i) the expression of these miRNAs in CD34$^+$ HPCs during cord blood development, as compared to the corresponding adult peripheral blood cell population; ii) the effects of overexpression or knockdown of these miRNAs on erythropoiesis and HbF content in erythroid culture of cord blood HPCs. The gradual increase of miR-221/222 expression levels from pre- to mid-full term cord blood up to adult peripheral blood HPCs is in line with the progressive decline of kit activity during the perinatal phase through the adult age. Moreover, the functional studies on miR-221/222 overexpression or knockdown show either a decline of kit expression and HbF synthesis or a rise of both parameters, respectively. Particularly, we observed that miR-221/222 overexpression in erythroid progenitors lowered the kit expression, thus inhibiting the response of these cells to KL in terms of cell proliferation and HbF synthesis.

In addition to c-kit, miR-221/222 regulates the expression of other important cellular proteins, including p27(Kip1).[44] Overexpression of these two miRs in various tumor cell types usually resulted in an increased cell proliferation.[45] However, in mast cells, miR/221/222 overexpression

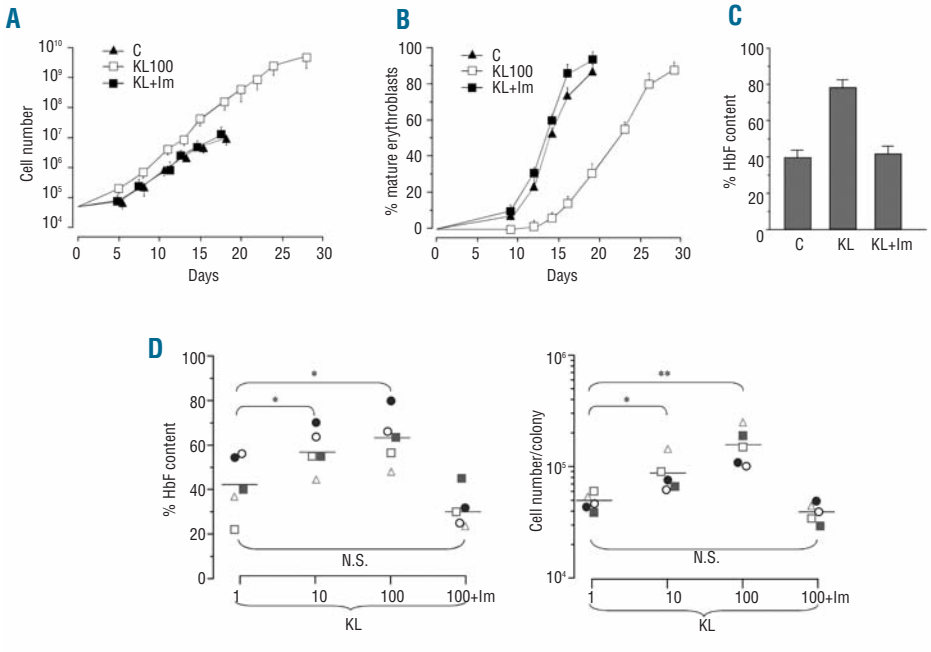

Figure 3. Effect of imatinib (IM) on HbF reactivation in CB erythroid culture. (A) Growth curve of minibulk HPC erythroid cultures supplemented or not with KL (100 ng/mL)±Gleevec (2x10^{-6}M). Mean ± SEM values from 5 separate experiments. (B) Percentage of mature (polychromatophilic + orthochromatic) erythroblasts in the erythroid cultures. (C) Percentage of HbF content in mature erythroblasts. (D) Left panel. Percentage of γ-globin chains in single sibling BFU-E colonies grown in unilineage erythroid cultures supplemented with KL (1, 10 and 100 ng/mL) ± Im (2x10^{-6}M). Results from 5 experiments, each including 4 sibling colonies are presented (each experiment represented by symbol; mean values are indicated). Right panel. Cell number/colony values from 5 different clones, each including 4 sibling BFU-E colonies. $^{**}P<0.01$, $^{*}P<0.05$, N.S. (not significant) when compared to the indicated groups.

caused a reduced cell proliferation with accumulation of cells in G_0/G_1.[46] In line with these last findings, we also observed that miR-221/222 overexpression caused an inhibitory effect on erythroid cell proliferation, coupled with G_0/G_1 cell accumulation. Importantly, both mast and erythroid cells express c-kit and are responsive to KL in terms of cell proliferation and differentiation. Altogether, this second series of findings indicate a significant role of miR 221 and 222 in human Hb switching through modulation of kit receptor level.

The molecular mechanisms controlling miR-221/222 expression in the hematopoietic system are still unknown. It is of note that remarkable changes in miR-221/222 expression have also been observed during embryonic

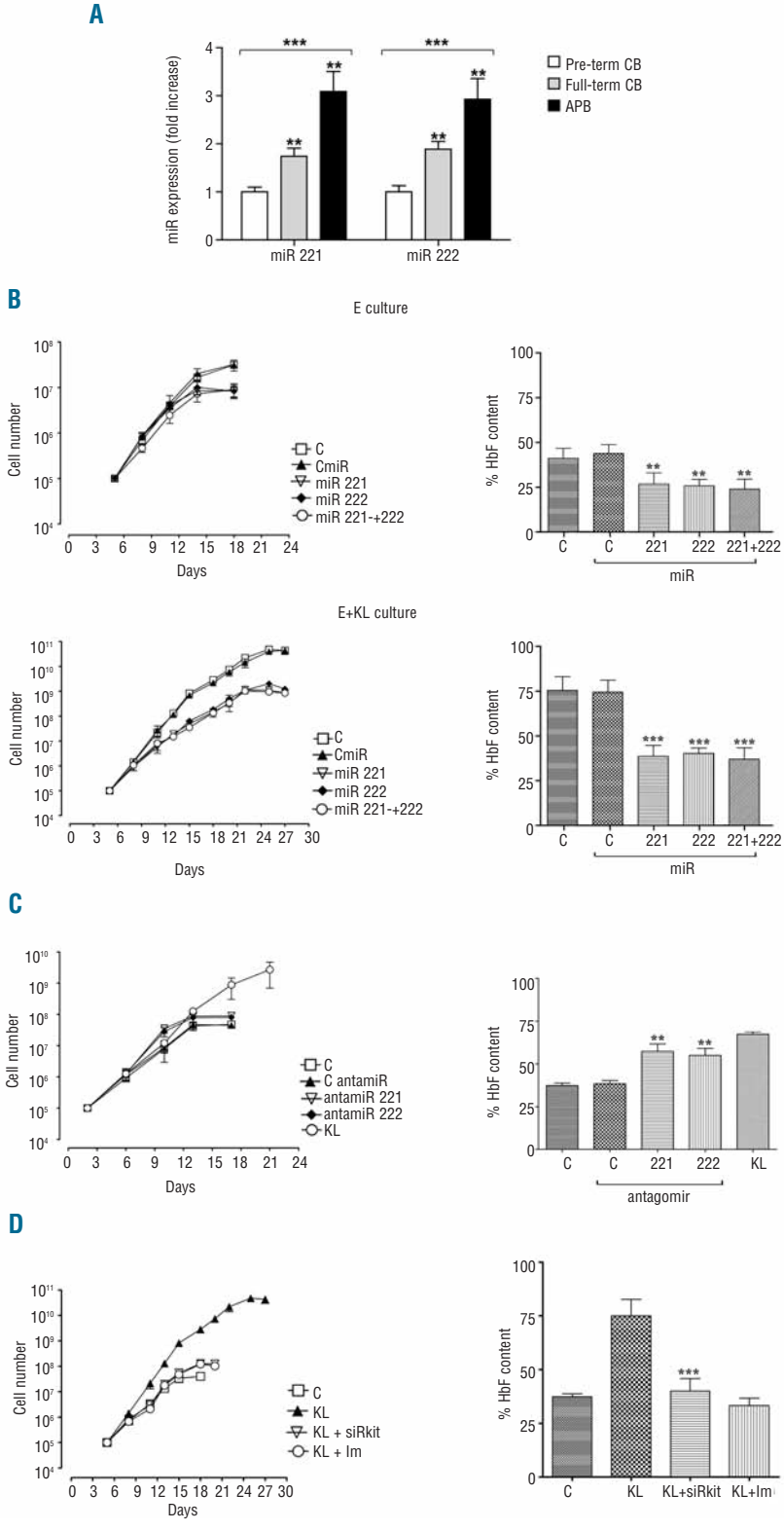

Figure 4. Analysis of miR-221/222 expression in CD34$^+$ HPCs. (A) miR-221/222 expression in pre-term CBs (white), mid-full-term CBs (gray) and APB (black). Fold increase of miR-221 and miR-222 levels as compared to pre-term CBs (set as 1) is indicated. Mean ± SEM (n=9 pre-term CB; n=12 mid-full-term CB; n=20 APB). ***P<0.001 when compared to pre-term CB group, **P<0.01 when compared to mid-full-term group. (B) Growth curve (left panel) and percentage of HbF (right panel) in HPC erythroid cultures untransfected (C) or transfected with a control miR (CmiR) or with miR-221 and miR-222 alone or in combination (miR-221+222). Cells were grown in absence (upper panels) or in the presence (lower panels) of KL. Mean ± S.E.M. (n=3). (Upper panels) P<0.05 when miR-221 and -222 growth curves are compared to C groups (left panel) and **P<0.01 when miR 221 and -222 HbF contents are compared to C groups (right panel). (Lower panels) P<0.01 when miR-221 and -222 growth curves are compared to C groups (left panel) and ***P<0.001 when miR 221 and -222 HbF contents are compared to C groups (right panel). (C) Growth curve (left panel) and percentage of HbF (right panel) in HPC erythroid cultures untransfected (C) or transfected with a control antagomiR (C antagomiR), antagomiR 221 or antagomiR 222. Cells grown in the presence of KL are included (KL). Mean ± SEM (n=3). **P<0.01 when compared to C groups (right panel). (D) Growth curve (left panel) and percentage of HbF content (right panel) in HPC erythroid cultures untransfected (C) or transfected with c-kit siRNA and grown in the presence of KL. Cells grown in the presence of KL alone or in combination with imatinib are included as positive and negative controls of HbF induction. Mean ± SEM (n=3). P<0.001 when KL+ siRkit growth curve is compared to KL curve (left panel) and ***P<0.001 when compared to KL HbF content (right panel).

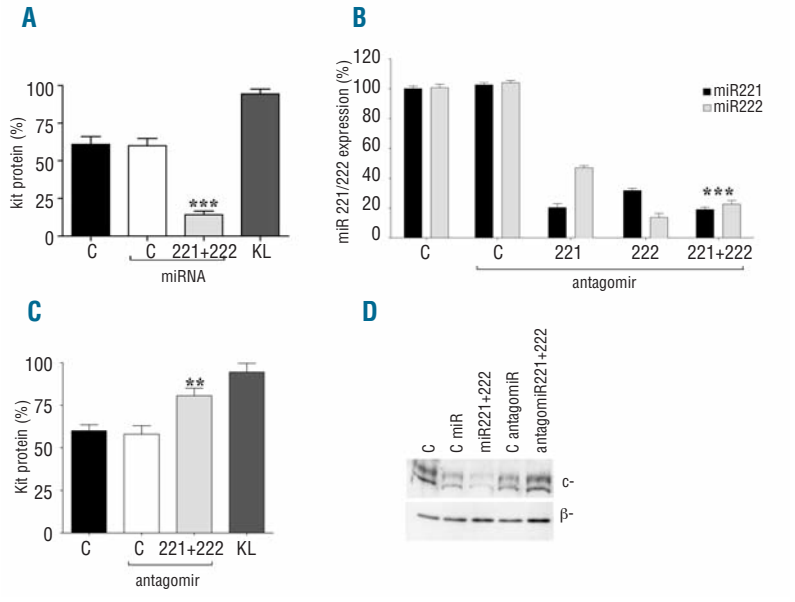

Figure 5. Effect of miR-221/222 overexpression/knockdown on kit levels. (A) Kit protein levels evaluated by Western blot in HPC erythroid cultures untransfected, C, or transfected with a control miR (C miR) or with miR-221 plus miR-222 (miR-221+222). Cells grown in the presence of KL are included (KL). Mean ± SEM (n=3). (B) miR-221/222 expression and (C) kit protein levels in HPC erythroid cultures untransfected (C) or transfected with a control antagomiR (C antagomiR) or with antagomiR 221 and antagomiR 222 alone or in combination (antagomiR 221+222). Cells grown in the presence of KL are included (KL). Mean ± SEM (n=3). ***P<0.001 and **P<0.01 when compared to C groups. (D) A representative Western blot analysis of the kit protein evaluated in erythroid HPC cultures 96 h after transfection with a control miR (C miR), control antagomiR (C antagomiR), miR-221 plus miR-222 (miR-221+222) or antagomiR 221 plus antagomiR 222 (antagomiR 221+222), respectively. β-actin controls are also included.

development of chick immune organs.[47] Altogether, it is apparent that miR-221/222 may act as regulators of multiple aspects of hematopoietic system development. Interestingly, a recent study[48] suggested that the hypoxia-associated miR-210 might be involved in increased expression of γ-globin genes in differentiating erythroid cells.

Future studies will further unveil the molecular mechanisms of Hb switching. In this regard, the potential role of glucocorticoids and the BCL11A HbF repressor has been mentioned above. Furthermore, in erythroid cultures of adult and cord blood HPCs, the HbF reactivation induced by KL may be mediated by Id2 and/or Tal1 transcription factors[19] (*results not shown*), as well as the MEK signaling pathway,[49] which in turn down-regulates the expression of the transcription factor COUP-TFII, a repressor of γ-globin genes.[50] These factors may mediate the KL stimulus on HbF synthesis, independently of or in relationship to the miR-221/222 mechanism hereby reported.

In conclusion, our studies indicate that human Hb switching is at least in part under the control of the kit receptor. At cellular level, the progressive decline of kit activity, particularly in the perinatal but also in the postnatal period, causes a gradual reduction of early erythroblast proliferation and hence of HbF production, thus leading to the HbF to HbA switch. At molecular level, the increasing levels of miR-221/222 from pre-term to mid-full term and adult HPCs down-modulate kit expression and thereby play a significant role in Hb switching. However, other mechanism(s), e.g. glucocorticoids[19,51] and the HbF inhibitor BCL11A,[20] may also contribute to the Hb switch. Finally, our results, in addition to demonstrating the involvement of c-kit in the mechanism of perinatal Hb switching, have also potentially interesting implications for therapy of β-thalassemia based on HbF reactivation. Specifically, these data suggest that HbF reactivation induced by exogenous KL therapy[16] may be potentiated by systemic administration of antagomir-221/-222[22] via enhanced kit receptor expression.

Authorship and Disclosures

MG and LF designed research, analyzed the data and wrote the paper; UT performed experiments and wrote the paper; OM, EP, ES, EP, GC, SG, GM, ME and AM performed experiments and analyzed the data; CMC contributed to the research strategy; CP planned the research strategy and wrote the paper.

The authors reported no potential conflicts of interest.

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