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ANNUAL REPORT: Role of Pleckstrin 2 in improved erythropoiesis of transferrin-treated β-thalassemic mice and effect of exogenous transferrin in 5q- mice

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I. Project aim/objectives

During erythropoiesis, multipotent hematopoietic stem cells proliferate, differentiate and ultimately produce red blood cells (RBCs). Terminal erythroid differentiation begins at the morphologically recognizable pro-erythroblast (pro-E) stage and is completed when orthochromatic erythroblasts (ortho-E) expel their nuclei to produce reticulocytes [1]. Progressive differentiation between these stages occurs in homologous cell division progressively doubling proportions of pro-E, basophilic (baso-E), polychromatophilic (poly-E), and ortho-E, and multiple signaling pathways are involved in differentiation and enucleation, including multiple steps requiring actin cytoskeleton reorganization. We have previously shown that β-thalassemic (th1/th1) mice demonstrate a disorder progression from pro-E to baso-E and that exogenous transferrin (apoTf) therapy restores normal proportion of early stage erythroid precursors in th1/th1 mice [1], resulting in more circulating RBCs, increased hemoglobin (Hb), reversal of splenomegaly, and improvement of ineffective erythropoiesis in th1/th1 mice [2].

To identify genes that play novel function and could play an important role in different stages of terminal erythropoiesis, we performed RNA sequencing analysis of sorted bone marrow pro-E from wild type (WT), th1/th1, and apoTf-treated th1/th1 mice. We identify pleckstrin-2

(plek2) as a gene of interest with a 15-fold increase in plek2 mRNA expression in th1/th1 relative to WT mice, normalized in apoTf-treated th1/th1 mice. The function of plek2 in erythropoiesis is incompletely understood but appears to impact both erythroid precursor apoptosis and membrane characteristics of RBCs [3]. We hypothesize that plek2 plays a central role in the effect of exogenous apoTf on erythroid differentiation and enucleation in th1/th1 mice.

Furthermore, we postulate that ineffective erythropoiesis in β-thalassemia shares many characteristics with that of low risk MDS, such as defective erythroid differentiation, anemia, splenomegaly, and systemic iron overload, suggesting that similar effects of exogenous apoTf may be relevant also in MDS. Low risk myelodysplastic syndrome (MDS) is associated with relatively longer survival and higher RBC transfusion requirements, resulting in a secondary iron overload. Heterozygous interstitial deletion of chromosome 5 is the most common cytogenetic abnormality in low risk MDS. The commonly deleted region in 5q- syndrome maps to a 1.5 Mb region between 5q31 and 5q32. We characterize a mouse model of 5q- syndrome with chromosomal engineering on the long arm of the chromosome 18, Cd74+/lox Nid67+/lox, Lmo2Cre+ mice (5q- mice) [4].

Our aim for year 1 was to characterize 5q- mice as a model of human 5q- syndrome low risk MDS. Our findings reveal for the first time that 5q- mice exhibit macrocytic anemia (Hb 6±0.8 vs. 12±0.7 g/dL, $P<0.0001$; MCV 61±0.6 vs. 48±0.6 fL, $P<0.0001$), splenomegaly (0.007 vs. 0.003 organ / body weight, $P<0.0001$), and expanded and ineffective erythropoiesis in the bone marrow, spleen, and liver. Furthermore, 5q- mice exhibit evidence of iron overload relative to WT mice with increased transferrin saturation and liver iron content, but no difference in hepcidin expression. These findings provide strong preliminary evidence of a robust mouse model with characteristics mimicking human disease. In year 2, we aim to evaluate **(1)** the role of plek2 in apoTf-treated th1/th1 erythroblasts and **(2)** the effectiveness of exogenous apoTf in 5q- mice.

II. Background

A. Normal and Ineffective Erythropoiesis and Enucleation: Erythropoiesis is the process of differentiation of hematopoietic stem cells to mature erythrocytes. This stepwise process includes the formation of committed erythroid burst-forming units (BFU-E) followed by rapidly dividing erythroid colony-forming units (CFU-E). Differentiation from CFU-Es to mature RBCs, generally termed terminal erythropoiesis, is driven by multiple erythropoietin (Epo)-induced signalling transduction pathways [5]. Epo and its receptor EpoR are essential for terminal erythropoiesis.

Binding Epo to EpoR triggers the activation of Jak2, which is followed by the induction of Stat5, PI3K/Akt, and Ras-MAP kinase pathways [6]. During the late stage of terminal erythropoiesis, erythroblasts exit the cell cycle, undergo nuclear chromatin condensation, and enucleate. Recent studies reveal that multiple signalling pathways are involved in enucleation [7,8], including histone deacetylation [9,10], actin cytoskeleton re-organization [11,12], cytokinesis [13,14], cell-matrix interactions [15], specific microRNAs regulation [16], and vesicle trafficking [17].

At steady state, the bone marrow maintains sufficient numbers of erythroid precursors to replace senescent RBCs needed to meet oxygen demand in tissues. Blood loss and hemolysis trigger stress erythropoiesis, resulting in increased Epo production, erythroid proliferation, and reticulocytosis to temporarily increase RBC production. Ineffective erythropoiesis occurs in conditions in which erythroid precursors either fail to mature, undergo apoptosis prior to enucleation, or enucleate to form RBCs with significantly shortened survival. Although the erythron is expanded, diseases of ineffective erythropoiesis are characterized by limited number of RBCs, far fewer than the same number of erythroid progenitors cells could generate in non-ineffective erythropoiesis conditions [18].

β-thalassemia, one of the most common congenital anemias, arises from homozygous mutations or deletions in the β-globin gene that result in partial (intermedia) or complete (major) lack of β-globin synthesis. The ineffective production of RBCs has been attributed to death of erythroid precursors during maturation, mediated by apoptosis and / or hemolysis. The proposed etiology of cell death is precipitation of α-globin chains or hemichrome aggregates which impair erythroid precursor maturation triggering apoptosis [19]. However, several observations call into question the view that cell death is the only cause of ineffective erythropoiesis in β-thalassemia [20], and consensus on the definition of ineffective erythropoiesis or measurable molecular mechanisms thereof are not currently available.

B. Role of Activated Caspase 3 and RacGTPase in Differentiation, Apoptosis and Enucleation:

Activated Caspase 3:

In addition to erythroid maturation and enucleation, chromatin condensation also occurs during apoptosis, and proteins typically involved in apoptotic pathways are known to play important roles in erythropoiesis [21]. Apoptotic pathways have been implicated in the enucleation of mammalian lens epithelia and keratinocytes [22, 23]. Several lines of evidence indicate that caspase activation also occurs during erythroid maturation and may play a critical role in erythroid differentiation. A

transient activation of caspase-3 (and caspase-7) is observed in early stages of erythroid maturation (i.e. pro-E and baso-E), regressing in late stages (i.e. poly-E and ortho-E) [24]. Transient caspase activation occurs through the mitochondrial pathway, cleaving proteins involved in nucleus integrity (LaminB) and chromatin condensation (acinus), without inducing cell death [24]. *In vitro* inhibition of activated caspase in human erythroblasts (e.g. using z-VAD) blocks erythroid maturation at the baso-E stage. This finding has also been confirmed in murine erythroblasts [25].

Rac-GTPase:

Rac-GTPases are a subgroup of the Ras superfamily of proteins which function as molecular switches in signal transduction pathways in eukaryotic cells. Specifically, Rac-GTPases control actin cytoskeleton; influence cell polarity and motility, microtubule dynamics, and vesicular transport pathways; and regulate cell survival, G1 cell cycle progression, and transcription [26,27]. Rac1- and Rac2-GTPases play unique and overlapping roles in hematopoietic stem cells, where they differentially control engraftment, marrow retention, cell survival, and proliferation [28]. Rac1- and Rac2-GTPase function in hematopoietic cells is mediated through regulation of actin cytoskeletal organization. Thus, RacGTPases play an essential role in enucleation through the activation of a downstream target, the formin mDia2. Deregulation of RacGTPases during the late stages of erythropoiesis completely blocked enucleation of cultured mouse fetal erythroblasts without affecting normal proliferation and differentiation [11].

C. Pleckstrin-2: Plek2 is a 353 amino acid protein similar to plek1. While plek1 expression is restricted to hematopoietic cells, plek2 is detected in various other tissues [3]. Both plek1 and plek2 contain two pleckstrin homology (PH) domains in their amino- and carboxyl-termini flanking a central Disheveled, Egl-10, and Pleckstrin (DEP) domain [3]. PH1 domains bind polyphosphoinositides (PI) and thus regulate protein function [29-30]. Frequently, the binding of PI to the PH domain localizes the molecules to the cell membrane [31]. Despite similarity in structure, plek2, unlike plek1, is a poor substrate for PKC and thus the 2 proteins have little expected redundancy or compensatory potential. Expression of plek2 induced PI 3-kinase-dependent F-actin re-organization and cell spreading in Jurkat cells, a T cell line [29]. However, only the effects of ectopic plek2 expression have been assessed, and plek2 *in vivo* function is incompletely understood [29]. Plek2 and Rac are found to co-localize; this co-localization was detected with any types of Rac, wild type Rac, constitutively activated Rac with poor GTPase activity (Vall2 Rac), or Rac with poor guanine nucleotide binding activity (N17 Rac). Rac and plek2 are likely proximal to one

another because plek2 co-precipitated with Rac after crosslinking [29]. Mutations within the effector loop of Rac1 do not prevent co-localization with plek2 [29], suggesting that plek2 function is central to the activity of Rac rather than its downstream effect.

Based on this information, we in Aim 1 will explore the role of plek2 and its relationship to RacGTPase in erythroid precursor differentiation and enucleation. We will also explore others pathways that could be or not related, such as the non-apoptotic functions of activated caspase 3.

D. MDS and 5q- syndrome: MDS is a group of neoplastic myeloid disorders characterized by ineffective erythropoiesis, cytopenias, qualitative disorders of blood cells and their precursors, clonal chromosomal abnormalities, and a variable predilection to undergo clonal evolution to acute leukemia (AML) [32]. Molecular genetic studies identify gene mutations in hematopoietic stem cells of approximately 60% of patients. Such mutations contribute to the proliferation of early progenitors, with defective maturation in multi-lineage precursors and loss of more mature precursors in the bone marrow. MDS patients are classified into prognostic groups based on clinical and pathologic characteristics. Refractory anemia (RA), refractory anemia with ringed sideroblasts (RARS), and 5q- subtypes of the World Health Organization (WHO) classification and the low / intermediate-1 subtypes of the International Prognostic Scoring System have a longer median survival and the lowest rate of progression to AML [33]. Deletion of long arm of chromosome 5 (5q-) is the most common cytogenetic abnormality in low risk MDS, with many potential culprit genes in the region [34]. While most patients with 5q- syndrome have large deletions, some smaller chromosomal deletions have enabled geneticists to identify common deleted regions (CDRs) minimally necessary for clinical phenotype. Two CDRs, 5q52-33 [35,36] and 5q3137,38], have been reported. Macrocytic anemia is the most prominent cytopenia in 5q- syndrome patients, generally resulting in transfusion-dependence. Given the relatively prolonged survival in 5q- syndrome patients, iron overload from chronic RBC transfusions results in morbidity and mortality [39], similar to other low risk MDS subtypes [40,41].

E. Iron overload and erythropoiesis: Multiple areas of research suggest that iron overload impedes erythropoiesis [42,43]. However, the mechanisms by which this occurs are not completely understood. Iron overload inhibits BFU-E formation and erythroblast differentiation *in vitro*, and excess iron leads to dysplastic changes in exposed cells [44], triggering apoptosis in erythroid precursors [45]. Erythroid precursor susceptibility likely results from impaired heme synthesis and

mitochondrial iron trapping, observed in RARS patients. Thus, it is reasonable to expect that iron depletion is beneficial for erythropoiesis in iron-loading diseases [46]. Data from multiple sources, including our laboratories, reveal that iron restriction improves erythropoiesis in β-thalassemia, a disease characterized by ineffective erythropoiesis similar to MDS [2,47].

F. Hepcidin regulation: Inappropriately low hepcidin is implicated in iron overload in patients with β-thalassemia [48-50]. Hepcidin, the main regulator of iron absorption and distribution in the body, is a peptide hormone produced predominantly in the liver. Hepcidin binds to ferroportin (FPN1), the iron exporter present on the surface of enterocytes and reticuloendothelial macrophages [51,52], resulting in FPN1 degradation and decreased release of cellular iron. As a consequence, hepcidin down-regulates dietary iron absorption and tissue iron recycling. Despite parenchymal iron overload, hepcidin levels are inappropriately low and do not increase in proportion to the degree of iron overload in transfused β-thalassemia patients [53]. Relatively low hepcidin mRNA expression in the liver is also characteristic of mouse models of β-thalassemia [54-56]. This lack of appropriate hepcidin response, despite increased parenchymal iron stores in β-thalassemia, suggests that a competing signal is suppressing hepcidin expression [57-59]. In diseases of concurrent iron overload and ineffective erythropoiesis, hepcidin suppression is thought to originate from the bone marrow and is mediated by certain signaling molecules (e.g. growth differentiation factor 15 (GDF15), twisted gastrulation 1 (TWSG1), and erythroferrone (ERFE)) [60-62]. The genes responsible for the erythroid regulation of hepcidin and their signaling pathways are active areas of investigation and are targeted for the development of novel therapeutics in iron disorders [63].

G. Transferrin, preliminary results, and hypothesis: Transferrin functions as the main transporter of iron in the circulation, where it exists as iron-free apo-transferrin (apoTf), monoferric transferrin or diferric holo-transferrin. Typically, iron is bound to 30% of all transferrin binding sites in circulation. Patients who develop iron overload exceed the iron-binding capacity of transferrin, in which case non–transferrin-bound iron (NTBI) can be found in circulation [64]. NTBI is a redox-active form of iron taken up by cells in an unregulated manner, can cause free radical damage resulting in morbidity and mortality in iron overload diseases, and is unavailable for Hb synthesis during erythropoiesis. Transferrin bound iron uptake by transferrin receptor-1 (TfR1) is the only known means of iron delivery for erythropoiesis [65,66]. We previously demonstrated that exogenous apoTf in a frequently used mouse model of β-thalassemia results in relatively iron restricted erythropoiesis, markedly ameliorating ineffective erythropoiesis, decrease in NTBI, and

increasing hepcidin expression [2]. We hypothesize that exogenous apoTf would exhibit similar benefit in MDS. Because MDS is characterized by heterogeneous clinical symptoms and mouse models of MDS are complex, we selected an available model of 5q- syndrome as representative of low risk MDS. **.**

H. Reactive Oxygen Species: Oxidative stress is due to the effects of reactive oxygen species (ROS). DNA-damaging events are known to occur in every cell of the human body every day, a significant portion caused by ROS [67]. Mitochondria are an important source of ROS within most mammalian cells [68]. This ROS production contributes to mitochondrial damage in a range of pathologies and is also important in cellular redox signaling [69]. Hematopoietic cells appear to be particularly vulnerable in the presence of unchecked accumulation of ROS because deficiencies in several ROS scavengers result in anemia and/or malignancies of hematopoietic tissues [70-75]. Regulation of oxidative stress is particularly important for erythropoiesis as erythroid precursors synthesize and accumulate Hb [76]. In addition, insertion of iron to heme in mitochondria of erythroid precursors provides potential for redox activity and circulating RBCs carry oxygen and as such are highly prone to oxidative damage [75]. Consequently, erythroid precursors and RBCs are exposed to one of the highest levels of oxidative-stress conditions in the body. In agreement with this, compromised protection against ROS results in RBC diseases, with shortened RBC survival and hemolysis, leading to anemias [70, 71,73,75]

I. p53 overexpressing mice (model of 5q- syndrome): The del (5q) in 5q- syndrome is widely believed to result in MDS through a loss in a tumor suppressor gene important in the regulation of hematopoiesis. Through comprehensive chromosomal engineering on the long arm of chromosome 5, a clone (Cd74+/lox Nid67+/lox, Lmo2Cre+ mice) with a substantial hematopoietic phenotype was found [4]; an increased MCV anemia was observed and associated with a reduced total bone marrow cellularity, an increased proportion of erythroid progenitors, an increase expression of p53, and more apoptosis. We acquired p53 overexpressing (5q-) mice in our laboratory to better characterize erythroid-specific and iron-related parameters and use this mouse model in experiments to test the pre-clinical utility of exogenous apoTf in MDS.

In Aim 2, we will explore further the erythropoiesis phenotype and assess the effect of exogenous apoTf in 5q- mice.

III. Aim 1: To evaluate the role of plek2 in apoTf-treated β-thalassemic erythroblasts

a) Specific role of plek2 in individual stages of terminal erythropoiesis: We will sort all bone marrow populations (pro-E, baso-E, poly-E and ortho-E) from WT, PBS-injected th1/th1 and apoTf-treated th1/th1 mice. We will perform qRT-PCR and Western Blot analyses to evaluate any difference in mRNA and protein expression. We will also perform immunofluorescence staining on individual stages of sorted erythroid precursors to evaluate the different sub-localization of plek2 during erythroid maturation.

b) Plek2 function during enucleation in th1/th1 mice: We will evaluate Rac2GTPases and erythropoiesis in plek2 knockout mice and cross them with th1/th1 mice to evaluate the effect of plek2 insufficiency on ineffective erythropoiesis in β-thalassemia. Although the involvement of RacGTPase in plek2-mediated erythroid differentiation has not been fully explored, we hypothesize that plek2 activation triggers RacGTPase and prevents enucleation in th1/th1 mice. We will also explore the role of RacGTPase in improved erythroblast enucleation in apoTf-treated th1/th1 mice.

IV. Aim 1: Experimental Plan and Method

Flow cytometry: Bone marrow cells were processed as described previously [77,78] with minor modifications. Briefly, cells were incubated with anti-CD45 magnetic beads, and erythroid lineage-enriched cells that flowed through the column were collected. Following bead separation, cells were counted and incubated with anti-mouse TER119-phycoerythrin Cy7 (PE-Cy7), CD44-allophycocyanin (APC), and CD71-PE. Non-erythroid and necrotic cells were identified and excluded from analyses using anti-CD45, anti-CD11b, and anti-Gr1 (APC-Cy7) antibodies along with 7-amino-actinomycin D (7AAD, BD Pharmingen). Erythroid precursors were selected by gating and analyzed using TER119, CD44, and forward scatter as previously described [58,59]. Cells were collected on a MoFlo® XDP High-Speed Cell Sorter (Beckman Coulter, Miami, FL) using Summit Software (Beckman Coulter, Miami, FL). A subset of collected cells was used to check for purity post-sort to confirm the flow cytometric profile of the target populations and to confirm morphology by microscopic evaluation of cytospins.

Quantitative real-time PCR: RNA bone marrow and spleen was prepared using RNeasy Kit (Qiagen) according to the manufacturer's instructions. Single pass complementary DNA was synthesized using 5μg total RNA, Superscript III RNase H−reverse transcriptase, and anchored oligo(dT) (both from Invitrogen). We performed QRT-PCR with the ABI 7900HT Sequence

Detection System using a 384-well setup (Applied Biosystems) with SYBR green. Mouse plek2 mRNA was amplified from appropriate tissue samples as previously described. Control GAPDH was amplified using primers GAPDH F and GAPDH R (Qiagen). Concentrations of plek2 mRNA was normalized to GAPDH as described [60].

Immunofluorescence: 1x10^5 bone marrow cells were fixed with 2% paraformaldehyde (PFA) at room temperature (RT) for 20 min, permeabilized with ice-cold 0.1% Triton X-100 in PBS for 10 min. Samples were stained with rabbit anti-plek2 (1:100, Proteintech), rabbit anti-activated caspase3 (1:250, Cell Signaling) overnight at 4C. After washing in PBS, Alexa Fluor®488 (1:100, Invitrogen) were added for 1 h. Actin filaments were stained with anti-phalloidin (1/500 Sigma Aldrich) and nuclei with DAPI-Antifade (Molecular Probes).

Western blot: Bone marrow and spleen cells were lysed in cell lysis buffer (Cell Signaling Technology), and protein concentration was determined by BCA protein assay method (Thermo Scientific). Samples were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) using 12% polyacrylamide gels, and transferred to nitrocellulose membrane (Biorad). Membranes were blocked for 1 hour in TBST (10 mMTris-HCl, pH 8.0; 150 mM NaCl; 0.05% Tween 20) containing 5% skim milk, followed by overnight incubation with commercially available primary antibodies (Plek2 1/1000, Proteintech; RacGTPase 1/1000, Cell signaling). Blots were washed and incubated for 1 hour at RT with the secondary antibody (horseradish peroxidase [HRP] conjugated (Thermo Scientific). Immunoreactive bands were visualized by the enhanced chemiluminescence (ECL) method (Amersham Bioscience, Sunnyvale, CA) according to standard procedures.

V. <u>Aim 1</u>: Results

a) Our data demonstrates a statistically significant increase in plek2 mRNA in th1/th1 relative to WT mice, with the highest expression of plek2 in poly-E (**Fig. 1A**), normalized in apoTf-treated th1/th1 mice. A similar pattern of increased protein concentration in th1/th1 relative to WT mice and normalization in apoTf-treated th1/th1 mice is evident in sorted bone marrow samples (**Fig. 1B**).

b) Prior *in vitro* studies demonstrate that membrane localization of plek2 is required for erythroid differentiation [3]. Thus, we performed sub-cellular fractionation in bone marrow erythroid precursors and determinate for the first time that plek2 is found exclusively in the

cytoplasm as actin filaments in pro-E and is co-localized with actin filaments on the membrane from baso-E to ortho-E in sorted erythroblasts from WT bone marrow. In contrast, sorted erythroblasts from th1/th1 bone marrow reveal co-localization with actin filaments on the membrane starting from pro-E, suggesting an earlier activation of plek2 and consequent actin cytoskeleton re-organization during erythroid differentiation in th1/th1 mice, normalized in apoTf-treated th1/th1 mice (**Fig.2**).

c) We explore RacGTPase in light of its role in enucleation and hypothesize that a direct relationship exists between plek2 and RacGTPase that influences enucleation. Our data demonstrates that RacGTPase concentration is increased in sorted bone marrow erythroid precursors from th1/th1 relative to WT mice and normalized in apoTf-treated th1/th1 mice (**Fig. 1B**).

VI. Aim 1: Conclusions and Future Directions

Taken together, these results confirmed a defect in differentiation between pro-E and baso-E in th1/th1 mice and suggest that plek2, alone or involving other players (e.g. RacGTPase), has an important role in erythroid differentiation and possibly enucleation. Based on these preliminary results we will investigate the role of iron and ROS in mechanisms of plek2- and RacGTPase-related regulation of differentiation, especially between pro-E and baso-E. We will also evaluate the *in vivo* role of plek2 in erythropoiesis in th1/th1 mice by crossing them with plek2 knockout mice.

Figure 1: (A) Plek2 mRNA (A) and plek2 and RacGTPase protein (B) expression in all stages of sorted bone marrow from WT, PBS injected th1/th1, and Tf-treated th1/th1 mice (plek2 = pleckstrin 2; WT = wild type; Tf = transferrin).

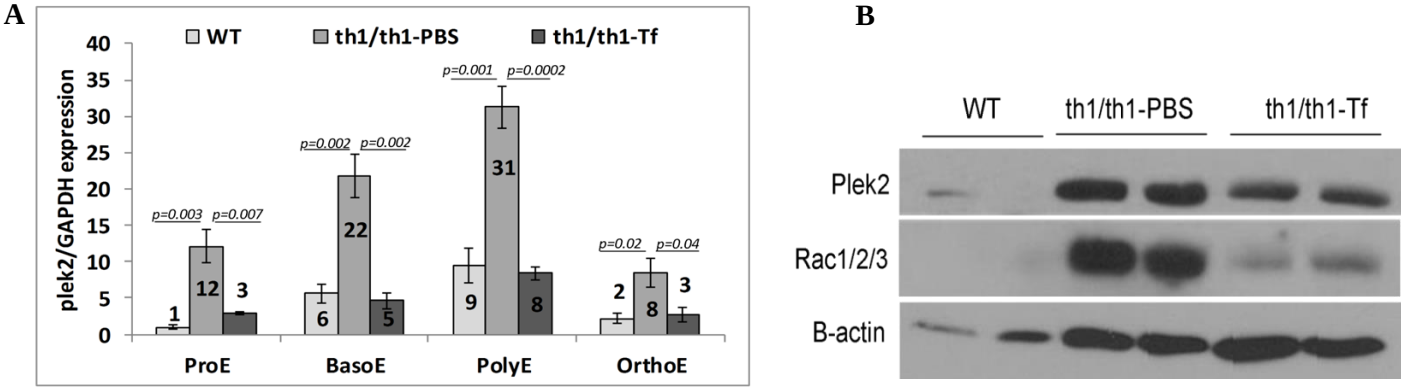
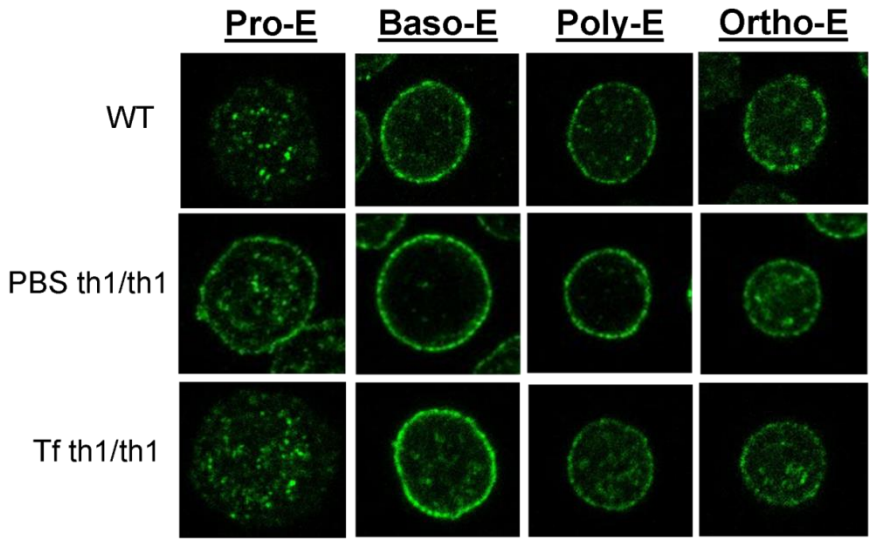


Figure 2: Plek2 sub-localization in all stages of sorted bone marrow from WT, PBS injected th1/th1, and Tf-treated th1/th1 mice (plek2 = pleckstrin 2; WT = wild type; Tf = transferrin).



VII.

Aim 2: To evaluate the effectiveness of exogenous apoTf in 5q- mice

a) ApoTf treatment: We will treat mice with apoTf vs. PBS starting at 2 month of age, 5 mice / group, 20 days of injection with either PBS or 10 mg apoTf (200 uL IP daily x 5/week x 4 weeks).

b) Bone marrow transplant to increase numbers of homogeneous mice: To enable statistically robust outcome assessment and avoid the potentially wide range of phenotypic severity, we plan to perform bone marrow transplants. A single bone marrow provides cells to enable bone marrow transplant of approximately 10 mice. Our intention would be to perform 1 (plus a confirmatory repeat) transplant for each of the extremes in phenotype severity. To accomplish this, we would breed mice, assess genotype, and measure Hb to determine whether the particular 5q- mouse belongs to the relatively mild or severe phenotype. The mouse will then be sacrificed and the bone marrow harvested for transplant into sub-lethally irradiated 8 week old WT recipient mice. Our laboratory has extensive experience with transplanting mice in this manner. Mice will be pre-treated with antibiotics, allowed to recover for 3-4 weeks following transplant, and will then start a course of PBS and apoTf injections as previously delineated.

c) Determine the molecular etiology of wide phenotype variability in 5q- mice: In order to pursue in parallel the molecular etiology of the wide variability observed in 5q- mice, we will evaluate RPS14 mRNA expression and p53 protein concentration in sorted bone marrow samples.

We anticipate that variability is either a consequence of variable efficiency of Cre recombinase activity or of the dynamic growth period between 2 and 4 months of age in 5q- mice.

VIII. Aim 2: Experimental Plan and Method

Both male and female 2 month old 5q- mice, 4 per group, were treated with daily IP injections of human apoTf (Kamada, Israel), 10mg in 200 ul PBS, 5 days per week for 4 weeks. We analyzed all erythroid- and iron-related parameters

Hematopoietic parameters: RBC indices and reticulocyte counts were measured using a flow cytometry-based hematology analyzer, Advia 120 Hematology System (Bayer Diagnostics) as per manufacturer's protocol for mouse specimens. Blood collected via tail vein blood (40 μL) was suspended in saline containing heparin or EDTA.

Serum parameter analyses: Anesthetized mice were bled via retro-orbital sinus. Serum samples were separated by centrifugation, stored at -20C until processed, and analyzed on an Integra 800 Automated Clinical Analyzer (Roche Diagnostics, Indianapolis, IN). Transferrin saturation was calculated using serum iron and total iron binding capacity (TIBC) in a standard formula (serum iron/TIBC) x 100. TIBC was calculated from the sum of measured unbound iron-binding capacity (UIBC) and serum iron, where UIBC is inversely proportional to the amount of unbound excess iron found in solution after the addition of a standard amount of iron, as measured by an increase in absorbance at 552 nm. Serum Epo and hepcidin were measured by ELISA (Quantikine, R&D Systems, Minneapolis, MN; Intrinsic LifeSciences, LLC, La Jolla, CA, respectively) according to the manufacturer's instructions.

Flow cytometry: Bone marrow and spleen cells were processed as described previously [77,78] and in Aim 1 above. In addition to bead separation and incubation with anti-mouse TER119-phycoerythrin Cy7 (PE-Cy7), CD44-allophycocyanin (APC), and CD71-PE, apoptosis with Annexin V-fluorescein and ROS were detected using commercially available kits per the manufacturer's instructions (Invitrogen).

Non-heme iron spectrophotometry: Quantification of non-heme iron was performed using the Torrance and Bothwell method [79]. Briefly, desiccated tissue samples were digested in acid-digestion mixture. An appropriate dilution of each acid extract was mixed with chromogen reagent

and absorption measured at 540 nm on a spectrophotometer (Multiskan MCC Microplate Reader, Fisher Scientific).

Quantitative real-time PCR: RNA from liver, bone marrow and spleen was prepared using RNeasy Kit (Qiagen) according to the manufacturer's instructions. Mouse hepcidin (Hamp1), ERFE and TWSG1 mRNA was amplified from appropriate tissue samples as previously described [80], normalized to GAPDH .

Histology and Immunohistochemistry: Mice were sacrificed by cervical dislocation 72 hours after the last apoTf injection. Organs of interest were fixed, paraffin embedded, sectioned to 5µm, and stained with hematoxylin/eosin or Perls' Prussian blue. Immunohistochemical staining was performed using anti-mouse TER119 (eBioscience) overnight at 4C (TER119, 1 µg/mL) with secondary antibody detection using biotin-conjugated and DAB (DakoCytomation). Images were acquired with an AxioCam HRC camera mounted on a Zeiss Axioskop 2 microscope using Plan-Neofluar objectives 20X/0.5 and Axiovision software.

Statistical analyses: All data are reported as means ± s.e.m and $p<0.05$ was considered statistically significant. Significance of differences was determined with Student's unpaired t test. All analyses and correlations were performed using Microsoft Excel. Fisher exact test was used to compare proportions.

Transplant mild and severe 5q- mice: To generate a cohort of 5q- mice with similar disease severity, 8x10^6 bone marrow cells from 2 month old mild and severe 5q- mice were transplanted into sub-lethally irradiated 8 week old WT mice. Transplanted mice were allowed to recover and started apoTf injections IP (10mg/day or equal volume PBS (200 uL) 2 weeks post-transplant. After completing 20 days of injection, mice were sacrificed and all erythroid- and iron-related parameters analyzed. All mice were bred and housed in the Animal Facility under AAALAC guidelines. The experimental protocols were approved by the facilities Animal Institute Committee. All mice had access to food and water ad libitum.

IX. Aim 2: Results

Our aim for year 1 was to characterize 5q- mice as a model of human 5q- syndrome low risk MDS. Our findings reveal for the first time that 5q- mice exhibit macrocytic anemia, ineffective erythropoiesis, and iron overload, suggesting that this model has similar characteristics to human

low-risk MDS. However, we observed a wide range of severity and thus variability in 5q- phenotype at 3 months of age.

a) Although we observe no difference in reticulocyte counts, serum Epo is significantly higher in mice with a more severe phenotype (**Fig. 3A**). The RBC count, Hb, HCT, and MCHC decreased but RDW increased in severe relative to mild 5q- mice (**Fig.3B**).

b) We quantified the fraction of erythoid precursors in the bone marrow and spleen (CD45- cells) and analyzed ERFE expression in bone marrow erythroid precursors from 5q- mice. Our data reveals that the fraction of bone marrow erythroid precursors is decreased in severe relative to mild 5q- mice (**Fig.4A**); no difference in spleen erythroid precursors is evident (data not shown). In addition, ERFE expression increases in severe relative to mild 5q- mice (**Fig. 4B**). This later finding suggests that phenotype severity correlates strongly with erythroid expansion in 5q- mice.

c) Because of the higher Epo level in the sera as the increase expression of ERFE in bone marrow precurosors, we found no difference in serum hepcidin concentration, liver hepcidin expression, and non-heme liver iron, but transferrin saturation is significantly higher in severe relative to mild 5q- mice (**Fig.5**), suggesting that phenotype severity correlates strongly with transferrin saturation in 5q- mice.

d) In our bone marrow transplant experiments, apoTf-treated mild 5q- mice exhibit statistically increased RBC count (10 vs. 8 x10^6 cells/uL, P<0.0001) and lower MCV (41 vs. 53 fL, P<0.0001) and MCH (12 vs. 16 pg, P<0.0001) compared to PBS- injected mild 5q- mice (**Fig. 6A**). Results demonstrate an increase in reticulocyte count (676 vs. 477 x 10^9 cells/L, P=0.003) and platelet count (549 vs. 364 x 10^3 cells/ul, P=0.02) (**Fig. 6B**) in the circulation and an increase in ortho-E (19.3 vs. 16.1%, P=0.04) and reticulocytes (32.8 vs. 26.6%, P=0.04) in the bone marrow (**Fig.7**). No changes in ERFE expression were observed (data not shown). Although TfR1 mRNA expression is unchanged, membrane TfR1 MFI is increased in late stage erythroid precursors in both bone marrow (11 vs. 7 x 10^3 MFI, P=0.01) and spleen (16 vs. 12 x 10^3 MFI, P<0.05) (**Fig. 8A-B**) from apoTf-treated relative to PBS-injected 5q- mice. Interestingly, intracellular ROS is increased in late stage of splenic erythroid precursors from apoTf-treated relative to PBS-injected 5q- mice (**Fig.9**).

X. Aim 2: Conclusions and Future Directions

Taken together, our data demonstrates erythropoiesis- and iron-related characteristics of 5q- mice consistent with human disease. Although these preliminary experiments using exogenous apoTf

reveal no increase in Hb, improved ineffective erythropoiesis, or reversal of iron overload, our data suggests that TfR1 and ROS are intimately involved in the effect of exogenous apoTf on erythropoiesis.

We will continue to expand our breeding to enable a sufficiently large cohort of mild and severe 5q- mice (without employing the transplant approach) and evaluate iron and erythroid parameters with and without exogenous apoTf treatment.

We are also working on better understanding the molecular mechanism for such a wide variability in phenotype within the same genotype (confirmed) and hypothesize that Lmo Cre results in inefficient cleavage and only the severe phenotype is adequate to evaluate the model for our purposes. In addition, we will evaluate RPS14 expression in bone marrow and select using CD44 / Ter119 / FSC erythroid precursors.

Figure 3: (A) Higher Epo concentration in 5q- severe mice sera (B) Decrease Hb concentration and MCHC, but increase RDW in 5q-severe mice

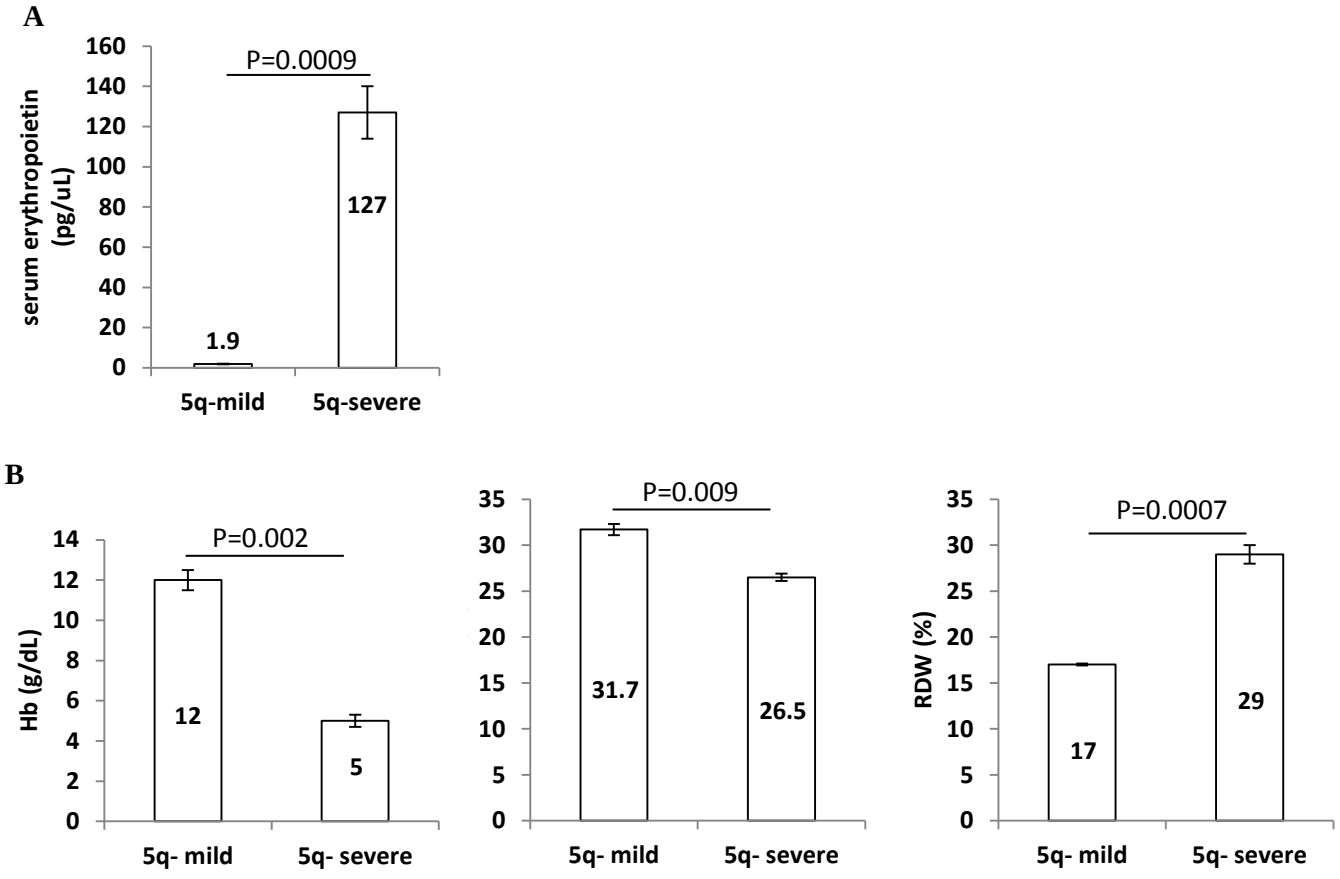


Figure 4: (A) Decrease bone marrow cells count in 5q- severe mice sera with high (B) ERFE mRNA expression

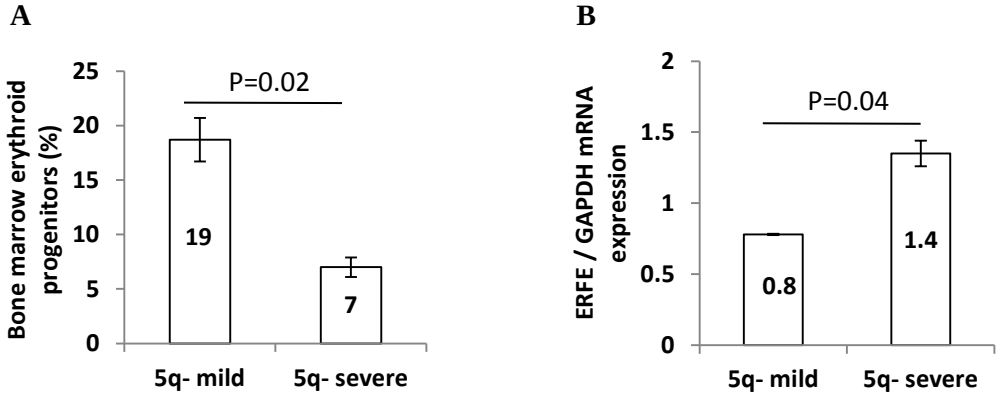


Figure 5: Increment of transferrin saturation in 5q- severe mice sera compare to 5q-mild mice

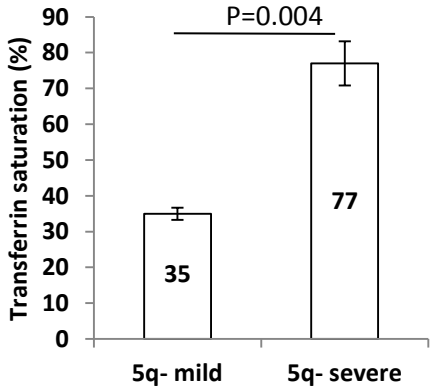
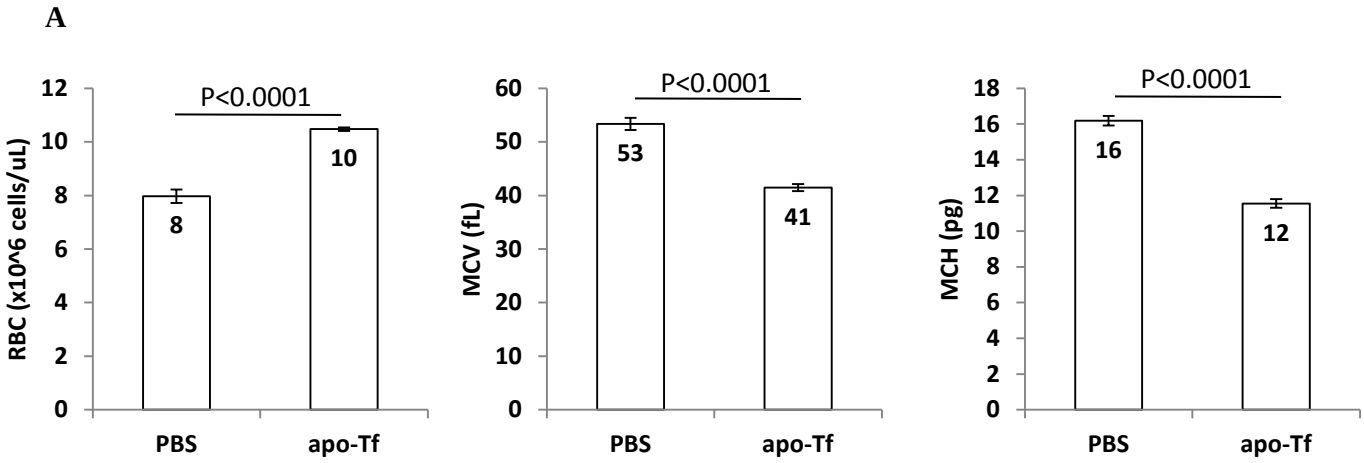


Figure 6: (A) Increase RBC and decrease MCV and MCH and increment (B) of reticulocytes and platelet counts in apo-treated 5q-mice



B

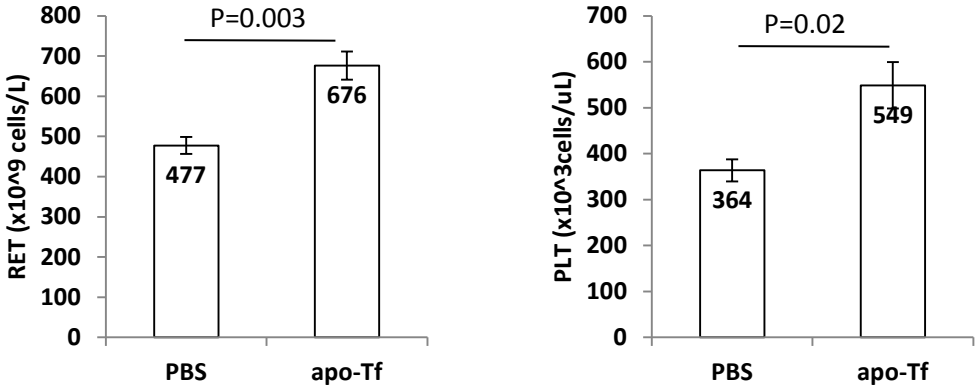


Figure 7: Increase in ortho-E and reticulocyte count in apo-treated 5q-mice

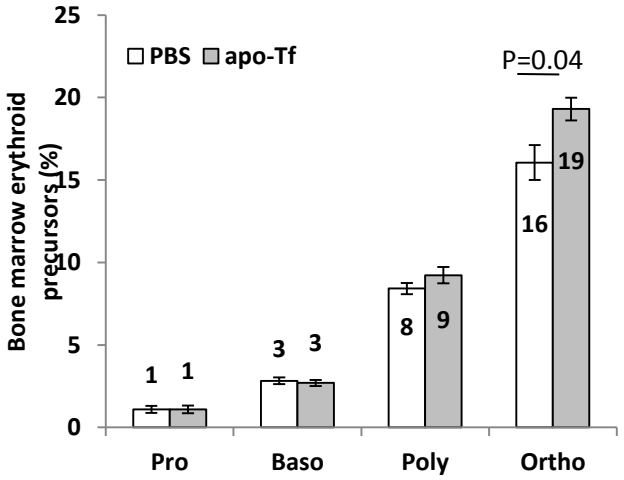
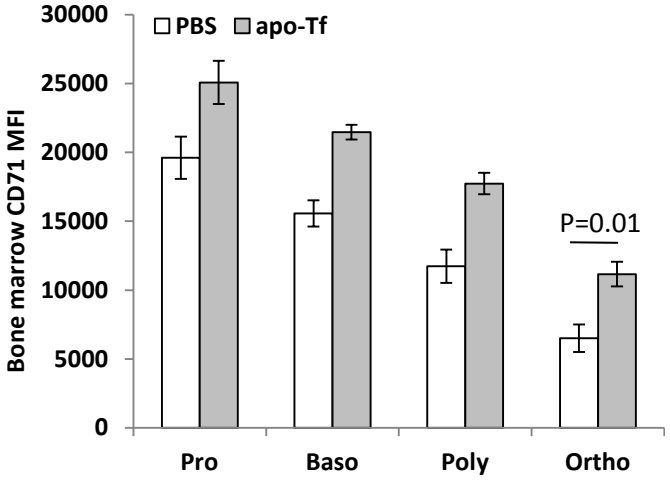
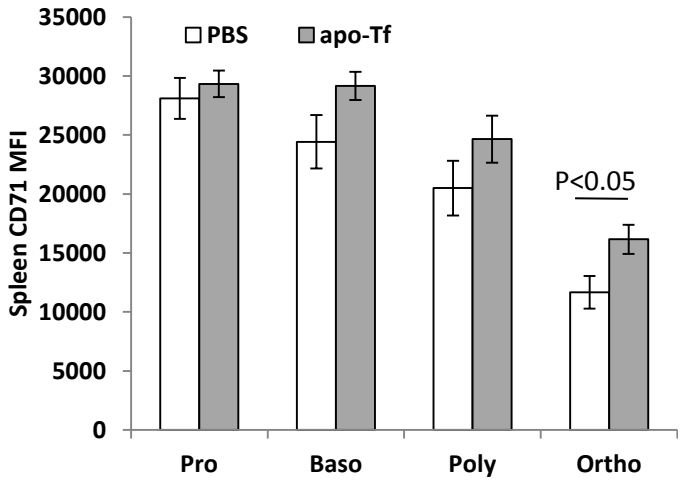


Figure 8: (A) Increase membrane TfR1 MFI in late stage of erythroid precursors both in bone marrow and (B) spleen in apo-treated 5q-mice

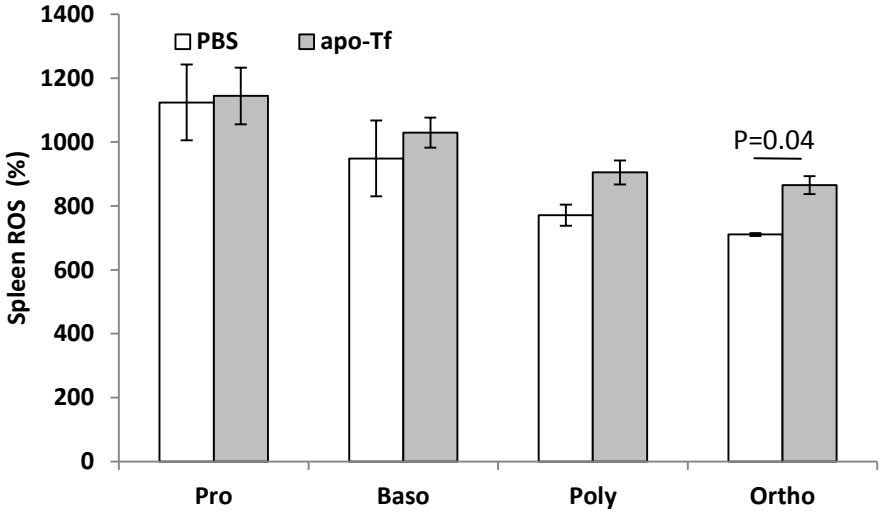
A



B



<u>Figure 9</u>: Increament of intracellular ROS in late stage of splenic erythroid precursors in apo-treated 5q-mice



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B.5b Participation at courses or summer schools organized by others than the PhD program

Course title and location	Organizer(s) and affiliation(s)	Number of hours	
Microtome training	Albert Einstein College of Medicine	4	

B.8b

Publication (articles, poster, abstract)	**Extrahepatic sources of factor VIII potentially contribute to the coagulation cascade correcting the bleeding phenotype of mice with hemophilia A.** Zanolini D, Merlin S, **Feola M**, Ranaldo G, Amoruso A, Gaidano G, Zaffaroni M, Ferrero A, Brunelleschi S, Valente G, Gupta S, Prat M, Follenzi A. Haematologica. 2015 Jul;100(7):881-92. doi: 10.3324/haematol.2014.123117.

	57th ASH Annual Meeting and Exposition **December 5-8, 2015** Orlando, FL Poster session **Characteristic macrocytic anemia, ineffective erythropoiesis, and iron overload and the effect of exogenous transferrin in 5q- mice** **Feola M**, Choesang T, Bao W, Li H, Chen H, Pham P, Li G, Verma A, Follenzi A, Ginzburg Y
	56th ASH Annual Meeting and Exposition **December 6-9, 2014** Moscone Center, San Francisco, CA Oral presentation **Changes in TfR1 expression and trafficking correlate with erythroid effectiveness in β-thalassemic mice** Li H, Blanc L, Choesan T, Chen H, **Feola M**, Bao W, Pham P, Follenzi A, Li G, Ginzburg Y
	56th ASH Annual Meeting and Exposition **December 6-9, 2014** Moscone Center, San Francisco, CA Oral presentation **Increased hepcidin expression in β-thalassemic mice treated with apo-transferrin is associated with increased Smad1/5/8 and decreased Erk1/2 pathway activation** Huiyong Chen, Tenzin Choesang, Petra Pham, Weili Bao, **Maria Feola**, Huihui Li, Mark Westerman, Guiyuan Li, Antonia Follenzi, Lionel Blanc, Stefano Rivella, Robert Fleming, Yelena Ginzburg
	56th ASH Annual Meeting and Exposition **December 6-9, 2014** Moscone Center, San Francisco, CA Poster session **Exogenous Apo-transferrin Increases Monoferric Transferrin, Decreasing Cytosolic Iron Uptake**

	and Heme and Globin Synthesis in β-thalassemic Mice Choesang T, Li H, Dussiot M, Maciel T, Breda L, Santos D, Chen H, **Feola M**, Bao W, Pham P, Follenzi A, Li G, Moura I, Ponka P, Fleming R, Rivella S, Ginzburg Y

B.XX Attended seminars

TOPIC	SPEAKER
"Erythropoiesis Lab's projects"	Huyong Chen, **Ph.D.** Huihui Li, **MS** Maria Feola, **MS**
"Complement Biology"	Karina Yazdanbakhsh, **Ph.D.**
Cell and Molecular Biology	Paul A. Ney, **M.D.**
"Apo-transferrin induced iron restriction in ß-thalassemia"	Yelena Ginzburg, **M.D.**
"Myeloproliferative Disorders"	Rona Weinberg, **Ph.D.**
"Clinical Research Sickle Cell Disease"	Patricia Shi, **M.D.**
"Viral Immunology"	Lanying Du, **Ph.D.** Shibo Jiang, **M.D., Ph.D.**