



RoFAR

Foundation for Anemia Research

Bi-annual report

December 2007

RoFAR is an independent foundation run by an international Board of Trustees and funded by an unrestricted grant from Roche. All submitted applications are peer reviewed by an independent Scientific Advisory Board.

Mission

The Roche Foundation for Anemia Research (hereinafter “the RoFAR”) is a registered Medical Research Charity with the mission of “encouraging innovative research that will open new avenues of exploration in the study of anaemia, its mechanisms and outcomes.” Individuals eligible for grants are members of academic staff in universities, dialysis centres and research institutes.

The RoFAR was established by the Roche Group in 2004 under Swiss law and incorporated in Basel, Switzerland. The Roche Group is committed to providing funding of four million Swiss Francs (CHF) annually for at least four years from inception to a total of at least 16 million CHF . The RoFAR is a non-profit, autonomous and legally independent charitable organisation.

The RoFAR encourages the exploration of new basic and clinical research in areas associated with the study of anaemia, its mechanisms and outcomes. The Board of Trustees will set the focus of research for the specific cycle.

In addition to focusing on anaemia related to kidney disease and oncology, the RoFAR also will encourage research into:

- Anaemia of chronic disease
- Anaemia related to congestive heart failure and stroke
- Effects of erythropoietin and erythropoietin-like substances as protective drugs in various target organs
- Central resistance to erythropoietin
- Biology of anaemia and its outcomes

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1 Preface

On behalf of the RoFAR Board of Trustees, it is a pleasure to announce that a total of 1.38 million Swiss Francs (CHF) have been awarded to seven outstanding research projects in the second half of 2007.

Seven applications received during the seventh cycle of competition have been selected to receive RoFAR research grants of up to 200,000 CHF each, distributed over two years. As for the previous RoFAR competition cycles, the high expectations for the quality of research projects and applicants have been met. Since 2004, RoFAR has awarded forty-six regular grants and three special cycle grants, totalling over 11.3 million CHF or 10.2 million USD.

There are two cycles of RoFAR awards each year. Timelines for the cycles and the submission deadlines for applications – usually in June and November – are published on the Foundation's website (www.rofar.org). The first step in the application process is to submit a Letter of Intent (LOI), which is reviewed by our Scientific Advisory Board (SAB). Applicants who are considered by the SAB to have submitted the most compelling LOIs are then invited to proceed to the next stage and submit a full application. Full applications are considered in detail by the SAB, and final decisions on award winners are confirmed by the Board of Trustees (BT), which undertakes to notify applicants of their decision within six months after submission of the LOI.

In addition to the regular competition cycles, RoFAR re-extended an invitation to scientists and institutions to submit applications for a special grant to support ground-breaking scientific work, basic and clinical. Topics included investigation of anaemia and erythropoietin, ranging from hypoxia-sensing to the organ-protective role of erythropoietin and understanding of iron metabolism. RoFAR was particularly interested in innovative proposals, which provide proof of principle and/or are of translational nature, that is, studies which have potential for translation into clinical practice. The submission and selection procedure, similar to the one in use for regular grants, has resulted in two awards totalling 1.6 million CHF. The 2007 special grant recipients are announced in this report.

To inform a broader scientific community about the funding opportunities RoFAR provides, our promotional campaign in the second half of 2007 has included

- distribution of brochures to major nephrology, oncology and cardiology centres
- media releases about the second special competition cycle and the regular cycles

- information booths at the 14th European Cancer Conference (ECCO) held in Barcelona (Spain) and at the 49th Annual Meeting of the American Society of Hematology (ASH) held in Atlanta (USA)
- advertisements in major scientific journals
- increased presence at targeted scientific congresses in the form of posters and leaflet distribution

RoFAR is committed to its mission of fostering innovative anaemia-related research and sincerely hopes to make a major contribution to the scientific community by encouraging scientists to apply their skills and intellect to furthering knowledge and understanding in this field.

RoFAR administers an initial donation of 16 million CHF provided by F. Hoffmann-La Roche between 2004 and 2007. As a sign of Roche's long-term commitment to RoFAR, a further donation of 3 million Swiss Francs has been provided for 2008. For its generous gift to the anaemia research community and enduring commitment to anaemia and related avenues of research, the BT and the SAB of RoFAR join in expressing their gratitude to Roche.

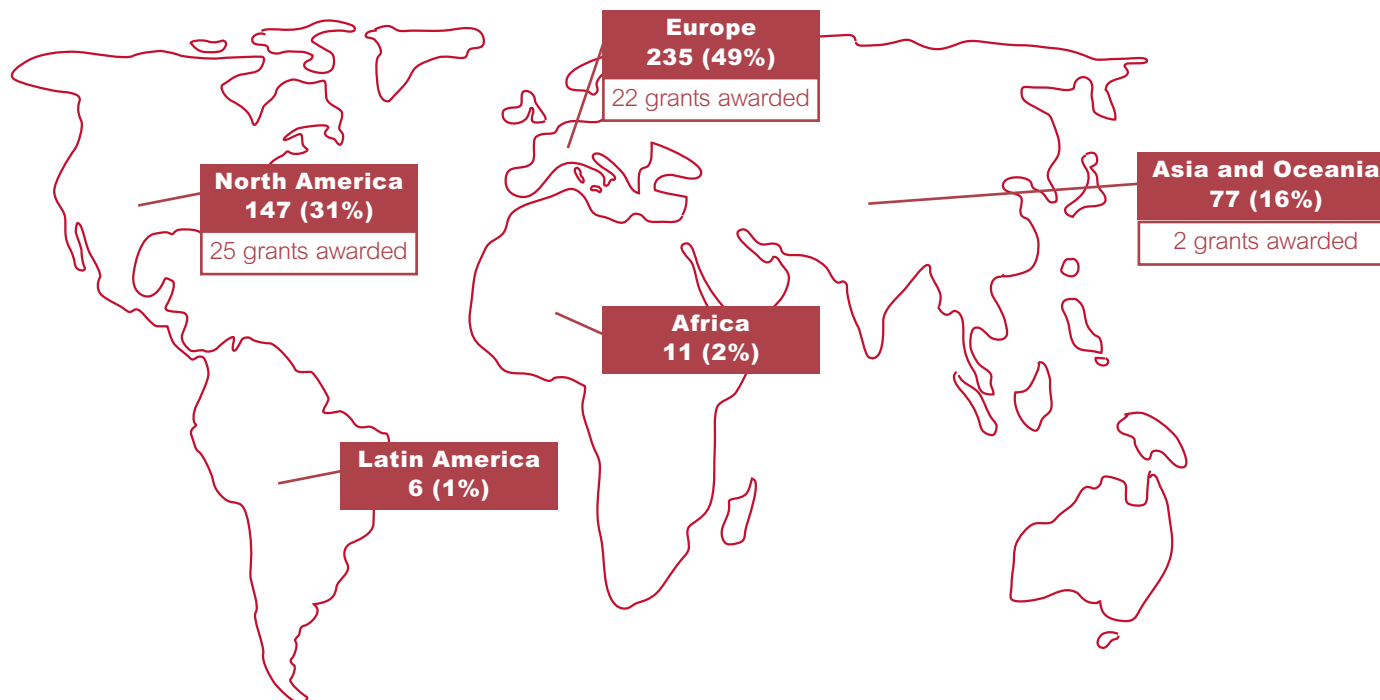
RoFAR welcomes any feedback or suggestions to assist us in accomplishing our stated mission.

On behalf of the Board of Trustees



Dr Nathan W. Levin
Chairman of the Board of Trustees
Roche Foundation for Anemia Research

Geographical breakdown of submitted research proposals



Applicants in the first eight cycles of competition including two special cycles represent a range of institutions in 48 countries. Approximately half (49%) of all the LOI applications have been submitted from Europe, primarily from Germany, UK, Italy, Switzerland and Israel. Over one-third (31%) of the applications have been submitted from the United States and Canada. About 27% of the applicants are female scientists. The great majority (99%) of applicants work in universities or university-affiliated institutions. Research proposals are distributed among clinical studies (47%), animal trials (44%) and basic science projects. Submitted projects focus on nephrology and diabetology (41%), haematology (44%), oncology (16%) and cardiology (12%) with some overlap between areas.

In cycles I to VII, twenty-two regular grants have been assigned to European applicants, twenty-three to North American applicants and one to Oceania (Australia). Special grants have been awarded to two North American applicants and one to an applicant from Australia. Cycle VIII grants have not yet been awarded.

Submitted research proposals by

Study type

- Human trials (47%)
- Animal studies (44%)
- Others (41%)

Research focus

- Nephrology (41%)
- Haematology (44%)
- Oncology (16%)
- Cardiology (12%)
- Others (22%) (multiple allowed)

Gender of main applicant

- Males (73%)
- Females (27%)

Institution type

- Universities & related (99%)
- Others (1%)

3 Overview of awarded grants

				Grant awarded	Progress report published	Final report published
Cycle I	Nancy C. Andrews	Children's Hospital Boston, USA	Hepcidin regulation in the anaemia of chronic disease	11/2004	07/2006	07/2007
	Martin W. Bergmann	Franz Volhard Clinic, Charité University, Berlin, Germany	Effect of 5,000 IU erythropoietin beta once weekly subcutaneously administered for six months in patients subjected to percutaneous coronary intervention displaying reduced LV- ejection fraction due to regional left ventricular wall motion defects	11/2004	12/2006	
	Andrew McKie	King's College, London, UK	Characterisation of a novel intestinal haem transporter	11/2004	07/2006	12/2006
	Marco Merlano	S. Croce General Hospital, Cuneo, Italy	In vitro analysis of tumor response to radiation in oxic and hypoxic conditions	11/2004	07/2006	07/2007
	Peter Mertens	University Hospital Aachen, Germany	Mechanisms for erythropoietin resistance in transformed and non-transformed cells	11/2004	07/2006	07/2007
	Chris D. Vulpe	University of California, Berkeley, USA	Characterisation of a family of putative mammalian haem chaperones	11/2004	07/2006	07/2007
Cycle II	Hans Ulrich Bucher	University Hospital of Zurich, Switzerland	Erythropoietin reduces brain, eye and lung damage in very preterm infants: Proof-of-concept study	05/2005	07/2006	12/2007
	Edward Debnam	Royal Free & University College Medical School, London, UK	Is inflammation an important factor in the anaemia of chronic renal failure?	05/2005	07/2006	12/2007
	Diana Gilligan	Puget Sound Blood Center, Seattle, USA	Regulation of gene expression during erythropoiesis	05/2005	12/2006	
	Alexander Maxwell	Queen's University Belfast, UK	Investigation of the role of JUNE-1 in erythropoiesis	05/2005	12/2006	07/2007
	Jun-ichi Nishimura	Duke University Medical Center, Durham, USA	Innovative drug design using RNA aptamers for various anaemias	05/2005	12/2006	12/2007
	Radek Skoda	University Hospital Basel, Switzerland	The role of SMAD4-dependent signalling in anaemia	05/2005	12/2006	07/2007
	Carole Soussain	Oregon Health and Science University, Portland, USA	Neuroprotective effect of erythropoietin on chemo- and radiotherapy-induced toxicity	05/2005	07/2006	07/2007
	Christina Warnecke	University Erlangen-Nürnberg, Germany	Molecular mechanisms underlying the hypoxic induction of erythropoietin by HIF-2 α	05/2005	12/2006	12/2007
Cycle III	Max Gassmann	Vetsuisse, University of Zurich, Switzerland	The impact of erythropoietin on the hypoxic ventilatory response of mouse and man	11/2005	12/2006	12/2007
	Peter J. Kirkpatrick	University of Cambridge, UK	Effects of systemic erythropoietin therapy on cerebral auto-regulation and the incidence of delayed ischaemic deficits in patients with aneurysmal subarachnoid haemorrhage	11/2005		12/2006
	Véronique Lefebvre	Cleveland Clinic Foundation, USA	Roles of Sox6 in erythropoiesis	11/2005	07/2007	
	Stephen Leib	University of Berne, Switzerland	Effect of erythropoietin on brain injury and regeneration in bacterial meningitis	11/2005	07/2007	
	Barbara Scheiber-Mojdehkar	Medical University of Vienna, Austria	Recombinant human erythropoietin: A new treatment for Friedreich's ataxia	11/2005	07/2007	
	Jürg Schifferli	University Hospital Basel, Switzerland	Erythropoietin or erythrocyte transfusion for anaemia?	11/2005	07/2007	
	Marcela Votruba	Cardiff University, UK	Erythropoietin neuroprotection in retinal neurodegeneration	11/2005	07/2007	

3 Overview of awarded grants

				Grant awarded	Progress report published	Final report published
Cycle IV	Christof Dame	Charité University of Berlin, Germany	Role of GATA transcription factors in regulating erythropoietin and its receptor in the heart	05/2006	12/2007	
	Ricarda Diem	University of Göttingen, Germany	Efficacy and safety of erythropoietin as an add-on therapy in subjects with acute autoimmune optic neuritis	05/2006	12/2007	
	Tomas Ganz	University of California, Los Angeles, USA	Pathogenesis of anaemia of chronic infection	05/2006	07/2007	
	Dirk Hermann	University Hospital of Zurich, Switzerland	Effects of human erythropoietin on brain plasticity and functional recovery following stroke	05/2006	07/2007	
	Stéphane Picot	Claude Bernard University of Lyon, France	Randomised trial of erythropoietin to prevent death from cerebral impairment during severe malaria	05/2006		
	Jerôme Rossert	Georges Pompidou European Hospital, Paris, France	Study of the characteristics and fate of erythropoietin-producing cells	05/2006		
Cycle V	Anne Angelillo-Scherrer	University Hospital of Lausanne, Switzerland	Role of growth arrest-specific gene 6 in anaemia of chronic disease	11/2006		
	Margaret H. Baron	Mount Sinai School of Medicine, New York, USA	Regulation of red blood cell enucleation	11/2006	12/2007	
	Michael Bulger	University of Rochester, Rochester (New York), USA	Function of Sox6 in β -globin gene silencing and definitive erythropoiesis	11/2006	12/2007	
	Sandra Juul	University of Washington, Seattle, USA	Mechanisms of erythropoietin-mediated neuroprotection	11/2006		
	Herbert Y. Lin	Massachusetts General Hospital, Boston, USA	Regulation of iron metabolism by soluble haemojuvelin. Fc fusion protein	11/2006	12/2007	
	Stefano Rivella	Weill Medical College of Cornell University, New York, USA	Identification of the genes responsible for the pleiotropic effects observed in β -thalassaemia	11/2006		
Special cycle 2006	Nicoletta Eliopoulos	Lady Davis Institute for Medical Research (McGill University), Montreal, Canada	Cell and gene therapy with erythropoietin-secreting marrow stem cells for kidney repair	11/2006		
Cycle VI	Nancy C. Andrews	Children's Hospital Boston, USA	Regulation of hepcidin expression	05/2007		
	Mark D. Fleming	Children's Hospital Boston, USA	The genetics of erythroid haem and iron metabolism	05/2007		
	David Johnson	Princess Alexander Hospital, Brisbane, Australia	A randomised, placebo-controlled trial of oxpentifylline on haemoglobin levels in patients with erythropoietin-resistant anaemia	05/2007		
	Zvonimir S. Katusic	Mayo Clinic, Rochester (Minnesota), USA	Role of antioxidant enzymes in vasculoprotective effect of erythropoietin	05/2007		
	Frank S. Lee	University of Pennsylvania School of Medicine, Philadelphia, USA	Prolyl hydroxylase domain protein 2, a physiologic regulator of erythropoietin	05/2007		
	Tonia S. Rex	University of Tennessee Health Science Center, Memphis, USA	Analysis of rhEPO processing in mouse tissue - implications for gene therapy of retinal degenerations	05/2007		

3 Overview of awarded grants

				Grant awarded	Progress report published	Final report published
Cycle VII	Clara Camaschella	University Vita-Salute San Raffaele, Milan, Italy	GLRX5 deficiency as a model of anaemia responsive to iron chelation	12/2007		
	Madeleine Carreau	Laval University, Quebec, Canada	Fanconi anaemia proteins as regulators of genes involved in haematopoietic stem cell function	12/2007		
	Wenbin Deng	University of California, Davis, Sacramento, USA	Protective effects of erythropoietin against hypoxic-ischaemic injury to developing oligodendrocytes	12/2007		
	Adam Goldfarb	University of Virginia School of Medicine, Charlottesville, USA	Iron regulation of erythropoiesis: Characterisation of a novel signalling pathway	12/2007		
	Véronique Lefebvre	Cleveland Clinic Foundation, USA	Erythropoiesis control by Sox6 and erythropoietin signalling	12/2007		
	John G. Quigley	University of Illinois at Chicago, USA	FLVCR protein trafficking and the regulation of haem export	12/2007		
	Li Zhong	University of Florida College of Medicine, Gainesville, USA	Recombinant parvovirus vectors for gene therapy of Fanconi anaemia	12/2007		
Special cycle 2007	Tomas Ganz	University of California, Los Angeles, USA	Regulation of the hepcidin-ferroportin axis in anaemia of inflammation	12/2007		
	Stephen M. Jane	Bone Marrow Research Laboratories, Parkville, Australia	Developing small molecule inhibitors of PRMT5 for treatment of thalassaemia and sickle cell disease	12/2007		

4 Grant awards in Cycle VII

Prof. Clara Camaschella (principal applicant)

Prof. Sonia Levi (co-applicant)
Prof. Giuliana Ferrari (co-applicant)
Dr Laura Silvestri (co-applicant)
Dr Alessandro Campanella (co-applicant)



University Vita-Salute San Raffaele, Milan, Italy

GLRX5 deficiency as a model of anaemia responsive to iron chelation

New models of inherited anaemias characterised by red cells of small size and excess of total body iron have been recently recognised based on animal models (mice and fish) with similar defects. Absence of the protein enzyme glutaredoxin-5 (GLRX5) was first described in fish models, where it is incompatible with life, because of the development of severe anaemia before birth. Reduction of GLRX5 has been recognised so far in a single patient of middle age with severe anaemia and iron overload. The story of this patient is unusual, because of the paradoxical effect of blood transfusions that worsened anaemia, which was significantly ameliorated only by therapeutic iron removal. This project is proposed to facilitate the study of this new type of anaemia. It aims to develop *in vitro* models of different cells (hepatocytes, erythroblasts) in which the effect of artificially decreasing GLRX5 enzyme activity is analysed, as well as to understand the molecular mechanisms induced by drug-mediated iron removal.

Using recombinant DNA technology, the gene expression of this enzyme will be downregulated in hepatic and erythroid cell lines, and the effect on cellular iron changes and the ability of the cell to respond to these changes will be evaluated, analysing proteins of iron metabolism in the different cell compartments.

The project might reveal new physiological mechanisms of cellular iron regulation and their derangements in GLRX5 deficiency, with potential therapeutic implications for patients affected by rare congenital and acquired anaemias secondary to defects of GLRX5 or related enzymes implicated in the same pathway.

Dr Madeleine Carreau (principal applicant)

Dr Georges Lévesque (co-applicant)



Laval University, Quebec, Canada

Fanconi anaemia proteins as regulators of genes involved in haematopoietic stem cell function

Children affected by Fanconi anaemia (FA) suffer from a life-threatening haematological failure, congenital abnormalities and a predisposition to cancer such as leukaemia. Although many genes associated with this disease have been identified, their function remains unknown. We know that when mutated or de-regulated, these Fanconi genes affect the normal development of haematopoietic stem cells leading to their progressive exhaustion. These stem cells are responsible for the generation of all blood cell types, thus their exhaustion leads to anaemia and bone marrow failure.

Our main objective is to identify the function of Fanconi genes and their role in haematopoiesis, specifically those related to stem cell function such as their maintenance and renewal. We have obtained data linking Fanconi genes to a signalling pathway well-known for its role in embryonic stem cell development, the Notch1/HES1 pathway. We have found that HES1 is a molecular partner of FA genes. We propose to characterise the role of this novel FA-HES1 interaction in haematopoietic stem cell function.

We believe that understanding the molecular basis of the Fanconi anaemia disease will lead to the development of new therapeutic approaches aimed at increasing haematopoietic stem cell numbers through regulation of their maintenance and renewal. We also believe that knowing molecular partners of FA genes and their role in regulating haematopoietic stem cells should help design new therapies for blood disorders such as aplastic anaemia. Since de-regulation of FA genes causes leukaemia, knowing their functions will inevitably help the design of new drugs that specifically target leukaemia cells.

4 Grant awards in Cycle VII

Dr Wenbin Deng



University of California, Davis, Sacramento, USA

Protective effects of erythropoietin against hypoxic-ischaemic injury to developing oligodendrocytes

Periventricular leukomalacia (PVL) is the predominant form of brain injury in the premature infant and the most common cause of cerebral palsy, yet no therapy currently exists for this serious human disorder. Injury to the oligodendrocyte precursor (termed “pre-OL”) is a major factor in PVL. We have recently shown that erythropoietin (EPO) attenuates pre-OL injury *in vitro*. This proposal aims to expand upon the preliminary results and further investigate the protective effect of EPO in pre-OL injury *in vivo*. We will characterise the oligodendrocyte-specific developmental regulation of the EPO receptor in the rodent white matter and in the human parietal white matter by using post-mortem human tissue. We will evaluate the effect of EPO on hypoxic-ischaemic pre-OL injury in an animal model of PVL that we have recently established. We will determine the mechanisms of EPO protection against pre-OL injury. Completion of this project will help to define the translational potential of EPO as a therapeutic agent for pre-OL injury underlying PVL, for which no therapy presently exists. The scientific knowledge to be acquired through this project is of likely benefit to the care of children with PVL leading to cerebral palsy.

Dr Adam Goldfarb



University of Virginia School of Medicine, Charlottesville, USA

Iron regulation of erythropoiesis: Characterisation of a novel signalling pathway

Human iron deficiency impairs the marrow response to the red cell growth factor erythropoietin (EPO). Conversely, intravenous iron infusion enhances the bone marrow response to EPO, even in anaemia patients with adequate pre-existing iron stores. Iron regulation of EPO-driven red cell production affects proliferation and differentiation of early bone marrow progenitors, prior to the onset of haemoglobin production. Thus, while iron is essential for all cells, red cell precursors manifest an exquisite sensitivity to iron deficiency, most likely as a rationing mechanism to protect other, more vital iron-dependent functions. Using a system with human bone marrow precursors cultured in defined levels of iron and EPO, we have confirmed the existence of a critical threshold of iron deprivation, at which erythroid progenitors display proliferative and maturation blockade while other blood cell types remain unaffected. Extensive pharmacologic and genetic screening for components of this iron response pathway have identified the aconitase enzymes as a critical signalling node. Mitochondrial and cytosolic aconitase (mAcon and cAcon) interconvert citrate and isocitrate as a key step in the Krebs cycle. The aconitase enzymes within red cell precursors are more sensitive to iron deprivation than aconitase enzymes in other cell types. Providing cells with the product of the aconitase enzymes, isocitrate, restores the growth and development of red cell precursors subjected to iron deprivation. In addition, treatment of mice with isocitrate enhances their red cell haemoglobin production. In an effort to identify how the aconitase enzymes are regulated within red cell precursors, we identified PKC α as a kinase whose activity is regulated by iron deficiency and which appears to control the activity of the aconitase enzymes. This project will delineate the function of PKC α in the response of red cell precursors to iron deficiency. This pathway has direct relevance for future clinical approaches to EPO-unresponsive chronic anaemias.

4 Grant awards in Cycle VII

Dr Véronique Lefebvre



Cleveland Clinic Foundation, USA

Erythropoiesis control by Sox6 and erythropoietin signalling

This project is designed to advance understanding of major mechanisms involved in red cell formation. We will focus on the gene called *Sox6*, which encodes a protein that helps several cell types activate the genes that they need to fulfil specialised functions. We recently found that these cell types include red cell precursors (called erythroid cells). We showed that *Sox6* helps these cells survive and proliferate, and is crucial in the final steps of their conversion into mature red cells. Mice that lack *Sox6* have mild anaemia under normal conditions of life, but are unable to quickly produce mature red cells when subjected to severe blood loss, and consequently, many die within a few days. We also found that *Sox6* works together with the hormone called erythropoietin (EPO), which has a key role in stimulating erythroid cell survival and proliferation and thereby in adjusting red cell production to body need. *Sox6* also works beyond EPO to ensure timely red cell maturation. The first aim of this project is to identify mechanisms whereby *Sox6* acts in erythroid cells. We will specifically ask how *Sox6* works together and beyond EPO to control expression of the gene for *Bcl-xL*, a protein essential for erythroid cell survival. The second aim is to identify the hormones and intracellular proteins that control expression of the *Sox6* gene in erythroid cells. In both aims, we will identify the DNA sequences that are needed for expression of the *Bcl-xL* and *Sox6* genes *in vivo*, and we will identify the proteins that bind to these sequences. By greatly increasing our understanding of the molecular mechanisms underlying red cell formation, we expect our study to provide solid foundations for uncovering the causes of various forms of anaemia diseases and for designing better therapies for these diseases.

Prof. John G. Quigley



University of Illinois at Chicago, USA

FLVCR protein trafficking and the regulation of haem export

FLVCR (Feline Leukemia Virus subgroup C Receptor) function, the export of haem, is required to permit normal erythropoiesis. Inhibition of this function leads to a block in erythroid differentiation and severe anaemia. Cell membrane expression of FLVCR, similar to other membrane transporters, is likely regulated through control of its trafficking to and from the cell surface via interactions with a PDZ domain-containing protein. A yeast two hybrid screen using the cytoplasmic tail of FLVCR identified a candidate protein, which contains a PDZ domain. The proposed experiments involve verification of interactions between FLVCR and the candidate protein, and characterisation of the interacting protein. In addition, the effects of deletions of domains of the interacting protein on FLVCR expression and trafficking in heterologous cells, including polarised cells, will be examined. An overriding aim however, is to determine the role of trafficking on FLVCR haem export function in epithelial and erythroid cell lines. This will improve our understanding of haem transport, relevant to diseases characterised by haemolysis and release of free (toxic) haem, including the haemolytic anaemias, sickle cell disease, the thalassaemias and malaria, situations where FLVCR likely functions to protect cells from haem toxicity.

4 Grant awards in Cycle VII

Dr Li Zhong



These studies will provide new insights into the development of safe and effective parvovirus-based vectors, and help to evaluate the *in vivo* efficacy and safety of these vectors prior to their potential use in gene therapy of human haematopoietic diseases, particularly in FA syndrome.

University of Florida College of Medicine, Gainesville, USA

Recombinant parvovirus vectors for gene therapy of Fanconi anaemia

Fanconi anaemia (FA) is an autosomal recessive disease that is characterised by congenital abnormalities, defective haematopoiesis, a high risk of developing acute myeloid leukaemia and certain solid tumours, and cellular sensitivity to cross-linking agents. Since none of the currently used therapeutic approaches is curative, the development of a safe and effective gene therapy approach involving the introduction of a functional FA gene into haematopoietic stem cells (HSCs) followed by autologous stem cell transplantation could achieve long-term benefits. Parvovirus vectors, including the non-pathogenic adeno-associated virus (AAV) and parvovirus B19 vectors have gained attention as a useful alternative to the more commonly used retrovirus- and adenovirus-based vectors in human gene therapy.

Our hypothesis is that by using novel serotype AAV and recombinant parvovirus B19 vectors and strategies that have been developed by us and others, highly efficient transduction of FA complementation C (*FancC*) gene in primary murine and human HSCs can be achieved. The optimal recombinant parvovirus vector-mediated *FancC* transduction will prove to be safe and effective in therapeutic correction of FA in animal models *in vivo*. The following specific aims will be pursued in this proposal: 1) to develop novel serotype AAV and recombinant parvovirus B19 vectors containing *FancC* cDNA driven by haematopoietic cell-specific promoters to achieve high-efficiency expression of the *FancC* gene in murine and human HSCs and alleviate complications of FA *in vitro* and 2) to evaluate the efficacy of novel serotype AAV and parvovirus B19 vectors for efficient transduction and long-term expression of the *FancC* gene to allow functional reconstitution and therapeutic correction of FA-C in mice *in vivo*. The safety of these vectors will also be evaluated.

5 *RoFAR grant awards in the Special Cycle*

Special Grants of 1.6 million Swiss Francs have been awarded to investigators in the United States and Australia in 2007.

In addition to the regular competition cycles in 2007, RoFAR re-extended an invitation to scientists and institutions to submit innovative proposals, which provide proof-of-principle and/or are of translational nature, that is, studies which have potential for translation into clinical practice.

RoFAR is very proud to announce that two special grants have been awarded in 2007:

Professor Tomas Ganz and his co-investigators Drs Seth Rivera and Elizabeta Nemeth of the David Geffen School of Medicine at the University of California in Los Angeles (UCLA), United States, have been awarded a grant for a three-year project aimed at 1) identifying the mediators and pathways that regulate hepcidin as well as ferroportin during inflammation, 2) analysing the effect of inflammation on homeostatic regulation of hepcidin by iron and erythropoietic activity and 3) examining the influence of sex hormones on hepcidin during inflammation.

Tomas Ganz graduated from UCLA with a B.S. in physics, obtained a PhD degree in applied physics at the California Institute of Technology in 1976, and received his medical degree in 1978 at UCLA. After an internship and residency in internal medicine and a fellowship in pulmonary medicine, he continued his scientific endeavours at UCLA as a postdoctoral fellow in the group of Prof. Harvey Hershman in the Department of Biological Chemistry. In 1983 Prof. Ganz became an Assistant Professor of Medicine at the David Geffen School of Medicine at UCLA and now serves as a Professor of Medicine and Pathology at the same institution. For the past several years, Prof. Ganz has been doing extensive work on hepcidin, studying its role in anaemia of chronic disorders and its deficiency in most forms of haemochromatosis. The proposed research builds on an earlier RoFAR grant in which the tools were developed for the study of hepcidin regulation during inflammation.

The second grant was awarded to **Professor Stephen M. Jane** of the Bone Marrow Research Laboratories at The Royal Melbourne Hospital, and his co-investigator Dr Ian Street from the WEHI Biotechnology Centre for a one-year project seeking to identify small molecule inhibitors of PRMT5 through a high throughput screen and to prevent the down-regulation of foetal globin chain synthesis as a means of treating sickle cell anaemia and beta thalassaemia.

Prof. Jane graduated from Monash University, Melbourne with a MBBS degree in medicine in 1981. Several years later in 1987, he received degrees in pathology and medicine at the Royal College of Pathologists of Australasia and Royal Australasian College of Physicians, respectively. Prof. Jane earned his PhD degree in medicine in 1990 also from Monash University and completed a postdoctoral fellowship under the auspices of Dr Arthur Nienhuis. Prof. Jane has been on the faculty of medicine at the University of Melbourne since 2000, and since 2006 the Director of the Bone Marrow Research Laboratories, Royal Melbourne Hospital.

5 *RoFAR grant awards in the Special Cycle*

Prof. Tomas Ganz (principal applicant)

*Dr Seth Rivera (co-applicant)
Dr Elizabeta Nemeth (co-applicant)*



University of California, Los Angeles, USA

Regulation of the hepcidin-ferroportin axis in anaemia of inflammation

Anaemia (called anaemia of chronic disease or anaemia of inflammation) commonly develops during infections and inflammatory diseases, and often contributes to their morbidity and possibly mortality. The host reaction that gives rise to this anaemia evolved as a part of host defence against microbial infections. Certain microbial molecules and inflammatory cytokines cause hepatocytes and macrophages to sequester iron, presumably to starve the invading microbes of this essential nutrient. As a side effect, less iron is available for haemoglobin synthesis and anaemia develops. Non-infectious diseases are the predominant cause of this type of anaemia in industrialised countries, and the reaction has become maladaptive. Recently, we and others have begun to identify the molecular pathways that cause anaemia of inflammation. The key molecules that link inflammation to iron restriction are the iron-regulatory hormone hepcidin, the hepcidin-regulating receptor haemojuvelin, and the iron channel and hepcidin receptor ferroportin. The synthesis of all three molecules is regulated during inflammation by both systemic and local mechanisms. Gender appears to influence these events strongly, suggesting that sex hormones participate in regulating the relevant pathways. Building on a foundation of our previous work in this area, we now aim to elucidate the pathogenic pathways in anaemia of inflammation.

We will 1) identify the mediators and pathways that regulate hepcidin synthesis during inflammation; 2) identify the mediators and pathways (systemic and autocrine in macrophages) that regulate ferroportin expression during inflammation; 3) analyse the effect of inflammation on homeostatic regulation of hepcidin by iron and erythropoietic activity, focusing on the role of haemojuvelin and 4) examine the influence of sex hormones on the regulation of hepcidin during inflammation.

This work will increase our understanding of the pathogenesis of anaemia of inflammation and identify potential targets for pharmaceutical intervention.

Prof. Stephen M. Jane (principal applicant)

Dr Ian Street (co-applicant)



Bone Marrow Research Laboratories, Parkville, Australia

Developing small molecule inhibitors of PRMT5 for treatment of thalassaemia and sickle cell disease

Haemoglobin, the major protein in red blood cells is essential for the transport of oxygen from the lungs to the tissues. The disorders of haemoglobin production are the most common genetic diseases world-wide, and include the devastating disorders sickle cell anaemia and beta thalassaemia. Both these disorders lead to significant morbidity and early mortality in many patients. Treatment regimens are either unavailable to most or particularly arduous, with a life-long need for regular blood transfusions, coupled with medication to remove excess iron from the system.

Studies in rare patients have revealed that these diseases can be markedly improved through elevation of foetal haemoglobin, the form of haemoglobin produced by the developing embryo. Several drugs are currently in use to achieve this aim, but most are non-specific and burdened with unwanted side effects or lack of efficacy. Our laboratories have recently identified key factors important for the silencing of foetal haemoglobin production that normally occurs at birth. The identification of these factors has provided targets for drug development, which may lead to compounds with increased specificity and efficacy. Our proposal centres on a screen for small molecules that will target the factors that silence foetal haemoglobin production. Compounds identified in this screen that reverse this process will be modified to increase potency and reduce toxicity - standard processes in drug development. Modified compounds will then be tested for efficacy in our cellular models. We envisage that our findings could translate into novel therapies for the haemoglobin disorders.

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Prof. Hans Ulrich Bucher¹ (principal applicant)

Dr Joachim Riethmüller² (co-applicant)
(Cycle II)



**University Hospital of Zurich, Switzerland¹
and University of Tübingen, Germany²**

*Erythropoietin reduces brain, eye and lung damage in very preterm infants:
Proof-of-concept study*

Background

Since long-term disability remains a major problem in very preterm infants, novel strategies to protect developing organs, in particular the central nervous system, are of greatest interest in neonatal intensive care medicine. Erythropoietin, the primary regulator of red blood cell production, has been shown to be protective against hypoxic-ischaemic and inflammatory injuries in cell culture, animal models of brain injury and in two trials in human adults with stroke or schizophrenia.

Objective

We hypothesised that early administration of high-dose recombinant human erythropoietin (rhEPO) to very preterm infants reduces perinatal brain injury and improves neurodevelopmental outcome. In a first step, we investigated whether high-dose rhEPO administered shortly after birth and subsequently over the first two days to very preterm infants is safe in terms of short-term outcome.

Patients and Methods

Randomised, double-masked trial with a 2 to 1 allocation in favour of rhEPO. Preterm infants (gestational age 24 0/7 - 31 6/7 weeks) were given rhEPO ($n_r = 30$; 3,000 U/kg body weight) or NaCl 0.9% ($n_c = 15$) intravenously at 3, 12-18 and 36-42 hours after birth.

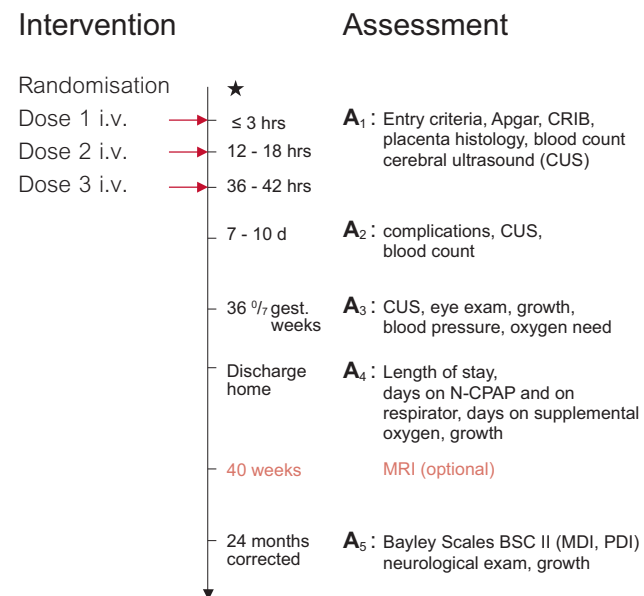


Figure 1. Diagram of study procedures.

Results

There were no relevant differences between both groups regarding short-term outcomes such as retinopathy, intraventricular haemorrhage (IVH), sepsis, necrotising enterocolitis, and bronchopulmonary dysplasia. In 5 infants of the rhEPO group with a gestational age below 26 0/7 weeks, withdrawal of intensive care was decided (3/5 with severe bilateral IVH, 2/5 with pulmonary insufficiency).

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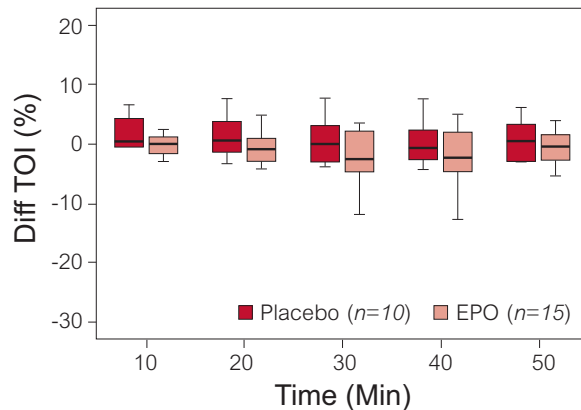


Figure 2. Clustered boxplots showing the median, 1st and 3rd quartile, minimum and maximum for cerebral tissue oxygenation index (TOI) in the EPO and placebo groups during the first five 10-minute intervals after injection.

Conclusions

No significant adverse effects of early high-dose rhEPO treatment in very preterm infants were identified. These results enable us to embark on a large multicentre study with the aim to determine if early high-dose administration of rhEPO in very preterm infants improves neurodevelopmental outcome at 24 months and 5 years corrected age.

A multicentre trial based on the same research protocol involving 420 infants has already started and is partly sponsored by the Swiss National Science Foundation.

These results have been and will be presented at the following scientific meetings:

1. *Effects of erythropoietin on cerebral blood circulation measured by near-infrared spectroscopy in preterm neonates.* European Academy of Pediatrics, 7-10 October 2006 in Barcelona, Spain
2. *Erythropoietin for neuroprotection in preterm infants: Feasibility and safety study.* World Congress on Pediatric Critical Care, 24-28 June 2007 in Geneva, Switzerland
3. *Erythropoietin for neuroprotection in preterm infants: Short-term outcome.* European Society of Pediatric Research, 7-9 October 2007 in Prague, Czech Republic

Dr Edward Debnam (principal applicant)

Prof. Robert J. Unwin (co-applicant)

(Cycle II)



Royal Free & University College Medical School, London, UK

Is inflammation an important factor in the anaemia of chronic renal failure?

Patients with chronic renal failure (CRF) often require systemic iron supplementation as well as erythropoietin (EPO) to correct anaemia, implying that intestinal uptake of dietary iron is suppressed during CRF.

We hypothesise that enhanced expression of hepcidin, a type II acute phase reactant that is known to suppress intestinal iron transport during CRF determines the responsiveness to EPO. Thus, raised circulatory levels of hepcidin during inflammation may override the effect of EPO treatment during CRF.

We aim to compare intestinal iron transport and the expression of known intestinal iron transporters with liver expression of hepcidin in a model of CRF, and to assess the role of hepcidin during treatment with EPO in CRF.

We used 5/6th nephrectomised rats as an experimental model for CRF. Some were treated acutely with turpentine (to induce an inflammatory response) or with EPO. The effect of the different treatment regimes on duodenal iron absorption was determined using a method that measured iron transport from intestinal lumen to blood. Changes in iron transport were compared with alterations in gene and protein expression of the intestinal iron transporters DMT1 and Ireg1. Gene expression of liver hepcidin was also measured for comparison with iron absorption in the different treatment groups.

Plasma levels of urea and creatinine were increased in 5/6th nephrectomised animals compared to sham-operated animals confirming CRF status, and were anaemic. Intestinal iron uptake was decreased by CRF but no change in expression of the BBM iron transporter, DMT1 or the basolateral membrane (BLM) transporter Ireg1 was seen. CRF increased hepcidin expression but not duodenal

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Ireg1. Thus the reduced iron transport across the intestinal BLM in CRF animals may be due to altered activity of Ireg1 induced by raised circulatory levels of hepcidin.

EPO treatment of nephrectomised animals increased the packed cell volume to beyond that seen in sham-operated animals. Iron absorption was increased by EPO but not fully restored to the level seen in non-CRF animals. Interestingly, treatment with EPO reduced liver hepcidin mRNA expression (Figure 1) but did not affect gene expression of the duodenal iron transporters. Acute inflammation in CRF animals significantly inhibited duodenal iron absorption (Figure 2) - an observation accompanied by decreased gene expression of DMT1 and Ireg1. Interestingly, there was no further enhancement by turpentine of liver hepcidin expression over that seen in CRF animals (Figure 1). In nephrectomised animals which received both EPO and turpentine, the positive effect of EPO on intestinal iron absorption was overridden by the inflammatory response induced by turpentine (Figure 2).

Thus, in patients with CRF, EPO treatment may reverse the increase in hepcidin levels and therefore, in part, restore the rate of duodenal iron absorption. However, other, yet unknown factors are involved in the inadequate iron uptake. The finding that turpentine abolished the effect of EPO treatment on iron absorption suggests that inflammation may be the key factor in the anaemia of CRF. Hepcidin may not be the sole inflammatory factor involved in suppressing iron absorption during CRF.

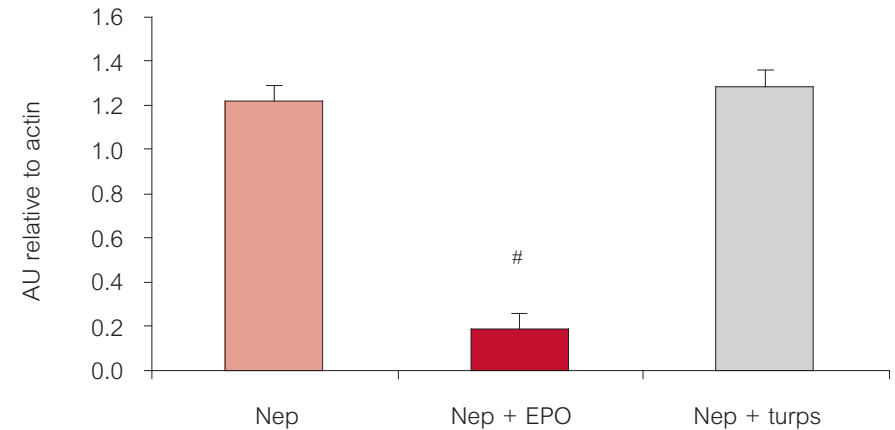


Figure 1. Gene expression of liver hepcidin using 5/6th nephrectomised (Nep), EPO and turpentine-treated (turps) CRF animals. Hepcidin mRNA levels are shown compared to β -actin mRNA levels. # $P < 0.05$ compared to nephrectomy.

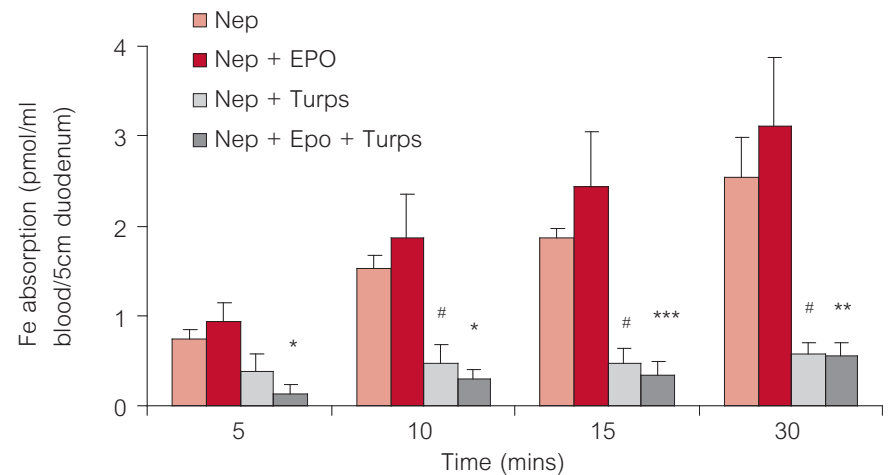


Figure 2. Duodenal iron absorption in 5/6th nephrectomised (Nep), EPO and turpentine-treated (turps) CRF animals. # $P < 0.05$ compared to nephrectomy, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared to nephrectomy+EPO.

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Dr Jun-ichi Nishimura (principal applicant)

Dr Marilyn J. Telen (co-aplicant)

(Cycle II)



Duke University Medical Center, Durham, USA

Innovative drug design using RNA aptamers for various anaemias

To prevent or treat vaso-occlusion in sickle cell disease (SCD), we have targeted three important adhesion molecules, including $\alpha V\beta 3$, P-selectin, and ICAM-4, using RNA aptamers.

Integrin $\alpha V\beta 3$

We have synthesised a high-affinity aptamer clone 17.16 (UUCAACGCUGUGAAGGGCUUAUACGAGCGGAUUAACCC) that binds to human integrin $\alpha V\beta 3$ ¹. To measure its anti-adhesion activity, an *in vitro* flow chamber assay was adopted. The anti-adhesion activity of aptamer clone 17.16 was tested using human umbilical vein endothelial cells (HUVEC) treated with thrombin (1 nM for 5 mins at 37°C) using a flow chamber assay. Aptamer clone 17.16 at 30 nM had strong anti-adhesion activity (Figure 1). Normalised percent inhibition of aptamer clone 17.16 at 30 nM at 2 dynes/cm² was 68%.

P-selectin

Aptamer clone **PF377** (ACGCUCAACGAGCCAGGAACAUCGACGUCAGCAAACGCGAGCGCAACCAGUAACACC) that binds to human P-selectin has been synthesised² and its high-affinity to P-selectin was confirmed by the binding assay. The anti-adhesion activity of aptamer clone PF377 was tested using primary HUVEC treated with IL-13 (50 ng/mL) for 48 hours, followed by stimulation with histamine (25 μ M for 12 mins at RT) immediately prior to a flow chamber assay. Aptamer clone PF377 at 60 nM also had strong anti-adhesion activity (Figure 2). Normalised percent inhibition of aptamer clone PF377 at 60 nM at 1 dyne/cm² was 99%.

ICAM-4

Co-aplicant, Dr Telen, previously found that epinephrine acted through erythroid signalling pathways (cAMP-dependent protein kinase A) to activate sickle

erythrocyte adhesion to endothelium via ICAM-4 (LW, CD242)- $\alpha V\beta 3$ interactions³. She recently further found that ICAM-4 activation by epinephrine via β -adrenergic receptor (AR) stimulation can promote SS RBC adhesion as well as vaso-occlusion *in vivo*. Therefore, ICAM-4 has replaced B-CAM, the original target, as a therapeutic target. Since recombinant ICAM-4 has been purified, the initial condition of the SELEX procedure for ICAM-4 was then determined by the double-filter nitrocellulose-filter binding method with various conditions. Target protein concentration was determined where the corrected fraction bound was 0.15; at 2.5 μ M with buffer F (20 mM HEPES, 150 mM NaCl, 2 mM CaCl₂) plus 4 mM MgCl₂. Human ICAM-4-binding aptamers are currently being screened using the SELEX method.

Thus, we have made significant advances in spite of various difficulties. Most notably, strong anti-adhesion activity of aptamer clones 17.16 and PF377 has been confirmed, and in *in vivo* experiments in mice, intravital microscopy is currently being employed to measure anti-adhesion activity of aptamer clones 17.16, PF377, and their combination. The development of combinatorial blocking aptamers against various adhesion molecules, including $\alpha V\beta 3$, P-selectin, as well as ICAM-4 represents a novel potential therapeutic option for patients with SCD.

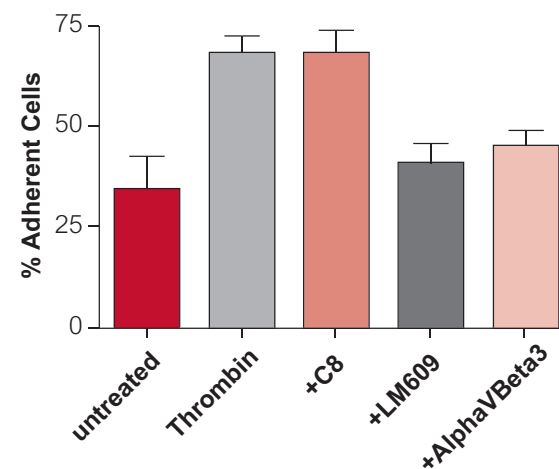


Figure 1. Anti-adhesion activity of $\alpha V\beta 3$ binding aptamer clone 17.16.

Aptamer clone 17.16 at 30 nM had anti-adhesion activity similar to LM609 (an inhibitory antibody to $\alpha V\beta 3$). However, human complement 8 aptamer² (negative control, 30 nM) did not have anti-adhesion activity.

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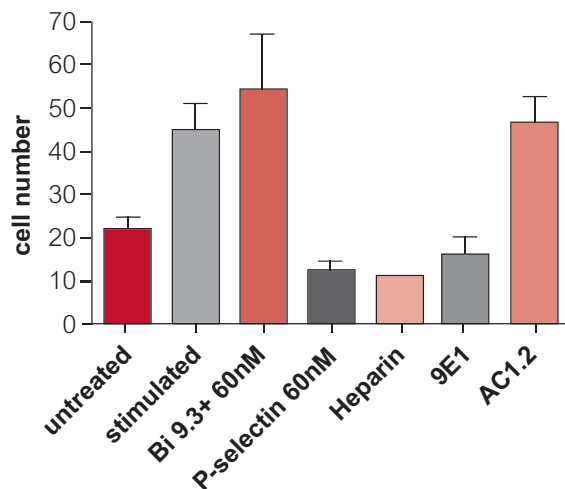


Figure 2. Anti-adhesion activity of P-selectin binding aptamer clone PF377.

Aptamer clone PF377 at 60 nM had anti-adhesion activity similar to heparin (a previously identified inhibitor of SS RBC adhesion to P-selectin) and 9E1 (an inhibitory antibody to P-selectin). However, Bi 9.3t (a non-functional aptamer to thrombin, used as a negative control, 60 nM) and AC1.2 (a non-inhibitory antibody to P-selectin), did not prevent adhesion.

References

1. Mi J, *et al.* Targeted inhibition of $\alpha V\beta 3$ integrin with an RNA aptamer impairs endothelial cell growth and survival. *Biochem Biophys Res Commun* 2005; 338:956-963.
2. Jenison RD, *et al.* Oligonucleotide inhibitors of P-selectin-dependent neutrophil-platelet adhesion. *Antisense Nucleic Acid Drug Dev* 1998; 8:265-279.
3. Zennadi R, *et al.* Epinephrine acts through erythroid signaling pathways to activate sickle cell adhesion to endothelium via LW- $\alpha V\beta 3$ interactions. *Blood* 2004; 104:3774-3781.
4. *Blood* 2005; 106:57a.

The latest data have been presented at the following scientific meetings:

1. 48th Annual Meeting of the American Society of Hematology (ASH), 9-12 December 2006 in Orlando, Florida, USA
2. Foundation Evening Symposium sponsored by RoFAR at the World Congress of Nephrology (WCN) 2007, 22-25 April 2007 in Rio de Janeiro, Brazil

Dr Christina Warnecke (principal applicant)

Prof. Kai-Uwe Eckardt (co-applicant)

(Cycle II)



University Erlangen-Nürnberg, Germany

Molecular mechanisms underlying the hypoxic induction of erythropoietin by HIF-2 α

Recent gene targeting studies confirmed our previous observation that erythropoietin (EPO) is a target of the transcription factor hypoxia-inducible factor 2 (HIF-2), but not HIF-1, as assumed previously. The mechanism of hypoxic HIF-1 α and HIF-2 α protein stabilisation appears to be similar, but the molecular determinants underlying target gene selectivity of the HIF- α subunits are not understood. We first performed an Affymetrix microarray with HIF-1 α and HIF-2 α siRNA-transfected Hep3B cells to identify other HIF-2 α target genes and to be able to compare their regulation by HIF-2 α in different cell types. The majority of identified HIF targets were HIF-1 α -dependent. HIF-2 α targets were scarce and, with the exception of EPO, only moderately affected by HIF-2 α knock-down. About half of them were also affected by HIF-1 α knock-down. In HepG2 cells, which exhibit a lower hypoxic HIF-2 α /HIF-1 α protein ratio than Hep3B cells, the number of HIF-2 α -responsive genes were reduced, and in HeLa cells no HIF-2 α target could be identified suggesting that the relative abundance of HIF-2 α affects its contribution to hypoxic gene regulation. In addition, cell culture conditions, such as the serum type used, influenced HIF-2 α effects in Hep3B cells and led to decreased EPO induction and shifts in the HIF- α isoform-dependencies for other HIF-2 α -targets (Figure 1A). Protein expression of HIF-2 α and potential interacting factors such as HNF-4 α , NEMO, ELK-1, CITED2 and c-Myc were not significantly altered (Figure 1B). In addition we could not demonstrate a direct physical interaction between HIF-2 α and these proteins.

In a second Affymetrix array experiment performed with two independent HIF-1 α and HIF-2 α siRNAs in parallel, the HIF-1 α siRNAs yielded largely identical results, whereas the congruence between the effects of the HIF-2 α siRNAs was limited, indicating off-target effects. Interestingly, EPO was more affected by the alternative HIF-1 α than by the HIF-2 α siRNA (Figure 2). Altogether the data

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suggest that HIF-2 α has, at least *in vitro*, a limited contribution to the total hypoxic response and that there is more functional redundancy between HIF-1 α and HIF-2 α than previously anticipated.

We also found that HIF-2 α transcriptional activity is merely affected by the asparagyl hydroxylase FIH, since mutation of the asparagyl hydroxylation site had no effect on HIF-2 α activity.

Mutational analysis of the 223-bp EPO enhancer revealed that only inactivation of the HNF binding site reduced the hypoxic induction of the luciferase reporter, whereas mutation of the HIF-1 ancillary sequence, the STAT, SMAD and NF- κ B binding sites had no effect. Although combined knock-down of both HIF- α subunits had additive effects, the EPO enhancer reporter responded more to HIF-1 α than to HIF-2 α knock-down suggesting that the mechanisms of enhancer activation differ from the activation of the endogenous EPO gene (Figure 2C).

In conclusion, we obtained deeper insight into the intricate HIF-2 α -dependent gene regulation. Still, the mechanisms underlying the partial selectivity of HIF-2 α function are not completely understood. The relative abundance of HIF-2 α compared with HIF-1 α , which may rise over stimulation time and may be increased in tumour cells, as well as cell type- and stimulus-dependent alterations in the activity of co-factors or repressors may affect the contribution of HIF-2 α to hypoxic gene induction.

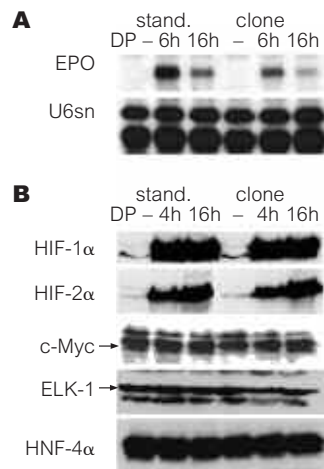


Figure 1. Effects of different serum types on the expression of EPO, HIF-2 α and potential co-factors. A) Two weeks of passaging in the presence of foetal calf serum 'clone' reduced EPO induction markedly compared with standard serum. RNase protection assay. U6small nuclear RNA (U6sn) served as loading control, DP=hypoxia-mimetic 2,2'dipyridyl). B) Protein levels of HIF-1 α , HIF-2 α and of factors potentially interacting with HIF-2 α in EPO induction were not altered.

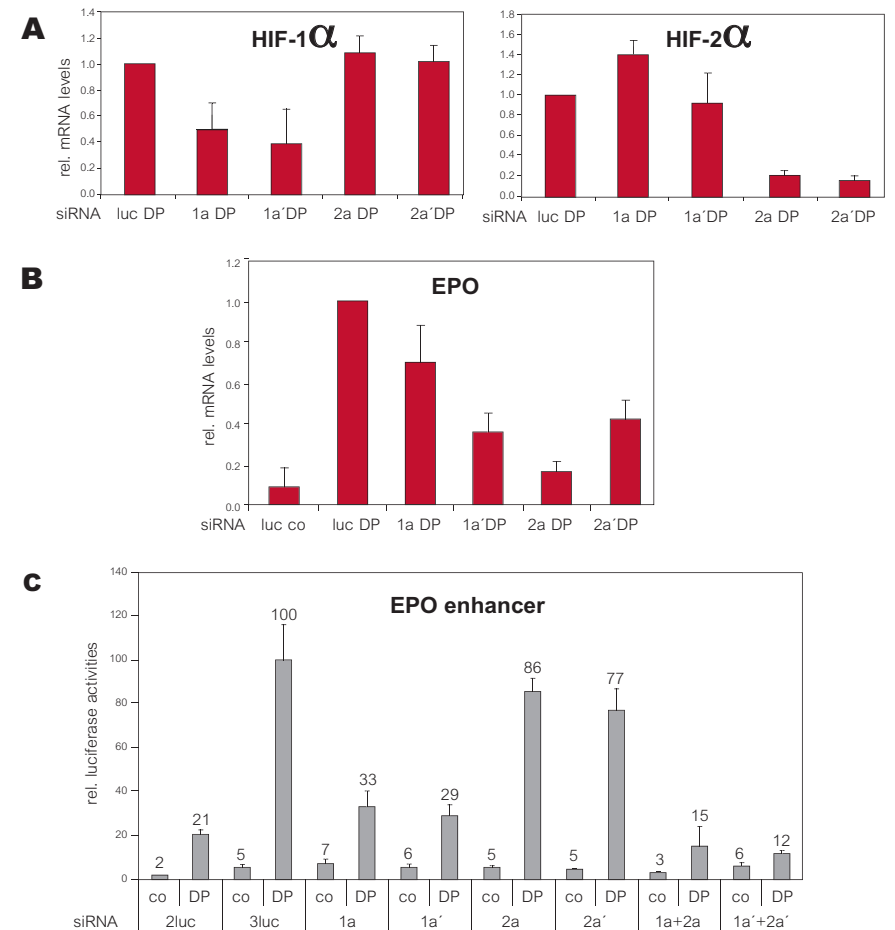


Figure 2. Effects of HIF-1 α and HIF-2 α knock-down with two independent siRNA pairs for each HIF- α subunit on EPO mRNA levels and EPO enhancer-driven luciferase activities. A) Efficiency of HIF-1 α and HIF-2 α knock-down at the mRNA level; DP=hypoxia-mimetic 2,2'dipyridyl, co = control, luc = luciferase control siRNA. B) Effect of the HIF- α knock-down on EPO mRNA levels in Hep3B cells. C) Effect of the HIF- α knock-down on a EPO enhancer luciferase reporter (pGL2luc vector). The synergistic effects of HIF-1 α and HIF-2 α knock-down were also observed at the mRNA level (not shown). The quantitatively different effects of the HIF- α knock-downs on EPO enhancer activity versus endogenous EPO mRNA levels suggest that sequence elements other than the EPO enhancer may contribute to the hypoxic regulation of the endogenous EPO gene. Data are means \pm SD of three independent experiments.

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Prof. Max Gassmann
(Cycle III)



Vetsuisse, University of Zurich, Switzerland

The impact of erythropoietin on the hypoxic ventilatory response of mouse and man

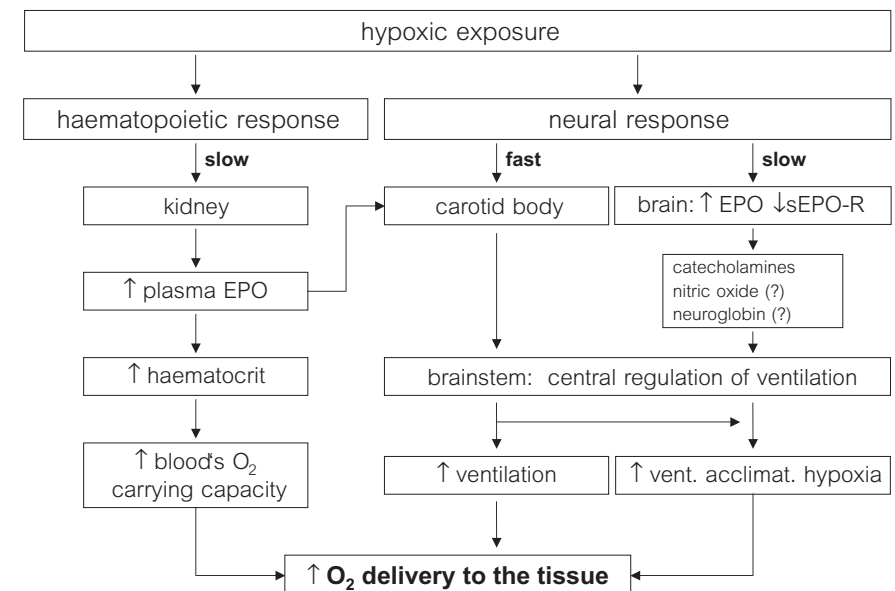
Acclimatisation to hypoxic exposure relies on elevated ventilation and erythropoietic activity. Back in 2005, we provided convincing evidence that erythropoietin (EPO) links both responses: apart from its well-known function on erythropoiesis, cerebral and plasma EPO interferes with the central respiratory centres (brainstem) and peripheral chemoreceptors (carotid bodies). Based on this novel function of EPO and considering that women cope better than men with reduced oxygen supply as observed, e.g. at high altitude, we intensified our investigations. As mentioned in the original RoFAR proposal, we developed the following three aims: sexual dimorphism of EPO's effect on the hypoxic ventilatory response (HVR), EPO's impact on carotid bodies and EPO's modulation of the HVR in human beings.

To compare the HVR in male and female animals, we used transgenic mice with elevated EPO levels in the brain only (Tg21), in the brain and plasma (Tg6), and wild-type animals with and without intravenous administration of recombinant human EPO (rhEPO). Exposure of these animals to moderate and severe acute hypoxia, as well as to hyperoxia (Dejours test) and intraperitoneal injection of domperidone, a potent peripheral ventilatory stimulant, revealed that the presence of transgenic EPO or rhEPO extensively increases the hypoxic ventilatory response in female mice compared to their male siblings. Accordingly, alterations of norepinephrine content and tyrosine hydroxylase activity in the brainstem's respiratory centres were also gender-dependent in transgenic animals. These data imply that catecholamines are involved in EPO's gender-dependent regulation of the HVR.

Another mechanism that allows EPO to modulate ventilation in the brain involves the soluble EPO receptor (sEPO-R). We demonstrated that sEPO-R, a negative

regulator of EPO's binding to the EPO-R, is present in the mouse brain and is downregulated after exposure to normobaric chronic hypoxia. Furthermore, while normoxic minute ventilation increased in control mice following hypoxic acclimatisation, sEPO-R infusion to the brain during hypoxic challenge effectively reduced brain EPO concentration and abolished ventilatory acclimatisation to hypoxia. These observations imply that hypoxic downregulation of sEPO-R is required for adequate acclimatisation to hypoxia.

To test whether our observations in mice are parallel in human beings, volunteers were injected 5000 IU rhEPO and subsequently exposed to 10% oxygen for 15 min. Compared to men, the HVR was significantly increased in women. We conclude that EPO exerts a gender-dependent impact on hypoxic ventilation most probably involving sex hormones. Moreover, our data might explain women's greater capacity to acclimatise to hypoxia and to be less susceptible to hypoxia-associated syndromes. We also foresee the therapeutic use of rhEPO to enhance ventilation in clinical situations.





The current RoFAR-funded project has been successful and has resulted in the following publications:

1. Soliz J, *et al.* Soluble erythropoietin receptor is present in the mouse brain and is required for the ventilatory acclimatization to hypoxia. *J Physiol* 2007; 15(583/Pt 1):329-336.
2. Soliz J, *et al.* Acute and chronic exposure to hypoxia alters ventilatory pattern but not minute ventilation of mice overexpressing erythropoietin. *Am J Physiol R* 2007; 293:R1702-1710.
3. Soliz J, *et al.* Gender-dependent regulation of hypoxic ventilation in mouse and man is mediated by erythropoietin (submitted).

Review articles:

1. Bavis RW, *et al.* Respiratory plasticity in response to changes in oxygen supply and demand. *Integr Comp Biol* 2007; 47:532-551.

7 Progress reports of RoFAR award winners

Prof. Christof Dame
(Cycle IV)



Charité University of Berlin, Germany

Role of GATA transcription factors in regulating erythropoietin and its receptor in the heart

Recombinant erythropoietin (EPO) has been shown to exhibit significant cardioprotective effects, mediated by binding to its specific receptor (EPOR) on cardiomyocytes. Thus, the specific aims were: 1) to describe the regulation of EPOR expression by the transcription factor GATA-4, in particular, in response to acute hypoxic-ischaemic stress and 2) to analyse EPO expression in the developing and adult heart, and to test whether GATA transcription factors are involved in silencing EPO gene expression in the myocardium.

Preliminary results

Specific aim 1: First, we confirmed high EPOR mRNA, but also GATA-4 and GATA-6 mRNA and protein expression throughout gestation and adulthood. *In vitro*, GATA-4 bound to 3 out of 6 potential binding motifs within the 5'-untranslated region of the EPOR gene (5'-EPOR-UTR), with the strongest affinity to the highly conserved GATA motif located within the minimal promoter. Since both positive and negative regulatory domains have been identified within the 5'-EPOR-UTR, we are currently performing reporter gene assays to clarify the function of the respective GATA motifs. Stress-induced domain decondensation (SIDD) analysis indicated that the GATA motifs are located in areas highly accessible for a variety of transcription factors. Chromatin immunoprecipitation analysis (ChIP) indicated binding of various transcription factors to regulatory domains within the 5'-EPOR-UTR, including Sp1, a well-known co-factor of GATA-4. Preliminary data on the effect of GATA-4 overexpression alone or in combination with its co-factor *friend-of-GATA 2* (FOG2) in cardiomyocytes did not shed enough light on the significance of GATA-4 in regulating EPOR expression. In an opposite approach, GATA-4 depletion by the anthracycline doxorubicin showed a significant, time-dependent reduction of EPOR mRNA expression in cardiomyocytes. Most importantly, this finding could be confirmed *in vivo*. Of note, 5 days after single

dose treatment with doxorubicin, endogenous GATA-4 and EPOR levels normalised. In accordance, recently developed transgenic mice expressing an interfering RNA against GATA-4 confirmed the significant reduction of EPOR expression caused by GATA-4 suppression¹.

Specific aim 2: Careful analysis indicated that the *EPO* gene is expressed at very low levels during early gestation (embryonic day e11.5), but silenced later on. As proposed, we tested the hypothesis that GATA-6 – known to act either synergistically or competitively with GATA-4 – is involved in silencing cardiac EPO expression. For technical reasons, we first analysed the effects of GATA-6 in human hepatoma cells (HepG2), an excellent model for analysing EPO gene regulation. Despite *in vitro* DNA-binding, GATA-6 did not affect EPO promoter activation. Both under hypoxia and normoxia, very efficient GATA-6 overexpression failed to change EPO mRNA expression. This phenomenon was also not limited by a lack of FOG-2 or changes in GATA-4 expression. A series of loss-of-function experiments confirmed that GATA-6 is not relevant for EPO silencing². Based on a SIDD analysis, we are currently investigating novel candidates for EPO regulation, including YY1, shown to bind to the EPO promoter in ChIP analysis.

In summary, preliminary data indicate that GATA-4 directly activates EPOR expression in cardiomyocytes. Since cardiac EPO gene expression is silenced after early embryonic development, the EPOR plays a predominant role in EPO-mediated cardioprotection.

Parts of this work have been presented at the following scientific meetings:

1. The 4th Annual C.E.R.A. Anaemia Academy Meeting entitled *Role of GATA transcription factors in regulating erythropoietin and its receptor in the heart*, 5-7 October 2007 in Frankfurt a.M., Germany

References

1. Schwerdtner-Hanske S, *et al.* Activation of the EPOR in cardiomyocytes by the transcription factor GATA-4. *In preparation.*
2. Sallmon H, *et al.* GATA-4, but not GATA-6 plays a predominant role in regulating hepatic erythropoietin expression. *Prepared for submission after final editing.*

7 Progress reports of RoFAR award winners

Dr Ricarda Diem (principal applicant)

Prof. Michael Knauth (co-applicant)

Dr Gunther Helms (co-applicant)

Prof. Reinhard A.W. Hilgers (co-applicant)

Prof. Jürgen Petersen (co-applicant)

(Cycle IV)



University of Göttingen, Germany

Efficacy and safety of erythropoietin as an add-on therapy in subjects with autoimmune optic neuritis

Optic neuritis is one of the most common clinical manifestations of multiple sclerosis (MS), an autoimmune inflammatory central nervous system (CNS) disease. Visual recovery after an episode of optic neuritis frequently remains incomplete due to degeneration of optic nerve axon fibres and secondary apoptosis of retinal ganglion cells, the neurons that form the axons of the optic nerve. Methylprednisolone (Mpred), the standard treatment for autoimmune optic nerve inflammation, targets the inflammatory component of the disease but does not influence the extent of neurodegeneration.

Our project aims to develop a neuroprotective add-on therapy in patients with optic neuritis. Therefore, we initiated a double-blind, placebo-controlled clinical trial which tests the neuroprotective potential of erythropoietin (EPO) administered during the acute phase of optic nerve inflammation. In this trial, patients are randomly assigned to one of the following treatment groups:

Treatment group	Mpred dosing regimen	Add-on regimen
E (EPO)	1000 mg in 250 ml normal saline given i.v. once daily for 3 days	3.3 x 10 ⁴ IU recombinant human EPO given i.v. once daily for 3 days
S (Saline)	1000 mg in 250 ml normal saline given i.v. once daily for 3 days	Normal saline given i.v. once daily for 3 days

The primary objective of this study is to determine the efficacy of EPO in combination with Mpred on nerve fibre loss in the optical nerve head. Nerve fibre loss is measured by optical coherence tomography using the change in retinal nerve fibre layer thickness around the optical nerve head and the change of neural tissue volume in the optical nerve head itself. The measurements are performed at baseline, week 4, week 8 and week 16.

Secondary objectives include:

- Visual acuity determined at weeks 1, 4, 8, and 16 compared with baseline.
- Visual field perception determined by automated perimetry at weeks 1, 4, 8, and 16 compared with baseline.
- Optic nerve atrophy assessed by volumetric MRI data. MRI measurements are performed at weeks 4, 8, and 16 compared with baseline.
- Recovery of latency and amplitudes of visual evoked potentials. Electrophysiological measurements are performed at weeks 4, 8, and 16 compared with baseline.

Additional study objectives are to determine the safety and tolerability of EPO in combination with Mpred in subjects with optic neuritis/MS.

Up to now, 9 patients have completed the follow-up period of 16 weeks. The efficacy and safety data will remain blinded until 40 patients have finished the study.

In order to shorten the time of recruitment and improve the scientific value of the study, we plan to open a second study centre at the Department of Neurology, Saarland University, Germany.

7 Progress reports of RoFAR award winners

Prof. Margaret H. Baron
(Cycle V)



Mount Sinai School of Medicine, New York, USA

Regulation of red blood cell enucleation

Using a transgenic mouse system in which green fluorescent protein (GFP) is expressed exclusively in primitive erythroid cells (EryP), we have found that EryP progress through previously unrecognised stages leading to their maturation¹. EryP proved to be a stable population present throughout gestation¹ and display distinct cell surface phenotypes on enucleated versus nucleated cells¹. They are also present in erythroblastic islands (EBIs) of the foetal liver (FL), where they upregulate a number of cell adhesion molecules (unpublished). We hypothesise that terminal steps in EryP maturation, including enucleation, occur in the FL EBIs and involve adhesive interactions with macrophages. As the FL is little more than a rudiment until approximately E11.5, this hypothesis provides a simple explanation for the finding that EryP do not enucleate before E12.5^{1,2}, even though they begin to form and enter the circulation much earlier (around E7.5 and E9.5, respectively). Interestingly, several cell adhesion molecules that are not detected on EryP at E9.5 begin to be upregulated by E12.5. We propose that upregulation at later stages, around the time the FL becomes a haematopoietic organ, allows EryP to enter the liver and become incorporated into EBIs, where they complete the final steps of maturation, leading to enucleation. Consistent with a function for cell adhesion molecules in EryP-macrophage interactions, E12.5 EryP bind more strongly to FL macrophages than do E9.5 EryP.

We proposed that the transcription factor erythroid Krüppel-like factor (EKLF) is a major regulator of EryP maturation. EKLF-deficient EryP are developmentally arrested, as indicated by Giemsa staining and FACS analysis. Nuclei condense but are not extruded from the deformed cells. These defects could be due, in part, to abnormalities in cell adhesion and cytoskeletal structure and function. Consistent with our preliminary data, a microarray analysis identified gene encoding membrane proteins CD71 (TfR), CD24a and ICAM-4 that fail to be upregulated normally in E12.5 FL in the absence of EKLF³. The maturational arrest is reflected in our simultaneous

FACS analysis of CD71 and Ter119: erythroblasts are almost exclusively confined to the progenitor populations corresponding to regions R1 and R2. This observation is consistent with previous reports that EKLF is essential for the last stages of erythroid differentiation but dispensable for the expansion of erythroid progenitors³⁻⁵. EKLF mutant EryP show greatly reduced expression of several cell adhesion molecules on their surface. In contrast, essentially wild-type levels of CD147 and CD55 were detected on the mutant cells. Thus, a subset of cell surface proteins is affected in the absence of EKLF.

References

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7 Progress reports of RoFAR award winners

Dr Michael Bulger (principal applicant)

Dr James Palis (co-applicant)

(Cycle V)



University of Rochester (New York), USA

Function of Sox6 in β -globin gene silencing and definitive erythropoiesis

Our work has focused on identifying motifs within the transcription factor Sox6 that are required for silencing of ϵ -globin transcription. We used a transient co-transfection assay for this purpose, in which a luciferase reporter under the control of the ϵ -globin promoter is transfected into mammalian cells along with an expression vector for Sox6 or one of a series of deletion mutants we have engineered for this factor. Using this assay, it was shown previously that deletion of the HMG-box DNA-binding domain of Sox6 eliminated silencing activity at the ϵ -globin promoter; we have systematically deleted regions that collectively encompass the remainder of the protein. Amazingly, no single deletion abrogated ϵ -globin silencing by Sox6. There are three possible explanations for this: 1) the necessary motif falls within one of the small (5-8 amino acid) gaps between the deletions we tested; 2) Sox6 interactions with co-repressors are redundant, so no single region is required for silencing or 3) the transient co-transfection assay does not accurately model the silencing of ϵ -globin by Sox6 as originally defined by genetic studies. We are currently engineering more extensive deletions of Sox6, including a mutant consisting solely of the DNA-binding domain, to test these possibilities.

The long-term purpose of identifying interaction motifs within Sox6 was to use non-functional mutants as controls for purification of FLAG-tagged Sox6 along with its interacting factors; that is, we hoped to eliminate factors that associate with both wild-type and non-functional mutant Sox6 as candidates for co-repressors. While this would be helpful, it is not strictly necessary, so we have proceeded to isolate murine erythroleukemia (MEL) cell lines expressing FLAG-tagged Sox6, with the ultimate aim of purifying Sox6 and identifying factors that co-purify with it by mass spectrometry. We are currently evaluating FLAG-Sox6 expression levels in a number of these lines to identify sub-clones that

express enough FLAG to be useful in purification, but not too much, in order to minimise abnormal interactions (i.e. 50–100% of endogenous Sox6).

As an alternative to FLAG-tag purification, we have also engineered a Sox6 protein linked to the Invitrogen capTEV tag, a motif that is biotinylated in mammalian cells and allows purification of tagged proteins using streptavidin beads. The major advantage of this procedure is that the biotin-streptavidin interaction is so strong, and thus purification so efficient, that even lower expression levels of the Sox6 protein (5–20% of endogenous) can be useful, thus minimising spurious results due to overexpression.

Finally, we have attempted to delete the erythroid-specific Sox6 promoter from the endogenous Sox6 locus by homologous recombination in murine embryonic stem cells. The purpose of this is twofold: 1) to demonstrate conclusively that this promoter is entirely responsible for erythroid expression of Sox6 and 2) to derive a new mouse line in which Sox6 is absent solely from the erythroid lineage. Thus far, we have engineered the construct to replace the promoter with a selectable marker, but our first transfection resulted in no correct recombination events. A second attempt is ongoing.

Dr Herbert Y. Lin
(Cycle V)



Massachusetts General Hospital, Boston, USA

Regulation of iron metabolism by soluble haemojuvelin.Fc fusion protein

Haemojuvelin is a co-receptor for bone morphogenetic protein (BMP) signalling, and BMP signalling positively regulates hepcidin expression. In the first year of the RoFAR grant, we have shown that soluble haemojuvelin.Fc fusion protein (HJV.Fc) can selectively inhibit BMP induction of hepcidin expression *in vitro*, and that administration of soluble HJV.Fc decreases hepcidin expression *in vivo*. In normal mice treated with soluble HJV.Fc, we found an increase in splenic ferroportin protein expression, increased mobilisation of splenic iron stores, and increased serum iron levels, with transferrin saturation levels reaching 100%. In addition, soluble HJV.Fc can block IL-6-induced increases in hepcidin expression. These data support a role for modulators of the BMPs.

Specific Aim I

We have used soluble HJV.Fc to selectively regulate hepcidin expression induced by BMP/TGF- β superfamily members using a bio-inhibition assay. We found that soluble HJV.Fc could inhibit BMP2, BMP4 and BMP6 activity, but could not inhibit BMP7 or BMP9 activity. In the second year of the RoFAR grant, we will use a cell-free binding assay system using soluble HJV.Fc to determine the selectivity and affinity of soluble HJV.Fc for BMP family members.

Specific Aim II

We have purified milligram quantities of soluble HJV.Fc and treated normal mice to examine the effect on hepcidin levels and serum iron levels. We found that soluble HJV.Fc was a highly effective agent to inhibit hepatic BMP signalling, inhibit hepcidin expression, increase splenic ferroportin expression, mobilise splenic iron stores, and increase serum iron levels and serum transferrin saturation *in vivo*. In the second year of the RoFAR grant, we will treat anaemic mice with soluble HJV.Fc.

References

1. Babitt JL *et al.* Modulation of bone morphogenetic protein signaling *in vivo* regulates systemic iron balance. *Journal of Clinical Investigations* 2007; 117(7):1933-1939.

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Who is eligible for LOI submission?

RoFAR funds established members of academic institutions, dialysis units, and research centres. There are no age or geographical restrictions.

What kind of projects is RoFAR interested in?

RoFAR supports both clinical and basic science projects focused on anaemia related to kidney disease and oncology, effects of erythropoietin and erythropoietin-like substances as protective drugs in various organs, resistance to erythropoietin, anaemia of chronic disease, anaemia related to congestive heart failure and stroke, biology of anaemia and outcomes. RoFAR especially encourages innovative research that will open new avenues of exploration in the study of anaemia, its mechanisms and outcomes.

What will I need to provide RoFAR with if my project is funded?

Funds are paid in three instalments over a maximum of 2 years and are dependent on the delivery of an interim and a final report for public use. Additionally, RoFAR must be acknowledged in publications, on posters, etc. Applicants may be asked to attend events organised by RoFAR and present their results.

Are budget indications approximate or am I committed to them?

RoFAR assigns funds to awarded projects based on budget details given. It is not possible to renegotiate the amount after project approval. Indirect costs (institutional overhead, insurance, etc.) are the responsibility of the applicant. A maximum of 10% of the assigned funds can be used for the indirect costs.

Am I allowed to submit more than one project to RoFAR?

Applicants are allowed to hold only one grant at a time. Furthermore, you may not submit more than one LOI in the same cycle. This rule holds both for principal and co-applicants.

www.rofar.org



What kind of assistance does RoFAR give to awarded applicants?

The purpose of RoFAR is to provide awarded applicants with funds for the submitted project and to share outcomes with the scientific community. RoFAR will not provide any administrative assistance or scientific consultancy, nor recommend any preferential channels for the purchase of drugs or machinery necessary for the completion of the study.

Where can I find relevant information about RoFAR?

The RoFAR website (www.rofar.org) is the main channel of information, where you can find important announcements, future deadlines, submission forms, the RoFAR charter and regulations, as well as progress reports and funding history. If you have any specific questions, please do not hesitate to contact the secretariat (admin@rofar.org).

Projects are submitted electronically via our website**Projects are submitted as Letters of Intent (LOI)**

Submissions twice per year
(June and November)

You are asked to provide your personal details, indications about the budget, a short description of your experience and of the submitted project (latter two limited to 750 words). No figures, tables or extensive literature list can be submitted at this stage.

LOIs are evaluated by a Board of Scientific Advisors

6–9 weeks

LOIs are thoroughly reviewed by 3 members of the Scientific Advisory Board and judged based on relevance to RoFAR, originality, scientific excellence and feasibility. Applicants are informed of the outcome 6–9 weeks after submission. Declined applications are not provided with any feedback from the reviewers.

Top-ranked applicants are invited to submit a full application

4–6 weeks

Based upon the Scientific Advisors' evaluation, top-ranked applicants are invited to submit a full application with an approximate 50% chance of funding. Sample forms and guidelines are available in the Download section of the RoFAR website. Usually, 4–6 weeks are given for submission. Only completed applications are accepted and the stated deadline is final.

Full applications are evaluated by a Board of Scientific Advisors

8–10 weeks

Applications are thoroughly reviewed by at least 3 Scientific Advisors and judged based on relevance to RoFAR, originality, scientific excellence and feasibility. The Board of Trustees selects the projects to be granted based upon the evaluations made by the Scientific Advisors. Applicants are informed about the outcome 8–10 weeks after submission of the full application.

Notes



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