SCUOLA DI ALTA FORMAZIONE

DOTTORATO IN SCIENZE MEDICHE E BIOTECNOLOGICHE

PHD PROGRAM IN MEDICAL SCIENCES AND BIOTECHNOLOGIES

ANNUAL REPORT: Effect of exogenous apo-transferrin on erythropoiesis and iron metabolism in a 5q- syndrome mouse model

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CYCLE: XXIX YEAR: 2013/2014

1. Project aim/objectives

Low risk myelodysplastic syndrome (MDS) is associated with relatively longer survival and higher transfusion requirements, resulting in a secondary iron overload. We have previously shown that exogenous transferrin results in more circulating red blood cells (RBCs), increased hemoglobin (Hb), reversal of splenomegaly, and improvement of ineffective erythropoiesis in mouse models of β-thalassemia intermedia [1]. 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 transferrin may be relevant also in MDS. 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) [2]. 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 not 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 the effectiveness of exogenous transferrin in 5q- mice.

2. Background

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) [3]. Molecular genetic studies of patient cells identify gene mutations in hematopoietic stem cells of approximately 60% of patients. Such mutations contribute to the apparent maintenance of proliferation of early progenitors, the abnormalities in maturation seen in each hematopoietic lineage, and loss of mature cells in the marrow. This high frequency of deletions indicates a role for tumor suppressor genes in disease onset, but identification of these genes has been elusive [4,5].

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 [6]. Deletion of long arm of chromosome 5 (5q-) is the most common cytogenetic abnormality in low risk MDS. 5q- deletions encompass many genes in the region [7]. While most patients with 5q- syndrome have large deletions, some smaller chromosomal deletions have enable geneticists to identify common deleted regions (CDRs) minimally necessary for a clinical phenotype. Two CDRs have been reported, located at 5q52-33 [8,9] and 5q31 [10,11]. Macrocytic anemia is the most prominent cytopenia in patients with the 5q- syndrome, and patients are generally transfusion-dependent. Given the stability of the disease, with low rates of progression to AML, iron overload from chronic transfusions results in morbidity and mortality [12], similar to other low risk MDS subtypes [13,14].

Iron overload and erythropoiesis: Multiple areas of research suggest that iron overload impedes erythropoiesis [15,16]. However, the mechanisms by which this occurs are not completely understood. Iron overload inhibits burst-forming unit colony formation and erythroblast differentiation in vitro, and excess iron leads to dysplastic changes in exposed cells [17] and triggers apoptosis in erythroid precursors [18]. 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-overload diseases [19]. 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 [1,20].

Transferrin, preliminary results, and hypothesis:Transferrin functions as the main transporter of iron in the circulation, where it exists as iron-free apo-transferrin, 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, which non–transferrin-bound iron (NTBI) can be found in circulation [21]. 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 erythropoiesis. Transferrin bound iron uptake by transferrin receptor-1 is the only known means of iron delivery for erythropoiesis [22,23]. We previously demonstrated that exogenous apo-transferrin 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 [1]. We hypothesize that exogenous apo-transferrin 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.

3. Experimantal plan and method

Generation of transgenic 5q- syndrome mice

We generated the 5q- mouse [2] (with a deletion between Cd74 to Nid67 on chromosome 18, the mouse equivalent to 5q-) by crossing a Lmo2Cre knock-in mouse with a mouse engineered with loxP sites at both Cd74 and Nid67 loci ($Cd74^{+/lox}$ $Nid67^{+/lox}$). Using three specific primers, we analyzed genomic DNA by PCR to identify $Cd74^{+/lox}$ $Nid67^{+/lox}$ $Lmo2Cre^{+}$ mice heterozygous at all three alleles.

Characterization of 5q- mice

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 mouse erythropoietin, 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 [24,25] 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. Apoptosis was detected using Annexin V-fluorescein, and reactive oxygen species (ROS) were measured using a commercial kit per the manufacturer's instructions (Invitrogen). 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 [24,25]. Results were acquired by flow cytometry on a FACS Canto using FACS Diva version 6.1.2 software (Becton Dickinson) and cells 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.

Non-heme iron spectrophotometry: Quantification of non-heme iron was performed using the Torrance and Bothwell method [26]. 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. 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 hepcidin, ERFE and TWSG1 mRNA was amplified from appropriate tissue samples as previously described. Control GAPDH was amplified using primers GAPDH F and GAPDH R (Qiagen). Concentrations of hepcidin (Hamp1), ERFE and TWSG1 mRNA were normalized to GAPDH as described [27].

Histology and Immunohistochemistry: Mice were sacrificed by cervical dislocation 72 hours after the last 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.

4. Results

5q- mice have been bred, progeny assessed, and mice characterized to the specifications necessary for our experiments.

a) We generated the 5q- mouse [2] (with a deletion between Cd74 to Nid67 on chromosome 18, the mouse equivalent to 5q-) by crossing a Lmo2Cre knock-in mouse with a mouse engineered with loxP sites at both Cd74 and Nid67 loci (Cd74+/lox Nid67+/lox). Using three specific primers, we analyzed genomic DNA by PCR to identify Cd74+/lox Nid67+/lox Lmo2Cre+ mice heterozygous at all three alleles (**Figure 1**).

b) Characterization of 5q- relative to WT mice reveals splenomegaly and EMH in the liver (**Figure 2**), macrocytic anemia (**Figure 3**), increased serum erythropoietin (**Figure 4**), elevated transferrin saturation and serum iron, and increased liver non-heme iron (**Figure 5**). Although no difference in liver hepcidin expression or serum hepcidin concentration is evident, a trend toward decreased hepcidin:liver iron is observed in 5q- relative to WT mice (**Figure 6**).

c) This lack of appropriate increase in hepcidin expression despite increased parenchymal iron stores suggests that a competing signal is suppressing hepcidin expression in 5q- mice as in th1/th1 mice [28,29,30]. 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. twisted gastrulation 1 (TWSG1) and erythroferrone (ERFE)) [31,32,33]. We thus evaluate TWSG1 and ERFE expression in erythroid enriched (CD45-) bone marrow and spleen samples. ERFE expression is increased while TWSG1 expression is unchanged in 5q- relative to WT mice (**Figure 7**; spleen data identical (data not shown)).

d) Lastly, flow cytometry analysis reveals a decrease number of bone marrow erythroid precursors and expanded erythropoiesis in spleen (**Figure 8**). No differences in ROS are seen using flow cytometry analysis in bone marrow and spleen erythroid precursors from 5q- and WT mice.

Taken together, our data suggests that the mouse model shows characteristics that are consistent with MDS in humans and a good model in which to test effect of exogenous apoTf injection. Our aim for year 2 is to assess the above parameters in apoTf- relative to PBS-treated 5q- mice at several time points.

Figure 1: PCR analysis of triple positive heterozygous deletion of the region between Cd74 and Nid67

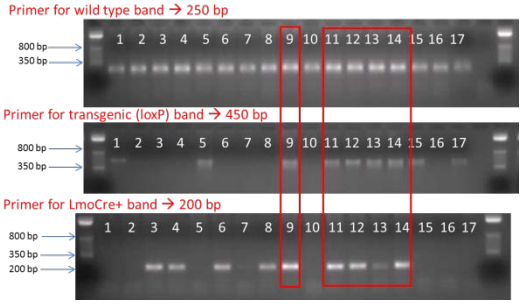


Figure 2: Splenomegaly (A) and liver EMH (B) in 5q- mice (Ter119 staining; 40x magnification)

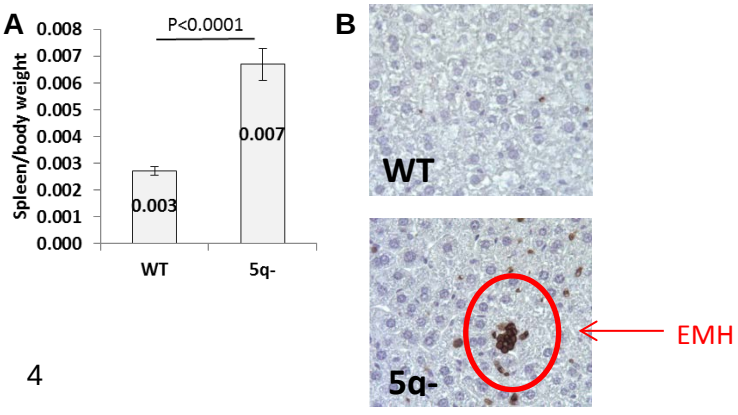


Figure 3: 5q- mice exhibit a macrocytic anemia relative to WT controls

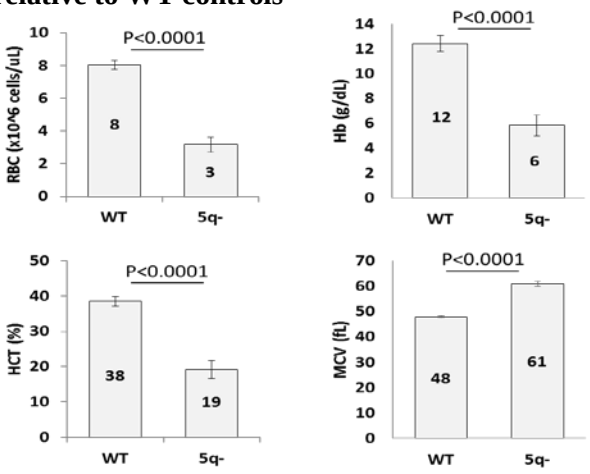


Figure 4: Serum epo concentration is increased in 5q- mice

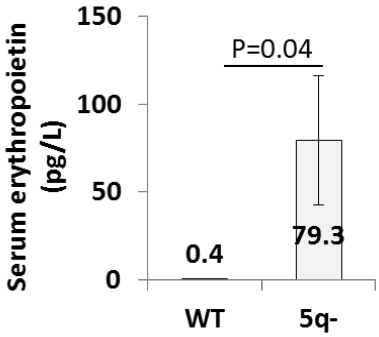


Figure 5: Liver non-heme iron concentration is increased in 5q- mice

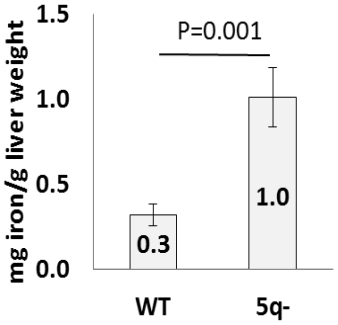


Figure 6: No change in hepcidin mRNA expression (A) and serum concentration (B), but a trend toward insufficiently elevated hepcidin relative to liver non-heme iron (C) in 5q-mice.

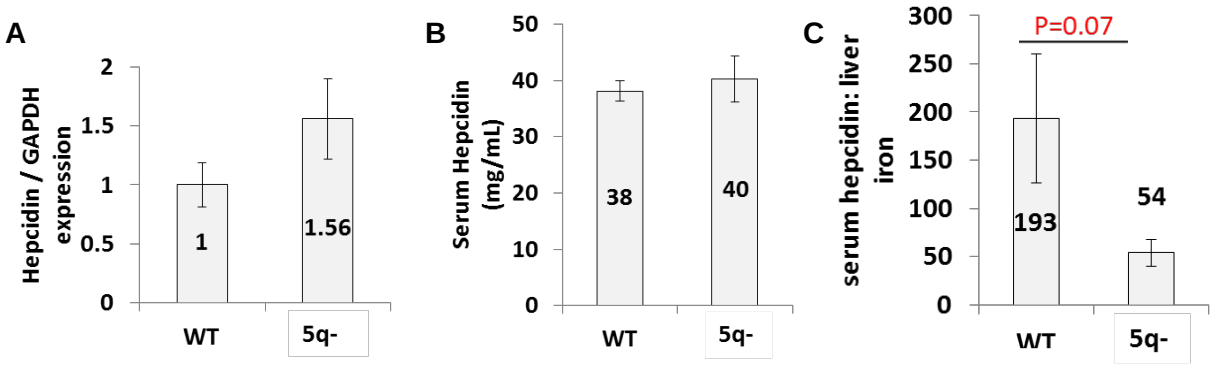


Figure 7: Increase in ERFE mRNA expression (A), but no change in TWSG1 (B) in bone marrow of 5q- mice

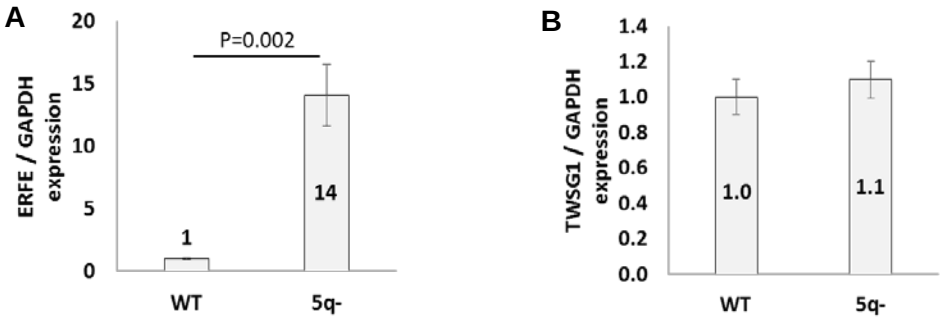
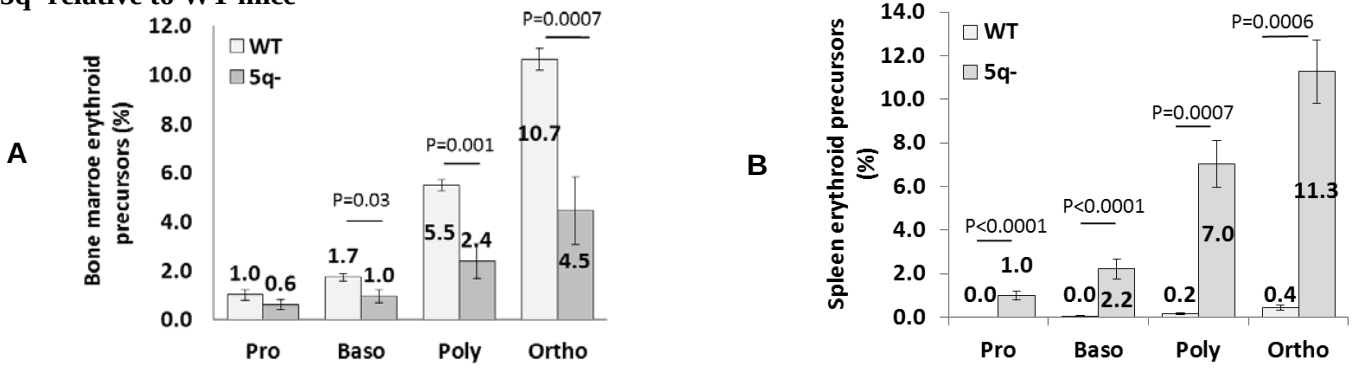


Figure 8: Decreased proportion of erythroid precursors in bone marrow (A) and expanded erythropoiesis in spleen (B) of 5q- relative to WT mice



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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)	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
"Genome-wide DNA demethylation: commonalities between erythroid progenitors and the zygote"	**Merav Socolovsky, M.D., Ph.D.** University of Massachusetts Medical School
"Human cell programming and genome editing for blood disease modeling and treatment"	**Linzhao Cheng, Ph.D.** Johns Hopkins University School of Medicine
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.**