



**RoFAR**  
Foundation for Anemia Research

# *Bi-annual report*

July 2007

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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 CHF four million annually for at least four years from inception to a total of at least CHF 16 million. The RoFAR is a non-profit, autonomous and legally independent charitable organisation.

The RoFAR encourages the exploration of new 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 outcomes

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# 1 Preface

On behalf of the RoFAR Board of Trustees, it is my pleasure to announce that a total of CHF 1.2 million in grants have been awarded to six outstanding research projects in the second half of 2007.

Six applications received during the sixth cycle of competition have been selected to receive RoFAR research grants of up to 200,000 CHF each, distributed over two years. As in the first two years of RoFAR, the high expectations for the quality of research projects and applicants have been met. Since 2004, RoFAR has awarded thirty-nine regular grants and one special cycle grant, totalling over CHF 8.3 million or USD 6.6 million.

There are two cycles of RoFAR awards each year. Timelines for the cycles and the submission deadlines for applications are published on the Foundation's website ([www.rofar.org](http://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 is similar to the one for regular grants. This 2007 special grant recipient will be announced at the end of the year.

To inform a broader scientific community about the funding opportunities RoFAR provides, our promotional campaign in 2006 and the first half of 2007 has included

- advertisements on major nephrology, oncology and cardiology web portals
- distribution of brochures to major nephrology, oncology and cardiology centres
- media release about the first special cycle award granted to Dr. N. Eliopoulos

- an information booth at the World Congress of Nephrology (WCN) held in Rio de Janeiro
- advertisements in major scientific journals
- increased presence at targeted scientific congresses in the form of posters and leaflet distribution

RoFAR had the pleasure of sponsoring a scientific symposium at the WCN in Rio de Janeiro on 24 April 2007, at which four nephrology-relevant RoFAR-awarded projects were highlighted. In the near future, RoFAR plans to continue presenting its programme at a number of scientific congresses.

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. 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 F. Hoffmann-La Roche Ltd.

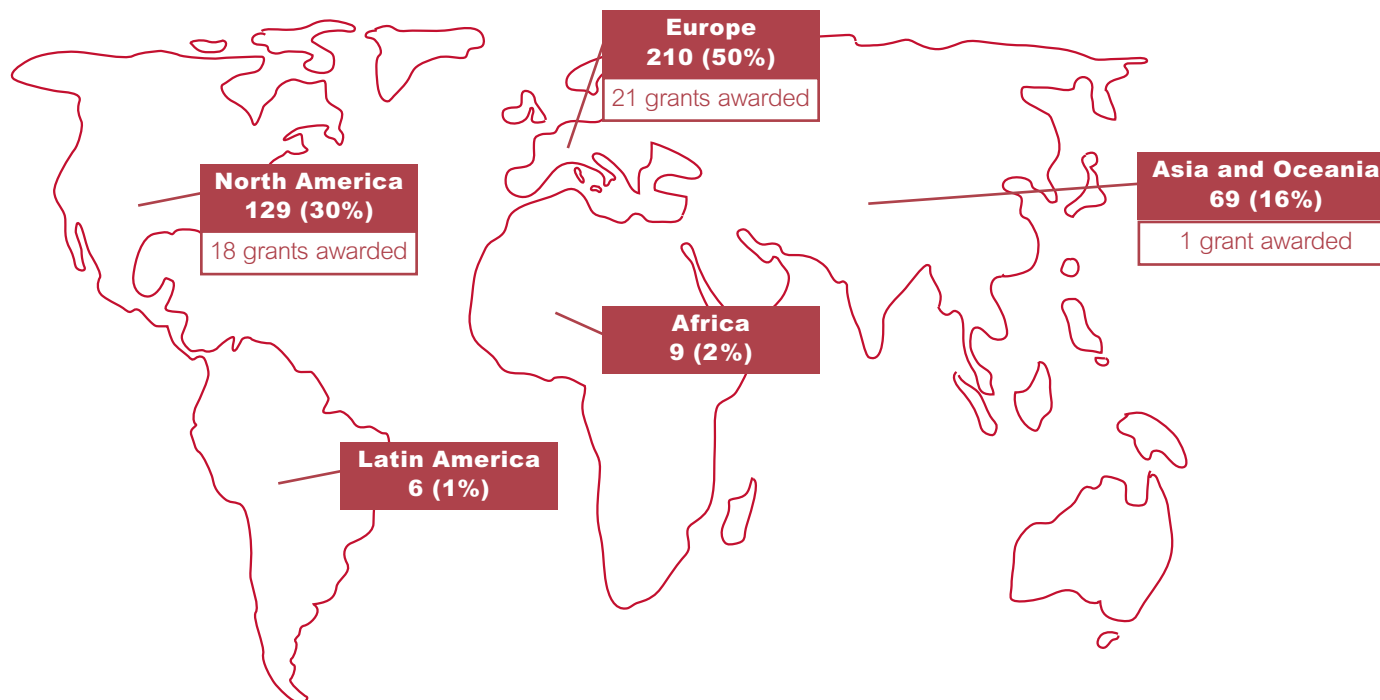
RoFAR welcomes any feedback or suggestions to assist us in accomplishing our stated mission.

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 seven cycles of competition including a special cycle represent a range of institutions in 44 countries. Half (50%) of all the LOI applications have been submitted from Europe, primarily from Germany, UK, Italy, Switzerland and Israel. One third of the applications have been submitted from the United States and Canada. About 25% 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 (48%), animal trials (43%) and basic science projects. Submitted projects focus on nephrology and diabetology (41%), haematology (44%), oncology (16%) and cardiology (13%) with some overlap between areas.

In cycles I to VI, twenty-one regular grants have been assigned to European applicants, seventeen to North American applicants and one to Oceania (Australia). One special grant has been awarded to a North American applicant. Cycle VII and special cycle 2007 grants have not yet been awarded.

**Submitted research proposals by**

**Study type**

Human trials	(48%)
Animal studies	(43%)
Others	(41%)

**Research focus**

Nephrology	(41%)
Haematology	(44%)
Oncology	(16%)
Cardiology	(13%)
Others	(22%)
(multiple allowed)	

**Gender of main applicant**

Males	(75%)
Females	(25%)

**Institution type**

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

### 3 Overview of awarded grants

				<b>Grant awarded</b>	<b>Progress report published</b>	<b>Final report published</b>
<b>Cycle I</b>	<b>Nancy C. Andrews</b>	Children's Hospital Boston, USA	Hepcidin regulation in the anaemia of chronic disease	11/2004	07/2006	07/2007
	<b>Martin W. Bergmann</b>	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	
	<b>Andrew McKie</b>	King's College, London, UK	Characterisation of a novel intestinal haem transporter	11/2004	07/2006	12/2006
	<b>Marco Merlano</b>	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
	<b>Peter Mertens</b>	University Hospital Aachen, Germany	Mechanisms for erythropoietin resistance in transformed and non-transformed cells	11/2004	07/2006	07/2007
	<b>Chris D. Vulpe</b>	University of California, Berkeley, USA	Characterisation of a family of putative mammalian haeme chaperones	11/2004	07/2006	07/2007
<b>Cycle II</b>	<b>Hans Ulrich Bucher</b>	University Hospital of Zurich, Switzerland	Erythropoietin reduces brain, eye and lung damage in very preterm infants: Proof-of-concept study	05/2005	07/2006	
	<b>Edward Debnam</b>	Royal Free & University College Medical School, London, UK	Is inflammation an important factor in the anaemia of chronic renal failure?	05/2005	07/2006	
	<b>Diana Gilligan</b>	Puget Sound Blood Center, Seattle, USA	Regulation of gene expression during erythropoiesis	05/2005	12/2006	
	<b>Alexander Maxwell</b>	Queen's University Belfast, UK	Investigation of the role of JUNE-1 in erythropoiesis	05/2005	12/2006	07/2007
	<b>Jun-ichi Nishimura</b>	Duke University Medical Center, Durham, USA	Innovative drug design using RNA aptamers for various anaemias	05/2005	12/2006	
	<b>Radek Skoda</b>	University Hospital Basel, Switzerland	The role of SMAD4-dependent signalling in anaemia	05/2005	12/2006	07/2007
	<b>Carole Soussain</b>	Oregon Health and Science University, Portland, USA	Neuroprotective effect of erythropoietin on chemo- and radiotherapy-induced toxicity	05/2005	07/2006	07/2007
	<b>Christina Warnecke</b>	University Erlangen-Nürnberg, Germany	Molecular mechanisms underlying the hypoxic induction of erythropoietin by HIF-2 $\alpha$	05/2005	12/2006	
<b>Cycle III</b>	<b>Max Gassmann</b>	Vetsuisse, University Hospital of Zurich, Switzerland	The impact of erythropoietin on the hypoxic ventilatory response of mouse and man	11/2005	12/2006	
	<b>Peter J. Kirkpatrick</b>	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
	<b>Véronique Lefebvre</b>	Cleveland Clinic Foundation, USA	Roles of Sox6 in erythropoiesis	11/2005	07/2007	
	<b>Stephen Leib</b>	University of Berne, Switzerland	Effect of erythropoietin on brain injury and regeneration in bacterial meningitis	11/2005	07/2007	
	<b>Barbara Scheiber-Mojdehkar</b>	Medical University of Vienna, Austria	Recombinant human erythropoietin: A new treatment for Friedreich's ataxia	11/2005	07/2007	
	<b>Jürg Schifferli</b>	University Hospital Basel, Switzerland	Erythropoietin or erythrocyte transfusion for anaemia?	11/2005	07/2007	
	<b>Marcela Votruba</b>	Cardiff University, UK	Erythropoietin neuroprotection in retinal neurodegeneration	11/2005	07/2007	

### 3 Overview of awarded grants

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

## 4 Grant awards in Cycle VI

**Prof. Nancy C. Andrews**



### **Children's Hospital Boston, USA**

#### *Regulation of hepcidin expression*

Genetic haemochromatosis is an iron overload disease that results from mutations in genes encoding hepcidin, ferroportin, HFE, transferrin receptor-2 (TFR2) and haemojuvelin (HJV). Hepcidin is a circulating peptide hormone that controls the activity of ferroportin, a cellular iron exporter. This control mechanism governs both dietary iron absorption and body iron distribution. HJV acts as a bone morphogenetic protein (BMP) co-receptor to stimulate hepcidin expression. However, the functions of HFE and TFR2 have remained enigmatic. Our preliminary results suggest that HJV, HFE and TFR2 interact to form a protein complex in transfected cells. HFE over-expression amplifies BMP signalling. Over-expression of TFR2 inhibits the cellular secretion of a soluble form of HJV that acts as a negative regulator of hepcidin expression. The studies proposed in this application will extend that work, and test the hypothesis that disease-associated mutations in HJV, TFR2 and HFE interfere with formation of the complex and/or its biological activities. This work will provide new insight into how hepcidin expression is regulated. This, in turn, will add to our understanding of the anaemia of chronic disease, a prevalent disorder attributable to excess hepcidin production in response to inflammation. Ultimately, a better understanding of HJV, TFR2 and HFE as potential therapeutic targets may lead to new treatments for iron overload disorders, iron deficiency disorders and the anaemia of chronic disease.

**Dr Mark D. Fleming**



### **Children's Hospital Boston, USA**

#### *The genetics of erythroid haem and iron metabolism*

Developing red blood cells need to make large amounts of haemoglobin, the oxygen carrying substance in the blood. Haemoglobin is comprised of a protein component, globin, and the red pigment haem, which is formed from the metal iron and a chemical compound called protoporphyrin IX (PPIX). All three - globin, iron, and PPIX - must be made or acquired in the proper proportion to make haemoglobin efficiently. A deficiency of any component leads to too little haemoglobin in the blood - anaemia - in which the red blood cells are small and pale. These so-called hypochromic, microcytic anaemias are among the most common diseases in humans, and include iron deficiency anaemia and the inherited globin disorders collectively called the thalassaemias. In order to meet the demand for globin and haem, red blood cell precursors have a number of proteins dedicated to their acquisition and synthesis that are turned on by other proteins during the course of red blood cell development. My laboratory is interested in understanding the basic physiology of haemoglobin production. In particular, we have focused on how red blood cells acquire iron and make PPIX in sufficiently large amounts to make the haem required for haemoglobin. To do so, we have used a technique called positional cloning to find the mutated genes and proteins responsible for inherited hypochromic, microcytic anaemias in mice. In this proposal we describe the anaemia and the gene responsible for a new mouse hypochromic, microcytic anaemia mutant that appears to have a defect in the early stages of PPIX biosynthesis; the anaemia is due to a mutation in a completely novel gene that may control the haem biosynthetic pathway in red blood cell precursors. Here, we propose experiments to determine the specific function of this protein in haem biosynthesis.

## 4 Grant awards in Cycle VI

**Prof. David Johnson**



### **Princess Alexander Hospital, Brisbane, Australia**

#### *A randomised, placebo-controlled trial of oxpentifylline on haemoglobin levels in patients with erythropoietin-resistant anaemia*

Low red blood cell counts (anaemia) occur in the vast majority of patients with chronic kidney disease. In most cases, these low red blood cell counts respond to treatment with medications, such as erythropoietin (Eprex®) or darbepoietin alpha (Aranesp®). However, about 10-15% of patients continue to be anaemic even with appropriate treatment. Such patients are at increased risk of being hospitalised, developing complications such as heart failure and death. A number of small studies have suggested that a drug called oxpentifylline (Trental®) is very good at safely raising red blood cell counts in persistently anaemic patients. However, this drug has not yet been properly tested in a controlled trial (that is, where the drug is given to one group of patients, but not another).

Eligible patients who choose to participate will receive either the oxpentifylline treatment or a placebo. Which treatment will be determined by random chance (similar to the toss of a coin) and neither the patient nor the doctor will know whether the treatment is oxpentifylline or a placebo. Apart from receiving oxpentifylline or placebo, usual treatment will not be affected in any other way. Information about blood test results, blood pressure, weight, any drug side effects, as well as limited clinical information will be recorded. The study will involve 4 months of daily treatment.

**Dr Zvonimir S. Katusic**



### **Mayo Clinic, Rochester (Minnesota), USA**

#### *Role of antioxidant enzymes in vasculoprotective effect of erythropoietin*

Erythropoietin (EPO) is widely used as a major therapeutic drug in the treatment of anaemia. The scientific community has recently recognised that EPO has beneficial effect in the prevention and treatment of diseases other than anaemia. Diseases of blood vessels are a major cause of mortality and disability. The inner lining of the blood vessel wall (endothelium) plays a key role in regulation of blood supply to the tissues. Previous studies suggest that EPO has beneficial effects on endothelial function. However, the exact mechanism of this effect is not completely understood. Experiments proposed in this project are designed to determine whether EPO stimulates repair of the injured blood vessel wall and what mechanisms are responsible for the effects of EPO. It is anticipated that the results may help to better understand the effects of EPO on the cardiovascular system in patients who are treated with this drug. Improved understanding of the effects of EPO may also help to harness the therapeutic value of EPO in the prevention and treatment of cardiovascular disease, including myocardial infarction and stroke.

## 4 Grant awards in Cycle VI

**Dr Frank S. Lee**



### **Univ. of Pennsylvania School of Medicine, Philadelphia, USA**

#### *Prolyl hydroxylase domain protein 2, a physiologic regulator of erythropoietin*

Recombinant erythropoietin (EPO) has proven to be a remarkably effective treatment for certain types of anaemia, such as that associated with chronic kidney disease or chemotherapy. If one were to be able to increase endogenous EPO, for example by controlling the activity of factors that regulate EPO, one might be able to avoid the necessity of EPO injections and routine clinic visits. This necessitates a detailed molecular understanding of the factors that regulate endogenous EPO levels and haematocrit. A family with abnormally high haematocrit was recently identified as having a novel mutation in a protein known as PHD2. This points to PHD2 as being a physiologic regulator of EPO and haematocrit in humans. We propose examining a mouse model in which the gene encoding this protein can be inactivated to test whether this leads to increased EPO and haematocrit. This would be an independent, and indeed, critical test of the physiologic role of PHD2 in red blood cell production. Should loss of PHD2 lead to increased haematocrit, it would make targeting this pathway and this particular protein a possible means to treating anaemia.

**Dr 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*

Erythropoietin (EPO) is a secreted cytokine with neuroprotective activity. Injection of EPO into the vitreous of the eye protects ganglion cells from axotomy-induced cell death. However, subretinal injection of AAV-EPO does not protect the photoreceptors from cell death induced by light-damage. Surprisingly, intramuscular delivery of AAV-EPO was neuroprotective to the photoreceptors. This implies that either EPO protected the photoreceptors via an indirect mechanism (i.e. increased oxygen), or that EPO produced in the retina is non-functional, while EPO produced in the muscle is functional. The fact that the ganglion cells are protected by direct injection of EPO would imply that there is a direct neuroprotective effect of EPO on retinal neurons. In addition, a recent study demonstrated that the isoelectric focusing pattern of EPO produced in the retina is different from that produced in the muscle. Therefore, the goal of the proposed study is to determine the differences between EPO produced in the retina and that produced in the muscle after transduction with AAV-EPO and to determine the ideal form of EPO to protect retinal neurons from degeneration. The sugar moieties on EPO are altered depending on the tissue from which it is expressed, and this, in turn, alters the ability of EPO to activate its receptor. Serum and anterior chamber fluid samples will be collected from primates previously injected with AAV-EPO. The samples will be analysed biochemically for differences in sugar moieties. Then, forms of EPO containing different amounts of glycosylation will be injected into the eyes of retinal degeneration mice to assess for neuroprotection by histological and physiological analysis. Finally, gene therapy will be optimised in two models of retinal degeneration using AAV to deliver a mutant form of EPO that has been shown to be neuroprotective, but not erythropoietic.

## 5 Final reports of RoFAR award winners

**Prof. Nancy C. Andrews**  
(Cycle 1)



### Children's Hospital Boston, USA

#### *Hepcidin regulation in the anaemia of chronic disease*

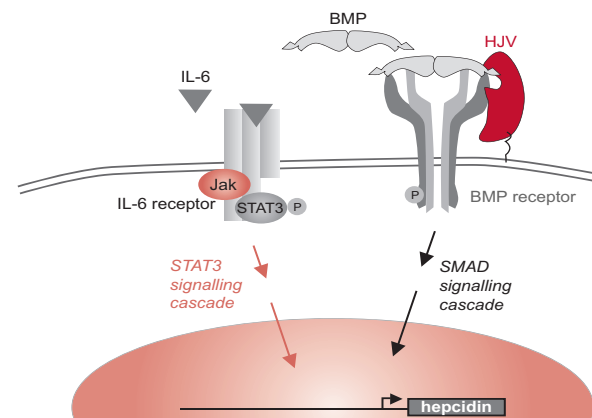
The anaemia of inflammation is an acquired condition that affects patients with a variety of disorders including infection, arthritis, inflammatory bowel disease, trauma, organ failure and cancer. It causes a measurable decrease in quality of life and general well-being. Hepcidin is a central mediator of the anaemia of inflammation. It is a circulating hormone that is induced by inflammation. Hepcidin blocks the release of iron from macrophages and interrupts intestinal iron absorption. Recently, we showed that mice expressing an inducible hepcidin transgene can serve as a faithful model of the anaemia of inflammation<sup>1</sup>.

Our studies funded by RoFAR have focused on understanding how hepcidin production is controlled. In the first year of this award, we described the molecular events involved in the induction of hepcidin expression in response to inflammation. We showed that interleukin-6, an inflammatory cytokine, triggers a JAK/STAT signalling cascade resulting in the binding of STAT3 to the hepcidin promoter<sup>2</sup>. Over the past year we have asked how other proteins might be involved in hepcidin regulation. We have focused on HFE, transferrin receptor-2 (TFR2) and haemojuvelin (HJV). Each of these proteins has been identified as a target of mutations that cause the genetic iron overload disease haemochromatosis.

In collaboration with Drs. Jodie Babitt and Herbert Lin, we showed that HJV acts as a co-receptor for bone morphogenetic protein (BMP) signalling<sup>3</sup>. We asked whether HFE and TFR2 might also be involved in that signalling pathway. Using a cell transfection system, we found that HFE, like HJV, stimulated transcription from an artificial promoter containing tandem BMP regulatory elements, but TFR2 did not have this property (unpublished data). Furthermore, HFE, but not TFR2, also stimulated transcription from a reporter construct including the normal human hepcidin promoter (unpublished data). We demonstrated that tagged forms of HFE and HJV formed a protein complex that could be isolated from transfected

cells (unpublished data). In a parallel line of experiments, we found that HFE and TFR2 also form a protein complex<sup>4</sup>. TFR2 appears to serve the function of removing HFE from an interaction with the homologous protein TFR1, particularly when ambient diferric-transferrin concentration is elevated<sup>4</sup>. Finally, we showed that HJV and TFR2 also interact (unpublished data).

Taken together, these results suggest possible mechanisms by which HFE, TFR2 and HJV participate in the regulation of hepcidin expression. Future studies will be aimed at fully understanding the molecular function of this complex. In addition, following up on recent studies of hepcidin regulation in response to ineffective erythropoiesis<sup>5</sup>, we hope to understand how iron homeostasis is regulated in response to systemic signals. Ultimately, we anticipate that a full understanding of hepcidin regulation will lead to new treatment strategies for the anaemia of inflammation and other disorders of iron homeostasis.



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## 5 Final reports of RoFAR award winners

**Dr Marco Merlano (principal applicant)**

Dr Silvana Ungari (co-applicant)  
(Cycle I)



### S. Croce General Hospital, Cuneo, Italy

#### *In vitro analysis of tumour response to radiation in oxic and hypoxic conditions*

Anaemia frequently occurs in head and neck squamous cell carcinoma (HNSCC) and has been associated with decreased quality of life, impaired treatment outcomes and shortened survival. Furthermore, anaemia is a causative factor of tumour hypoxia, which compromises the efficacy of radiotherapy (RT). In order to circumvent the negative effects of anaemia, several attempts have been made including the use of erythropoietin (EPO), particularly in HNSCC. However, recent clinical data suggest a detrimental effect of EPO on survival of HNSCC patients treated with RT plus EPO.

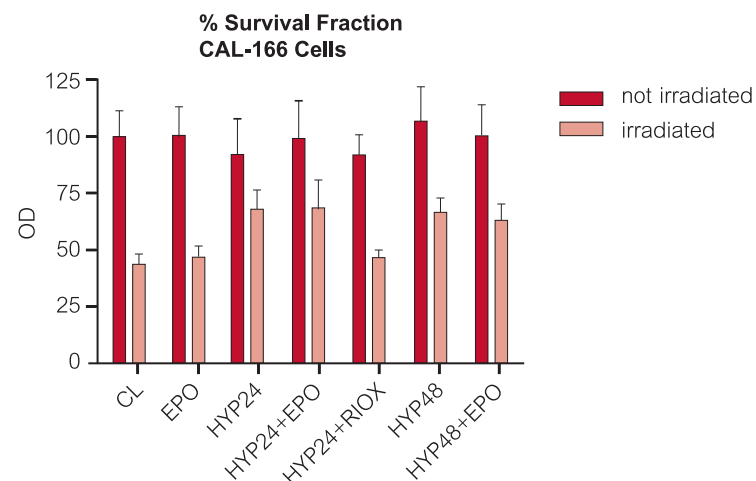
The HNSCC CAL-166 cell line, which is wild type p53 and expresses the EPO receptor (EPOR), was used in the present study to examine the effects of EPO on the cellular response to RT using a MTT test.

Extended studies were performed to standardise the experimental conditions (time of exposure to hypoxia and time to return of normal culture conditions) and the administration of radiation (fractionated or in single dose) to find the optimal ones in which the effect of the hypoxia on the radioresistance is most pronounced and the data obtained are likely to be more effective. Cells were exposed to an x-ray source at escalating doses from 2-20 Gy and showed a satisfactory dose-response curve. Hypoxia was mimed using a "Bug-Box" anaerobic workstation for 24-48 hours. After a single RT dose of 10 Gy, our results confirmed the negative effect of hypoxia, which is directly related to the duration of hypoxia (surviving cells when irradiated in normoxia,  $43.95 \pm 4.24\%$ ; when irradiated after 24 hours of hypoxia,  $62.4 \pm 8.3\%$ ; or after 48 hours of hypoxia, surviving cells  $70.8 \pm 7.4\%$ ).  $P = 0.001$  versus RT in normoxia. (Figure 1)

The addition of EPO, from 24 hours before RT to 72 hours after, until MTT test, did

not modify the cytotoxic effect of RT in hypoxic, as well as in oxic conditions. These preliminary data do not support a direct negative effect of EPO when treating EPOR positive tumour cells by irradiation.

In order to provide the molecular basis for better elucidating the effect of EPO in cancer patients under cytotoxic treatment, additional studies are ongoing. In particular, gene expression studies by quantitative RT-PCR and protein analysis by Western blot are currently being performed under the same experimental conditions in order to correlate the cellular data with the expression of EPOR, HIF-1 $\alpha$  and EGFR. Preliminary data, not yet presented, suggest that EGFR plays a major role in mediating radioresistance after EPO administration.



**Figure 1.** Amount of survival fraction, analysed by MTT test, after 10 Gy irradiation of CAL 166 cells in normoxia (CL), treated with EPO (30 U/ml) (EPO), after 24 hours of hypoxia (HYP24), after 24 hours of hypoxia plus EPO (HYP24+EPO), after 24 hours of hypoxia and then reoxygenated (HYP24+R10X), after 48 hours of hypoxia (HYP48), after 48 hours of hypoxia plus EPO (HYP48+EPO).

#### **Submitted for publishing**

The present data were submitted as an abstract entitled: "Impact of erythropoietin (EPO) on irradiation effect on the EPO receptor positive head and neck cancer", by Maffi M., Fischel J.L., Tonissi F., Milano G., Merlano M., Lo Nigro C. to the AACR (American Association for Cancer Research) Annual Meeting that was held in Los Angeles, 14-18 April 2007.

## 5 Final reports of RoFAR award winners

**Dr Peter Mertens**  
(Cycle I)



### University Hospital Aachen, Germany

#### *Mechanisms for erythropoietin resistance in transformed and non-transformed cells*

In our project we have focused on three main aspects:

#### **(i) Transcriptional regulation of Y-box protein-1 (YB-1) target genes and genes harbouring hypoxia-responsive elements (HRE)**

We evaluated YB-1 target genes putatively relevant in hypoxia-responses; example genes included matrix metalloproteinase-2 (MMP-2), SMAD7, epidermal growth factor receptor and DNA-polymerase- $\alpha$  (DPA). The promoter sequences of these genes were screened for YB-1 binding motifs including an incomplete *Y-box* (inverted *CCAAT-box*) and inverse repeat sequences (Figure 1). A similar motif is located within the HRE of the erythropoietin (EPO) gene. Detailed analyses revealed that YB-1 over-expression is accompanied by altered gene transcription<sup>1,2,4</sup>. DNA binding analyses revealed that the YB-1 protein impedes HIF-1 $\alpha$  binding to the HRE. YB-1 itself forms a tight complex with the HRE as demonstrated by testing with recombinant protein, supershift studies and competition analyses. These findings demonstrate for the first time that the transcription factor YB-1 is part of the HRE transcriptome<sup>3</sup>.

A *trans*-regulatory effect of YB-1 on the HRE was determined, the effect of which was highly dependent on the ambient oxygen tension and the cellular HIF-1 $\alpha$  content (Figure 2). Under normoxic conditions, the EPO-HRE was *trans*-activated 2-fold by forced YB-1 over-expression, whereas the *trans*-stimulatory effect was enhanced up to factor 10 under hypoxia. Taken together, our functional data on hypoxia-mediated gene regulation support the initial hypothesis of a crucial interplay between HIF-1 $\alpha$  and YB-1 in the context of the cellular "hypoxia programme".

#### **(ii) Evaluation of YB-1 subcellular shuttling and modifications dependent on oxygen-sensing**

Laser scanning microscopy was performed to assess the sub-cellular localisation of YB-1 under normoxia and hypoxia. A marked shift of endogenous YB-1, as well as GFP-tagged YB-1 protein from the cytoplasm into the nuclear compartment, was detected within 16 hours of hypoxia. This sub-cellular co-localisation of both proteins under hypoxia may be due to the occupancy of the same genes; alternatively, it may be due to direct protein interactions<sup>3</sup>. In preliminary experiments, such an interaction could not be confirmed by co-immunoprecipitation of recombinant HIF-1 $\alpha$  and YB-1 proteins. Indirect interactions of both proteins via adapter proteins such as CBP/p300 might also take place, as both proteins interact with CBP/p300.

#### **(iii) Setup of model systems to analyse the relevance of YB-1 in hypoxia-response of non-transformed and transformed cells**

Part of the planned project was to establish model systems with permanent *knockdown* of YB-1 expression. These model systems should ease the characterisation of YB-1 (and its crosstalk with HIF-1 $\alpha$ ) under hypoxia. In extensive analyses and by different approaches, we were able to effectively diminish YB-1 protein expression by up to 70% in transient transfection experiments. However, in numerous experiments with stable transfection of YB-1 siRNA, cells became senescent within 2 passages. These results indicate that the YB-1 protein is required for cell maintenance *in vitro*. When the cellular YB-1 content was transiently reduced in HepG2 cells, EPO gene transcription rates were down-regulated in normoxic and hypoxic cells by approximately 60%.

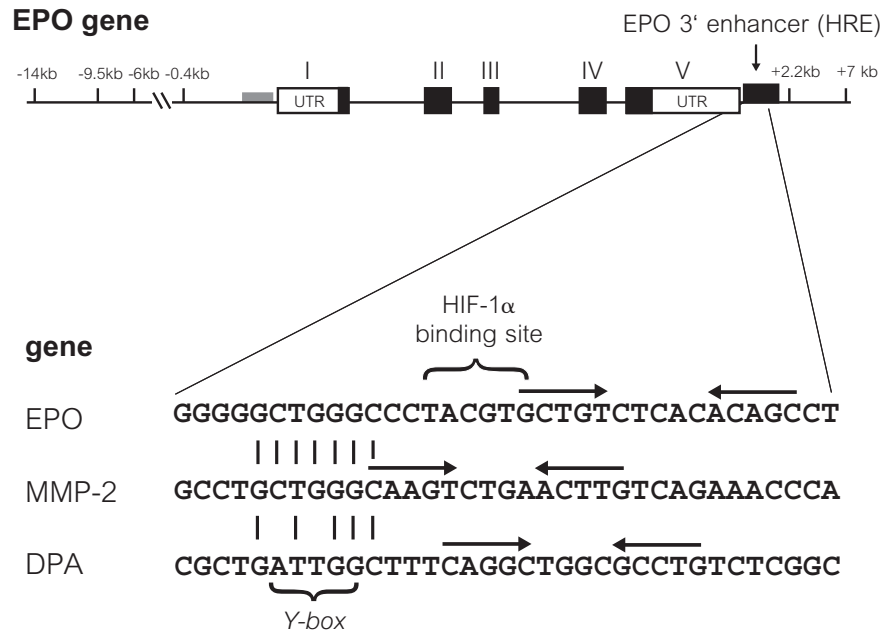
#### **Summary and outlook**

The described results establish YB-1 as a novel player in the hypoxia gene regulation programme. The binding characteristics indicate that YB-1 competes with HIF-1 $\alpha$  for the HRE sequence motif in most instances. Given the abundant expression of both proteins in tumour tissue and hypoxic inflammatory diseases, further assessment of their interplay *in vivo* will be of interest.

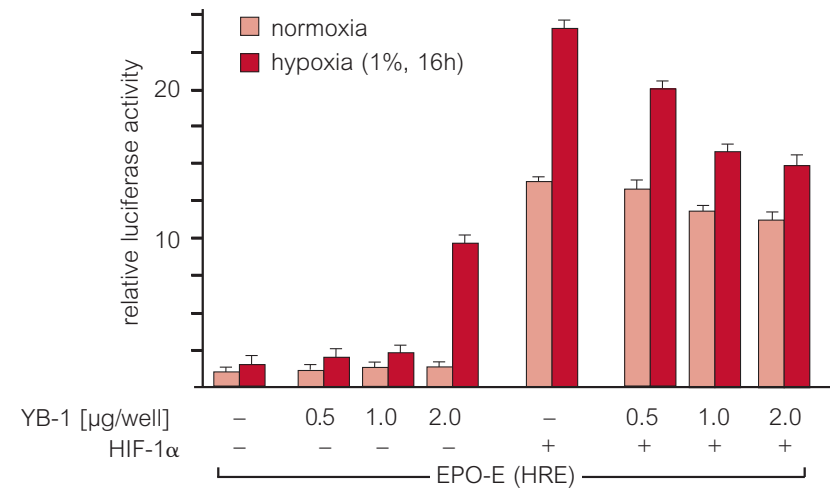
#### **References**

1. En-Nia A, *et al.* Serum-induced expression of the DNA-polymerase  $\alpha$  gene is mediated by transcription factor YB-1. *J Biol. Chem.* 2005; 280:7702-77012.
2. Dooley S, *et al.* YB-1 activation by IFN- $\gamma$  signalling leads to stimulatory and inhibitory effects on TGF- $\beta$ -dependent gene transcription. *J Biol. Chem.* 2006; 281:1784-1789.
3. Rauen T, *et al.* Y-box protein-1 is a master-switch for hypoxia-dependent gene regulation. (submitted)
4. Wu J, *et al.* Disruption of the Y-box binding protein-1 results in suppression of the epidermal growth factor receptor and HER-2. *Cancer Res.* 2006; 66:4872-4879.

## 5 Final reports of RoFAR award winners



**Figure 1. Y-box protein-1 binding elements within the hypoxia responsive element (HRE) of the EPO, MMP-2 and DPA genes.** The Y-box element and inverted repeat sequence are known binding motifs for YB-1.



**Figure 2. YB-1 trans-activates the HRE under hypoxia and interferes with the HIF-1 $\alpha$ -dependent trans-activation of the HRE.** YB-1 trans-activates the HRE in a concentration-dependent manner, up to 10-fold, under hypoxia. On the other hand the HIF-1 $\alpha$ -dependent trans-activation is diminished with forced over-expression of YB-1.

## 5 Final reports of RoFAR award winners

**Dr Chris D. Vulpe (principal applicant)**

Dr Ted Holman (co-applicant)

Dr Zhiwu Zhu (co-applicant)

(Cycle 1)

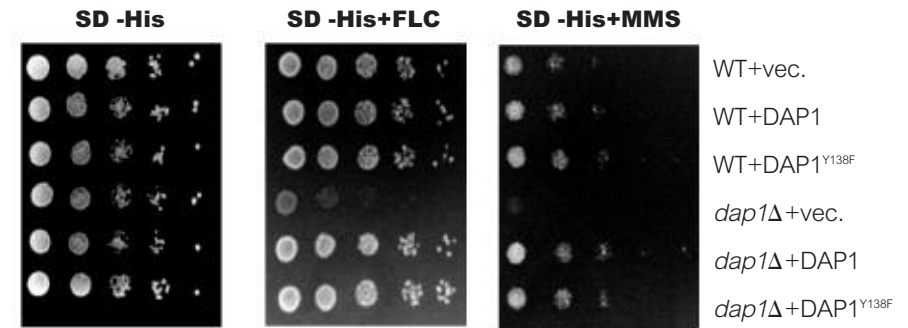


### University of California, Berkeley, USA

#### Characterisation of a family of putative mammalian haem chaperones

We hypothesised that yeast Dap1p is a haem chaperone which is responsible for the delivery of haem to haem proteins, such as the cytochrome p450 Erg11p, and that the mammalian homolog plays a similar role in mammalian cells. In the last progress report, we have described the characterisation of the haem binding of the Mammalian Cyk1p and Dap1p. We have found that these proteins have a cytochrome b5 haem-binding motif which lacks the classic histidine residues but rather binds haem via a conserved tyrosine residue (Y138 in Dap1p). We have shown that the *dap1Δ* mutant is sensitive to iron chelators (BPS) and others have demonstrated sensitivity of the *dap1D* mutant to mutagens and antifungal agents. We have shown that the mouse protein can functionally substitute for the yeast protein and rescue the iron chelator sensitivity phenotype of the *dap1Δ*.

We have shown that a tyrosine mutant of Dap1p, Y138F, could not rescue iron chelator sensitivity phenotype of the *dap1Δ*. We have now tested the ability of this mutant to complement the other phenotypes of the *dap1Δ* mutant (Figure 1). We found that the Y138F mutant was able to restore the wild type phenotype for sensitivity to Fluconazole (FLC) and methyl methanesulfonate (MMS). These results would suggest that haem binding is not essential for the Dap1p function as related to these phenotypes.



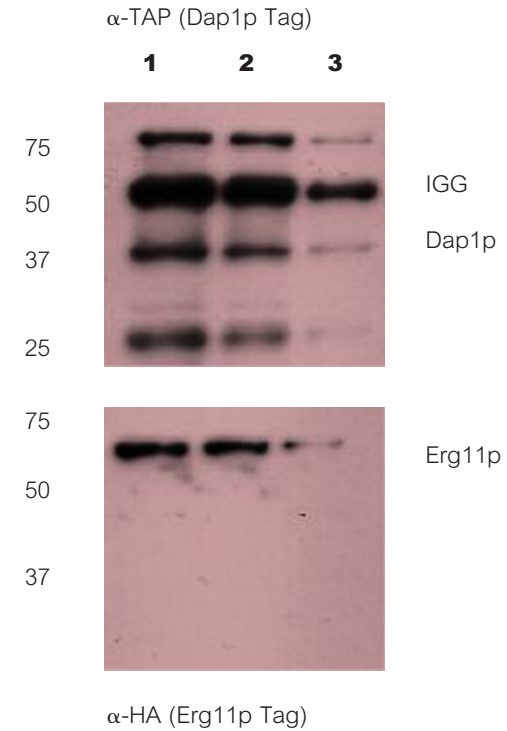
**Figure 1.** Dilution plate assay for growth on FLC and MMS. WT yeast (BY4743) with empty vector (WT+pRS313), WT yeast with vector expressing WT DAP1 Y138 WT (WT+DAP1), WT yeast with vector expressing mutant DAP Y138F (WT+DAP1<sup>Y138F</sup>), *dap1Δ* with empty vector (*dap1Δ* +vec.), *dap1Δ* with vector expressing WT DAP1 Y138 WT (*dap1Δ* +DAP1), or *dap1Δ* with vector expressing mutant DAP1 Y138F (*dap1Δ* +DAP1<sup>Y138F</sup>). Top left panel is SD-HIS, top middle is SD-HIS + 10 ug/ml FLC top right is SD-HIS + 3 % MMS.

We assessed whether Dap1p physically interacts with Erg11p by co-immunoprecipitation experiments (Figure 2). In order to carry out these studies, we created a yeast strain over-expressing Dap1p - TAP and Erg11p-HA. We performed an immunoprecipitation with either anti-TAP (Dap1p) or anti-HA (Erg11p) antiserum and then probed with antiserum to the other protein. As can be seen in Figure 2, our preliminary results indicate that Dap1p does co-IP with Erg11p which would be consistent with our hypothesis that Dap1p is a haem chaperone for delivery to Erg11p. We are currently confirming the identity of the immunoprecipitated proteins by sequencing.

Based on previously published work indicating decreased Erg11p protein levels in the *dap1Δ*, we decided to investigate whether Dap1p played a role in Erg11p protein stability. We developed a yeast strain lacking the endogenous Erg11p while expressing an inducible version of the gene. As a first step, we used this system to determine the effect of the presence or absence of Dap1p on the degradation rate of the Erg11p. Our preliminary results indicate that the degradation rates of Erg11p do not depend on Dap1p suggesting that increased degradation does not explain the previously observed decreased protein levels. We are currently investigating whether decreased Erg11p protein synthesis could play a role.

## 5 Final reports of RoFAR award winners

Our current studies are focusing on determining the  $K_d$  of haem-binding of both WT and Y138F Dap1. As mentioned above, Y138F does not bind haem, as isolated from *E. coli*. However, it does rescue yeast from both MMS and FLC. This indicates that either haem binding by Dap1p is not required for this function or that Y138F binds haem sufficiently in yeast such that it is still partly functional. We are currently investigating both possibilities. In addition, we are developing an assay to measure haem transfer from Dap1p to Erg11p directly using  $^{55}\text{Fe}$  labeled haem and native gel electrophoresis.



**Figure 2.** Western blot of immunoprecipitated Dap1p and Erg11 from yeast strain over-expressing both proteins. Decreasing amounts of immunoprecipitate with anti TAP antibody (specific for the DAP1p) were loaded in lanes 1, 2 and 3. The upper Western blot was blotted using anti-TAP and the lower Western blot using anti-HA (anti-Erg11p) antibody.

## 5 Final reports of RoFAR award winners

**Prof. Alexander Maxwell (principal applicant)**

Prof. Terence Lappin (co-applicant)  
(Cycle II)



### Queen's University Belfast, UK

#### *Investigation of the role of JUNE-1 in erythropoiesis*

This project has fully characterised a new erythropoietin-regulated gene, *JUNE-1* (now also known as *PHF23*) that was cloned by our group (Figure 1). The four main aims of the project have been achieved over the 2-year cycle of the grant.

#### **Aim 1: Examine the expression levels of *JUNE-1* splice variant**

- A. The expression of the splice variant follows a similar widespread tissue distribution pattern to full length *JUNE* with mRNA levels of the splice variant being approximately 40% lower than full length *JUNE*.
- B. Rat anti-mouse June monoclonal antibodies were successfully raised. The antibodies have been successfully used in immunoprecipitations and Western blot analysis to identify over-expressed June-1 and react with full length but not the splice variant June-1 isoform. Further optimisation of the novel antibodies for use in immunohistochemistry and Western blot detection of endogenous June-1 is on-going.

#### **Aim 2: Determine the cellular site of localisation of June-1**

- A. In Year 1, MEL cells proved too difficult to transfect, therefore, two alternative models were developed: the erythroleukaemia cell line K562 and the normal embryonic kidney cell line HEK293T. Both these models have been used successfully to analyse the localisation of June-1.
- B. A full length June-GFP fusion protein was expressed in K562 cells and exclusive nuclear expression of June-1 was clearly demonstrated (Figure 2). The alternatively spliced isoform of June-1 was successfully cloned into the NT-GFP vector and expressed in both K562 and HEK293T cells. The splice variant was also localised exclusively in the nucleus.

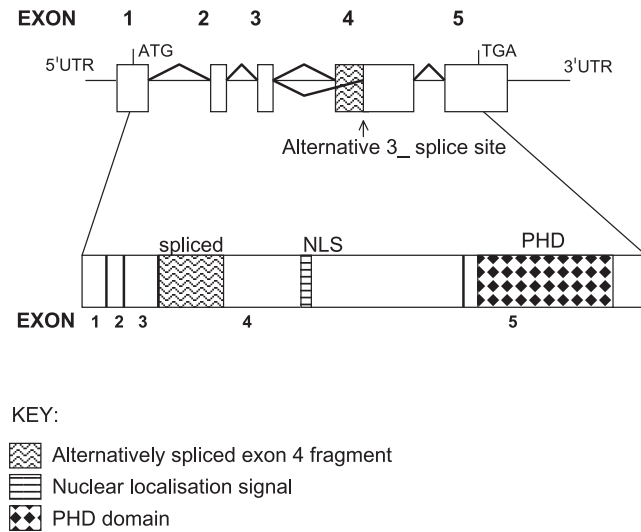
#### **Aim 3: Determine the effect of over-expression and under-expression of June-1 (siRNA knockdown) in a cell culture model of erythroid differentiation**

- A. June-1 and the alternatively spliced isoform have been successfully used for over-expression experiments in HEK293T cells to generate proteins for further analysis (see aim 4).
- B. Three *JUNE* siRNAs were tested alongside an appropriate scrambled control. Transfections were performed using Amaxa nuclear transfection system. Q-PCR demonstrates approximately 60% knockdown of *JUNE* mRNA. Results demonstrated a reduction in proliferation of K562 cells following *JUNE* knockdown.

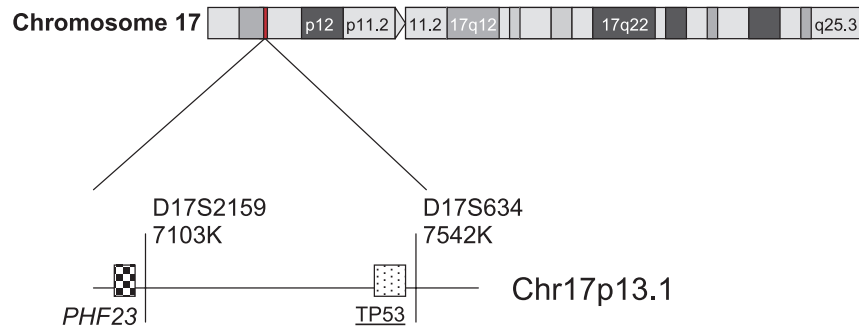
#### **Aim 4: Identify proteins that interact with June-1**

- A. In Year 1, generation of GST-tagged June was replaced by *in vitro* translation of June-1 yielding a FLAG-tagged protein and also mammalian expression of June-1 in HEK293T cells yielding a V5-tagged protein. These alternative systems, especially mammalian expression, were optimised in Year 2 and have proved invaluable in the study of June-1 interactions. June-1 protein has been successfully purified and used for mass spectrophotometry analysis.
- B. Co-immunoprecipitation assays:  
Four papers published *in Nature* in July 2006 suggested a subset of PHD-containing proteins, including ING2 and BPTF, recognised methylated histone residues, influencing transcription. The sequence of June-1, particularly the PHD domain, was aligned with ING2 and BPTF and the residues essential for methylated histone recognition were conserved. Further co-immunoprecipitation assays revealed that June-1 binds to histone H3, specifically trimethylated lysine 4 on Histone H3. Subsequent pull-down assays confirmed the interaction using specific histone peptides. These data suggest that June-1 is a novel member of the superfamily of PHD-containing proteins which can influence chromatin regulation through recognition of histone epigenetic marks.

## 5 Final reports of RoFAR award winners



**Figure 1A) Exonic structure of the PHF23 gene**



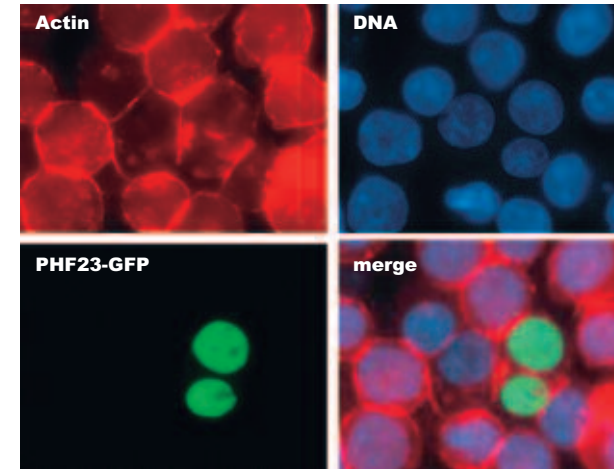
**Figure 1B) Chromosomal location of human PHF23.**

**Figure 1. Gene structure and chromosomal location of PHF23.**

A. PHF23 is a five exon gene with a nuclear localisation signal (NLS) in exon 4 and C-terminal PHD motif in exon 5. An alternative 3' splice site within exon 4 generates a variant isoform, which is shorter by 67 amino acids.

B. PHF23 maps to chromosome 17p13.1 <450kbp from the TP53 gene

### K562 cell line



**Figure 2. Localisation of full-length PHF23-GFP in K562 cell line**

Paraformaldehyde fixed cells are stained with Phalloidin-TRITC for filamentous actin (red fluorescence) and DAPI (blue fluorescence) for DNA, with PHF23-GFP showing green fluorescence.

### Oral presentations citing RoFAR support

1. A study of the novel protein June-1: Presented at the 7th International Lubeck Conference on the Pathophysiology and Pharmacology of Erythropoietin and other Hemopoietic Growth Factors in Lubeck, Germany from 6 - 9 September 2006.
2. The novel protein JUNE-1 binds Histone H3: Presented at the British Society of Haematology 47th Annual Scientific Meeting at the Bournemouth International Centre, UK from 30 April to 2 May 2007

### Submitted manuscripts citing RoFAR support

Hodges VM, *et al.* Cloning and characterisation of PHF23: a novel member of the PHD superfamily of H3K4 methylation-sensitive effectors (to be submitted June 2007)

Rainey S, *et al.* Pathophysiology of anaemia and erythrocytosis. *Haem Onc Reviews* 2007 (in review)

## 5 Final reports of RoFAR award winners

**Prof. Radek Skoda**  
(Cycle II)



### University Hospital Basel, Switzerland

#### *The role of SMAD4-dependent signalling in anaemia*

The tumor suppressor SMAD4 mediates signalling of the transforming growth factor-beta (TGF- $\beta$ ) superfamily of ligands. Previous studies showed that several TGF- $\beta$  family members play an important role in haematopoiesis. SMAD4 was therefore expected to be essential. We studied the role of SMAD4 in adult murine haematopoiesis using the inducible Mx-Cre/loxP system. Mice with induced homozygous SMAD4 deletion (SMAD4 <sup>$\Delta\Delta$</sup> ) developed severe anaemia causing death at 6-8 weeks after induction. Our initial findings of low serum transferrin (Tf) could not be confirmed and were, in retrospect, due to the lower sensitivity of the human Tf ELISA-assay for the mouse Tf protein. Despite only slightly decreased serum iron levels, we found a marked decrease of iron in liver of SMAD4-deficient mice 5-6 weeks after induction (healthy control: 11.5 $\pm$ 3.97 nmol/g, anaemia: 2.64 $\pm$ 0.34 nmol/g). This suggested that iron stores were depleted and transported to the bone marrow to maintain a hyperactive erythropoiesis. Consistently, serum erythropoietin levels and reticulocytes were strongly elevated.

Surprisingly, the anaemia was not transplantable, as wild-type mice reconstituted with SMAD4 <sup>$\Delta\Delta$</sup>  bone marrow cells had normal haematopoietic parameters in peripheral blood. These mice did not develop an inflammatory disease typical for mice deficient in TGF- $\beta$  receptors I and II, suggesting that the suppression of inflammation by TGF- $\beta$  is mediated by SMAD4 independent signalling. To confirm this result in a setting not requiring transplantation, we crossed the SMAD4<sup>fl/fl</sup> mice with mice that express Cre recombinase solely in the hematopoietic system under the control of the Vav promoter<sup>1</sup>. These VavCre; SMAD4<sup>fl/fl</sup> mice displayed complete deletion of the SMAD4 alleles and were healthy without abnormal blood parameters and without inflammatory symptoms.

In contrast, lethally irradiated SMAD4 <sup>$\Delta\Delta$</sup>  mice transplanted with wild type bone marrow cells developed anaemia similar to non-transplanted SMAD4 <sup>$\Delta\Delta$</sup>  mice.

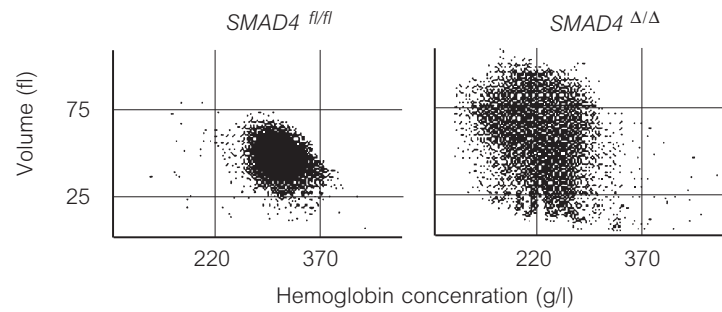
Blood was detectable in stool, indicating that the anaemia was due to blood loss, and polyps were found in stomach and colon representing a likely source of the bleeding. We conclude that SMAD4 is not required for adult erythropoiesis and that anaemia is solely the consequence of blood loss.

The manuscript describing our results has been submitted to the journal *Blood*.

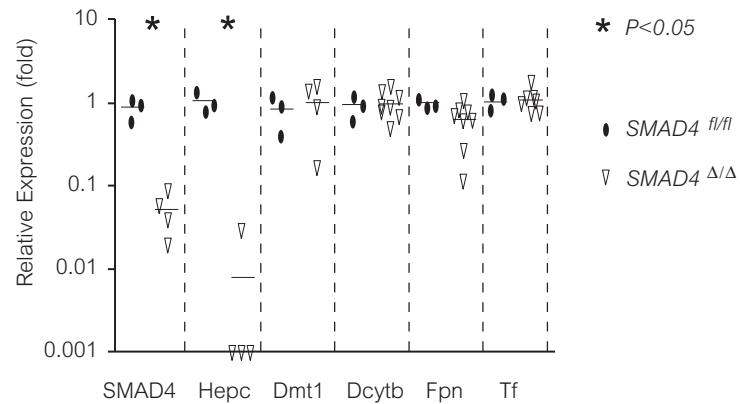
#### References

1. De Boer J, et al. *Eur J Immunol.* 2003; 33:314-325.

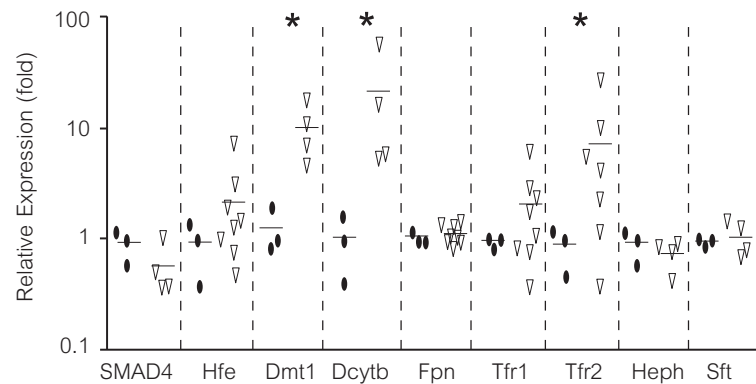
**A** Erythrocyte Volume and Hemoglobin Concentration **Figure 1**



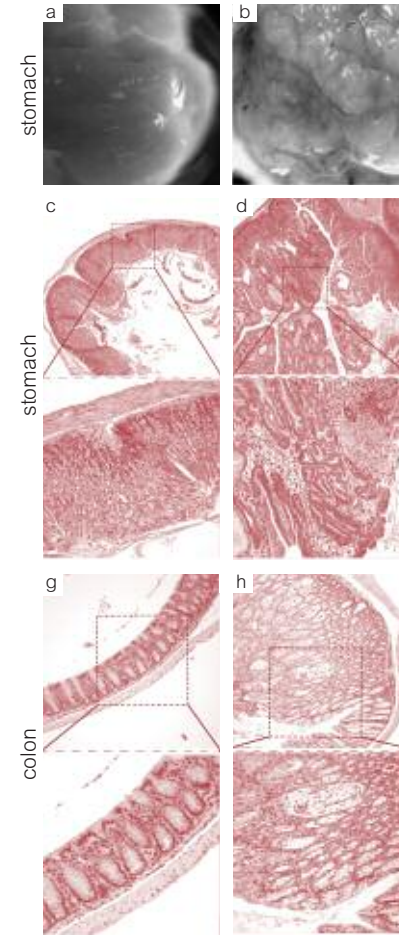
**B** Liver mRNA Expression



**C** Duodenum mRNA Expression



**SMAD4<sup>fl/fl</sup>** **SMAD4<sup>Δ/Δ</sup>** **Figure 2**



**Figure 1. SMAD4<sup>Δ/Δ</sup> mice display severe iron deficiency anaemia**

A) Hypochromic erythrocytes in SMAD4<sup>Δ/Δ</sup> mice. Cell volume was plotted against haemoglobin concentration. Left panel, normal control. Right panel shows hypochromic red blood cells (<220 g/L) and volume increase (> 75 fl) due to reticulocytosis in SMAD4<sup>Δ/Δ</sup>. B) In liver, SMAD4 and hepcidin (Hpc) expression are almost abrogated, and ferroportin 1 (Fpn) is slightly decreased. Cytochrome b reductase 1 (Dcytb), divalent metal transporter 1 (Dmt1) and transferrin (Tf) were unchanged. C) In Duodenum, SMAD4, Fpn, hephaestin (Heph), major histocompatibility complex class I-like protein (Hfe), transferrin receptor 1 (Tfr1) and stimulator of Fe transport (Sft) were unchanged, and Dmt1, Dcytb, and transferrin receptor 2 (Tfr2) were dramatically increased. SMAD4<sup>fl/fl</sup> littermates were chosen as controls. The p-values were calculated by Student's t-test.

**Figure 2. Stomach and colon polyp formation in SMAD4<sup>Δ/Δ</sup> mice.**

The left panel shows a SMAD4<sup>fl/fl</sup> control mouse, whereas the right panel shows a SMAD4<sup>Δ/Δ</sup> mouse. Gross macroscopy of stomach (a, b), histological haematoxylin-eosin staining of stomach (c,d) and colon (g,h) and corresponding magnified area.

## 5 Final reports of RoFAR award winners

**Dr Carole Soussain**  
(Cycle II)



### Oregon Health and Science University, Portland, USA

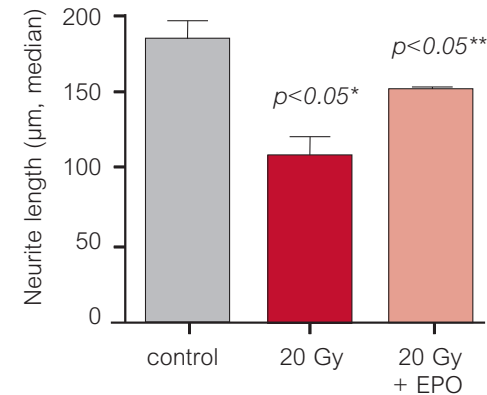
#### Neuroprotective effect of erythropoietin on chemo- and radiotherapy-induced toxicity

##### Objective 1:

We tested the effect of erythropoietin (EPO) on human B-lymphoma cells. The methodologies of a colorimetric cell viability kit and trypan blue exclusion assay showed no effect of EPO on the growth of human B-lymphoma (MC116 cells), nor provided protection from chemotherapy toxicity.

##### Objective 2: Neuroprotective activity of EPO *in vitro*.

A transfected subclone of PC12 rat pheochromocytoma cells was developed, which stably expresses green fluorescent protein (GFP). After exposure to nerve growth factor, the length of neurites of individual GFP-PC12 cells was used to monitor neurotoxicity. Median neurite length was significantly reduced 24 and 48 hours after irradiation. Neurite breakage/shrinkage was significantly reduced by pre-treatment with EPO (Figure 1). Irradiation did not induce apoptosis at these early time points as measured by flow cytometry (pre-G1 phase), and this was unchanged by EPO. Further studies are being done to show that the effect of irradiation on neurites is not mediated through cell death and can be minimised by EPO.

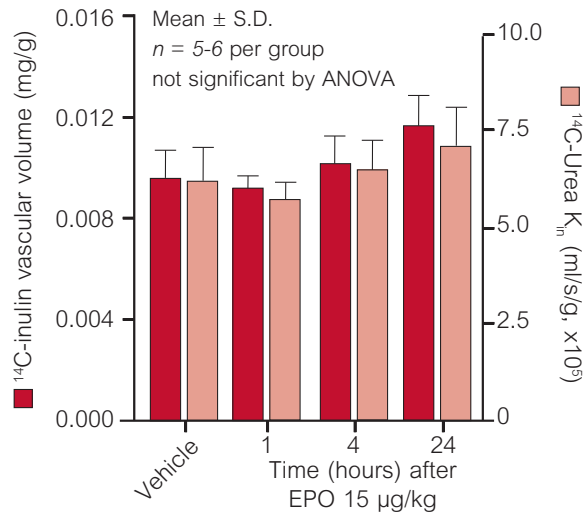


**Figure 1.** Effect of irradiation and EPO on neurite length in PC12 cells expressing GFP. Cells were differentiated with NGF for 8 days, then treated with or without EPO for 24 hours prior to irradiation at 20 Gy. Neurite length was measured on high resolution fluorescence micrographs using NeuronJ software.

##### Objective 3: Effect of EPO on blood-brain barrier (BBB) permeability.

Experiments were performed to evaluate drug delivery across the BBB. We used the well-established *in situ* rat brain perfusion model to determine the effects of EPO on the kinetics of  $^{14}\text{C}$ -inulin, a high molecular weight marker of extracellular space, and  $^{14}\text{C}$ -urea, a low molecular weight (60 g/mol) marker of passive permeability. Rats were pretreated with EPO (0, 5, 15 or 50 µg/kg) for 0, 1, 4, or 24 hours prior to infusion of the radiolabeled agents ( $n = 5-6$  rats per group). The amount of radioactivity in the vasculature and in the brain parenchyma was determined 60 seconds after infusion. Increasing doses of EPO did not enhance  $^{14}\text{C}$ -inulin vascular volume, nor the  $K_{in}$  permeability rate constant for  $^{14}\text{C}$ -urea. In the timecourse experiment, there was a trend towards increased brain permeability (<10% change) at 24 hours after treatment with 15 µg/kg EPO for both the low and high molecular weight markers, but the effect was not significant by ANOVA (Figure 2). We conclude that there was no significant effect of EPO on BBB permeability.

## 5 Final reports of RoFAR award winners



**Figure 2.** Effect of EPO on BBB permeability. Rats were treated with EPO (15 µg/kg) for 1, 4, or 24 hours prior to *in situ* brain perfusion of <sup>14</sup>C-inulin (red) or <sup>14</sup>C-urea (pink) for 60 seconds. No significant effect of EPO on drug delivery was found in this model.

### Objective 4: To characterise the *in vivo* neuroprotective activity of EPO.

We developed rat models of intracerebral and intraventricular B-cell CNS lymphoma (CNSL), and characterised these by MR imaging and immunohistochemistry<sup>1</sup>. In untreated CNSL, without EPO ( $n = 9$ ), mean volume was  $33.3 \pm 16.9 \text{ mm}^3$ . Two rats treated with EPO on day 19 had tumour volumes of 22.0 and 18.1  $\text{mm}^3$  on day 26, within the standard deviation of the controls. Three radiation-treated control rats had tumour volumes of 0.93, 0.55, and 0.80  $\text{mm}^3$ , while two rats pre-treated with EPO prior to irradiation had tumour volumes of 3.9 and 5.2  $\text{mm}^3$ . Although the EPO pre-treated tumours were larger, the tumour cells were necrotic/ apoptotic. We conclude that EPO does not effect CNSL growth or therapy, but additional animals are needed to confirm this finding. This CNSL model closely mimics human PCNSL in terms of imaging, histology, and treatment sensitivity, and will be useful for the development of future therapeutic strategies for PCNSL and neuroprotection.

#### Reference

Soussain C, *et al.* Characterization and magnetic resonance imaging of a rat model of human B-Cell central nervous system lymphoma. *Clinical Cancer Research* 2007; 13:2504-2511.

## 6 Progress reports of RoFAR award winners

**Dr Véronique Lefebvre**  
(Cycle III)



### Cleveland Clinic Foundation, USA

#### *Roles of Sox6 in erythropoiesis*

The major goal of this project is to uncover the roles of the Sry-related HMG box transcription factor Sox6 in erythropoiesis. Prior to this grant, we found that mouse fetuses lacking Sox6 (*Sox6*<sup>-/-</sup>) had anaemia with misshapen, short-lived red blood cells (RBC). Our preliminary data indicated that Sox6 is expressed in erythroid cells and enhances both the early development and the terminal maturation of these cells. In aim 1, we proposed to further characterise the importance of Sox6 in erythropoiesis, not only in fetuses, but also in adult mice under physiological conditions and in severe anaemia. In aim 2, we proposed to identify molecular mechanisms in which Sox6 controls erythropoiesis and, in particular, the erythroid cytoskeleton.

During the first year of this grant, we completed our analysis of the erythroid phenotype of *Sox6*<sup>-/-</sup> fetuses and also analysed fetuses lacking Sox6 only in erythroid cells (conditional knockout). We published our findings in an article entitled "Sox6 cell autonomously stimulates erythroid cell survival, proliferation, and terminal maturation and is thereby an important enhancer of definitive erythropoiesis during mouse development"<sup>1</sup>. Since then, we have determined that adult mice lacking Sox6 in erythroid cells look externally normal, but exhibit an erythroid phenotype similar to the fetal phenotype and fight anaemia by increasing their erythropoietic tissue volume. When injected with phenylhydrazine to induce severe haemolytic anaemia, they are dramatically impaired in their ability to quickly generate a massive erythropoietic response, and 25% of them die within 5 days. The others recover in 2 weeks, while control mice recover in 1 week. Sox6 thus plays a critical role in stress erythropoiesis.

We have also started to work on aim 2. We have screened gene expression microarrays to identify genes whose expression in erythroid cells is altered upon inactivating Sox6. We used RNA samples from erythropoietic tissues *in vivo* and

from erythroid cells at different stages of differentiation in culture. A few hundred genes were found to be either downregulated or upregulated in all Sox6-null samples compared to matching controls. This result is consistent with the ability of Sox6 to contribute to both transcriptional repression and transcriptional activation. These genes have roles in the erythroid cytoskeleton, apoptosis, cell proliferation, and other processes. We are now selecting a subset of highly relevant genes to confirm their expression change. We will then determine whether they are direct targets of Sox6.

In summary, our project has significantly progressed during the first year of this RoFAR grant. We will continue to work on our specific aims in the second year, and anticipate that our work will importantly contribute to a better understanding of the molecular control of erythropoiesis.

#### **The findings were published in:**

1. Dumitriu *et al.* *Blood*, 2006, 108:1198-1207

## 6 Progress reports of RoFAR award winners

**Prof. Stephen L. Leib**  
(Cycle III)



### University of Berne, Switzerland

#### *Effect of erythropoietin on brain injury and regeneration in bacterial meningitis*

##### **Objective**

The objective of the project is to evaluate the potential of exogenously administered erythropoietin for adjunctive therapy in bacterial meningitis. Specifically, an infant rat model of bacterial meningitis will be used to investigate the effect of erythropoietin therapy on clinical outcome, and damage to the brain and the inner ear, in the acute disease and on regenerative processes in long term survivors. The combined effect of erythropoietin on neuronal survival and regeneration will be evaluated in long-term survivors by assessment of brain repair function, i.e. cellular neurogenesis and neuro-functional performance (learning and hearing function).

During the first year of the project, research on aims 1 and 2 has been completed and results are presented in this progress report. Aims 3 to 5 are currently being investigated.

##### **Aim 1: To assess whether erythropoietin prevents acute brain damage in experimental pneumococcal meningitis**

The hypothesis to be tested here is that administration of erythropoietin at the time of infection and as subsequent adjuvant therapy, together with antibiotic therapy, will diminish cortical and/or hippocampal damage.

##### **Aim 2: To document whether the neuroprotective effect is still evident when therapy with erythropoietin is delayed until symptomatic disease**

This aim will test the effect of delayed erythropoietin administration 18 hours after infection. This time period corresponds to a clinically relevant situation, as when patients admitted to medical care facilities receive antibiotic and adjuvant therapies only after the appearance of the first symptoms.

##### **Materials and methods**

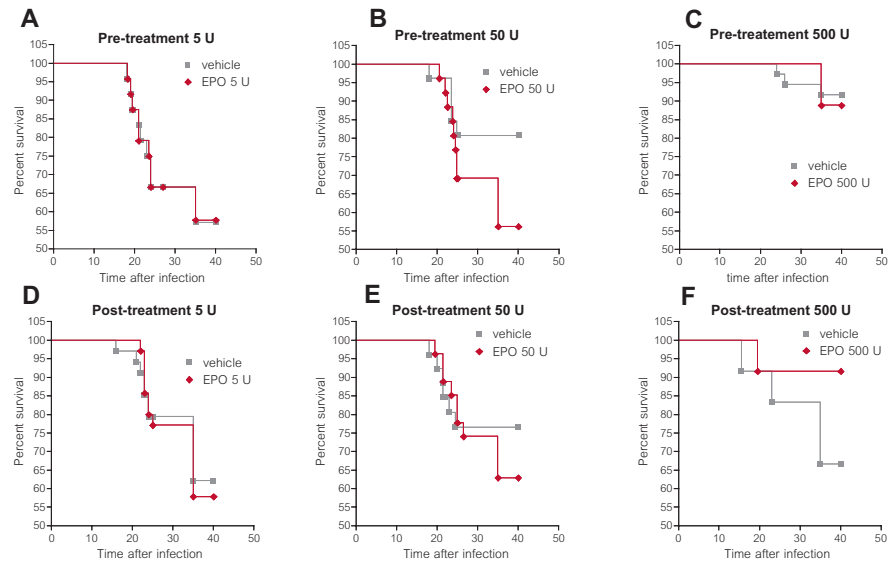
Pneumococcal meningitis was induced in 11-day-old Wistar rats by intracisternal injection of *Streptococcus pneumoniae* (serotype 3). Antibiotic therapy (ceftriaxone, 100 mg/kg s.c. bid) was initiated 18 hours after infection. Animals were randomised to either rhEPO or an identical volume (150 µl) of saline. Different treatment regimens were tested, e.g. pre-treatment studies (administration of rhEPO at the time of infection) and post-treatment studies (administration of rhEPO simultaneously with antibiotic therapy) and different doses were evaluated (5, 50 and 500 Units/kg/d rhEPO i.p). Survival was monitored and hippocampal apoptosis and cortical necrosis were assessed by histomorphometry at 36 hours after infection.

##### **Results**

Survival, evaluated by Kaplan-Meier curves, was not significantly influenced by administration of rhEPO, either given at the time of infection (pre-treatment, Figure 1 A-C) or delayed until the appearance of the first symptoms (18 hours after infection, post-treatment, Figure 1 D-F). While pre-treatment regimens showed a trend towards a decrease in hippocampal apoptosis, post-treatment regimens had no effect on brain injury (Figure 2). The extent of cortical damage was not significantly influenced by the administration of rhEPO, either as a pre-treatment or as a post-treatment therapy (Table 1).

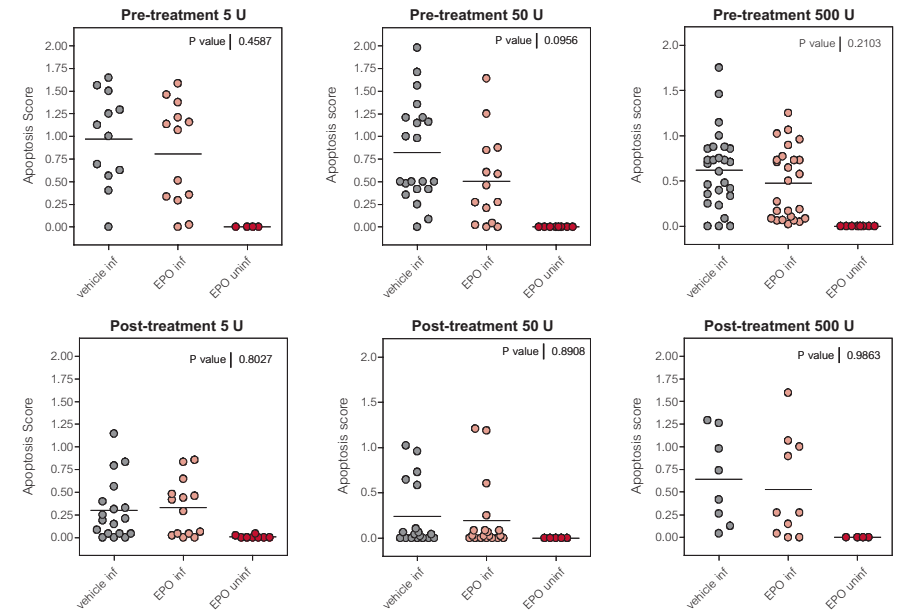
## 6 Progress reports of RoFAR award winners

### Kaplan-Meier survival curves of animals infected with *S. pneumoniae* and treated with rhEPO vs saline



**Figure 1.** Survival was not significantly influenced by the administration of rhEPO for all regimens tested, neither in the pre-treatment regimen (A to C), nor in the post-treatment study (D to F).

### Apoptosis in the dentate gyrus of the hippocampus during experimental pneumococcal meningitis



**Figure 2.** Apoptosis in the dentate gyrus of the hippocampus was determined according to the following scoring system: Score 0=0-5 apoptotic cells/microscope field, Score 1=6-20 apoptotic cells/microscope field, Score 2=>20 apoptotic cells/microscope field. No significant beneficial effect of EPO could be observed, although a trend ( $p < 0.1$ ) toward protection was documented for the 50 units pre-treatment regimen.

## 6 Progress reports of RoFAR award winners

### Extent of cortical damage during experimental pneumococcal meningitis

	Extent of cortical damage at 40 hpi (% total cortex area)		
	Vehicle	EPO	Mann Whitney
5 U pre-treatment	0.05±0.18	0.54±1.2	<i>P</i> =0.51
50 U pre-treatment	0	5.76±9.85	<i>P</i> =0.03*
500 U pre-treatment	1.01±2.85	0.88±2.2	<i>P</i> =0.84
5 U post-treatment	0.43±1.44	0.79±2.1	<i>P</i> =0.71
50 U post-treatment	3.84±8.39	1.09±2.53	<i>P</i> =0.8
500 U post-treatment	1.56±1.72	2.93±8.19	<i>P</i> =0.43

**Table 1.** rhEPO treatment did not significantly influence the extent of cortical damage for all regimens tested. For the 50 U pre-treatment group (\*), no cortical damage was detected in vehicle-treated animals. Therefore, we used a Wilcoxon signed rank test for statistic, by attributing a hypothetical value of 0 to the vehicle treatment group.

### CONCLUSION:

In contrast to other models of brain injury, rhEPO did not exhibit effective neuroprotection in experimental pneumococcal meningitis during the acute phase of the disease. The multifactorial disease mechanisms that include inflammatory, ischaemic, excitotoxic and pathogen-derived mechanisms may explain the lack of beneficial effect of EPO in this model. However, other potential beneficial effects of EPO during the regenerative phase cannot be excluded and will be investigated during the next period of the grant.

### Part of this work was presented at the:

1. 17<sup>th</sup> European Congress of Clinical Microbiology and Infectious Diseases and 25<sup>th</sup> International Congress of Chemotherapy, 31 March - 3 April 2007 in Munich, Germany
2. Swiss Society for Neuroscience (SSN), NCCR Neuro, Swiss Society of Multiple Sclerosis (SSMS) Joint Meeting, 9-10 March 2007 in Bern, Switzerland

## 6 Progress reports of RoFAR award winners

**Dr Barbara Scheiber-Mojdehkar**  
(Cycle III)



### Medical University of Vienna, Austria

#### *Recombinant human erythropoietin: A new treatment for Friedreich's ataxia*

Friedreich's ataxia (FRDA) is the most common of the inherited ataxias, affecting one in 50,000 people. Clinically, FRDA is characterised by multiple symptoms including progressive gait and limb ataxia, dysarthria, diabetes mellitus and hypertrophic cardiomyopathy. FRDA is caused by a GAA-trinucleotide expansion in the frataxin gene, resulting in a reduced expression of frataxin, a small mitochondrial protein.

Recently we found that additionally to its reported neuroprotective and cardio-protective capabilities, recombinant human erythropoietin (rhuEPO) significantly increases frataxin levels in isolated lymphocytes from FRDA patients *in vitro*. Additionally, rhuEPO significantly increased frataxin levels in primary human cardiomyocytes, cardiofibroblasts and neuronal cells. In an open-label, phase IIa study, 11 FRDA patients received 5,000 IU rhuEPO three times weekly subcutaneously for a period of 8 weeks. The primary outcome measure of the study was a stable increase of frataxin in FRDA patients over 8 weeks. Secondary outcome parameters included the clinical rating scale SARA (scale for the assessment and rating of ataxia) and measurements of indicators for oxidative stress (serum peroxide and urine 8-hydroxydesoxyguanosine). Iron metabolism was assessed at baseline and at the primary endpoint after 8 weeks. Safety was assessed by weekly measurement of haematocrit, haemoglobin, erythrocyte counts, reticulocytes and thrombocytes.

As primary outcome, frataxin levels were measured in isolated lymphocytes performed by ELISA at baseline and after 2 and 8 weeks (week 2 and week 8) of rhuEPO treatment (Figure 1). In 7 patients (3 female/4 male), there was an increase of frataxin levels during the 8 weeks of rhuEPO treatment. The increase varied among the patients from 15-63% compared to their individual baseline frataxin levels (baseline was set at 100%). Two patients (1 female, 1 male) showed no increase in their frataxin levels during the 8 weeks of rhuEPO treatment. Another male patient showed an increase in the frataxin level only after 8 weeks of rhuEPO treatment. In

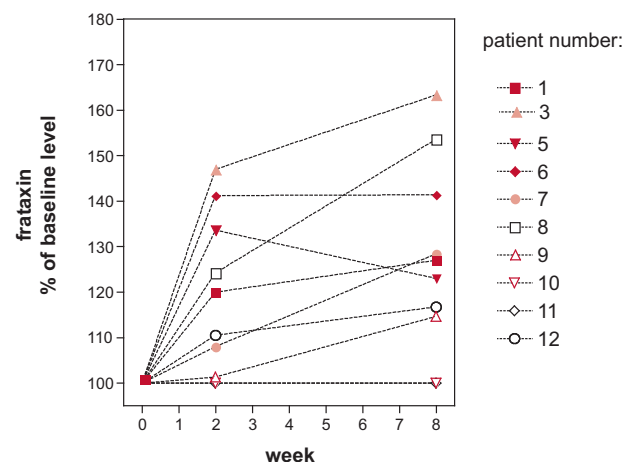
one male patient, blood samples for frataxin analysis were not available.

As secondary outcome, oxidative stress parameters were assessed. Urine 8-hydroxydesoxyguanosine (a marker for oxidative mitochondrial and nuclear DNA damage) and serum peroxides were significantly decreased after 8 weeks of rhuEPO treatment compared to baseline.

Serum ferritin levels significantly decreased during the 8 weeks of treatment with rhuEPO. Transferrin increased, whereas serum iron and transferrin saturation decreased after 8 weeks of treatment, compared to baseline.

Severity of ataxia was measured by SARA at baseline and after 8 weeks. The mean improvement in the SARA score in the whole study population was 6%, but this was not significant. After exclusion of those patients who did not show a stable increase in frataxin levels during the rhuEPO treatment, a significant reduction (11.5%) in SARA points was obtained. Nevertheless, major neurological improvement was not indicated after 8 weeks with rhuEPO.

In conclusion, we were able to show a stable increase of frataxin levels during 8 weeks of treatment with rhuEPO in the majority of our patients accompanied by a marked apparently independent reduction of surrogate markers for oxidative stress *in vivo*.



**Figure 1.** Baseline frataxin levels in 10 FRDA patients versus frataxin levels after 2 and 8 weeks of treatment with rhuEPO (individual values). Baseline frataxin level is set at 100%.

## 6 Progress reports of RoFAR award winners

**Prof. Jürg Schifferli (principal applicant)**

Dr Christoph Hess (co-applicant)

(Cycle III)



### University Hospital Basel, Switzerland

#### *Erythropoietin or erythrocyte transfusion for anaemia?*

The possible immunosuppressive properties of blood transfusions have been attributed to leukocytes or remnants thereof. The possibility that the so-called “microparticles” released by erythrocytes during storage are involved, has not been explored. In the first 12 months of the RoFAR project, we analysed the structure and properties of microparticles released by erythrocytes during storage before transfusion. Microparticles were purified by centrifugations from leukodepleted erythrocytes stored for 25 days in the blood bank. By FACS they expressed glycoporphin A (the marker for erythrocytes), with no platelet- or leukocyte-derived particle contamination. By electron microscopy these particles corresponded to vesicles with heterogeneous sizes ranging from 20 to 200 nm (Figure 1). The incorporation of an amphiphilic membrane dye confirmed the existence of a lipid membrane, suggesting that these vesicles bud out from the erythrocyte surface by ectocytosis. The pattern of membrane proteins expression by the vesicles/ ectosomes showed a specific sorting of proteins into and out as compared to erythrocyte membranes. In particular, the ectosomes expressed all three complement- inhibitors known to be expressed by erythrocytes (CR1, CD55 and CD59). However, the erythrocyte-derived ectosomes were able to fix C1q and activate complement with deposition of C3 fragments.

Of particular interest was the expression of phosphatidylserine (PS), as assessed by the calcium-dependent binding of annexin V, suggesting that ectosomes may react with and downregulate cells of the immune system. Indeed, liposomes expressing PS have been shown to inhibit the activation of dendritic cells and the immune response in mice. *In vitro*, zymosan is known to induce the production and release of different cytokines from monocyte-derived macrophages (e.g. TNF $\alpha$ , IL8 and IL10). When the erythrocyte-derived ectosomes were tested in this model, we could demonstrate that they inhibited the release of all three cytokines by zymosan-activated macrophages (80 $\pm$ 6% inhibition for TNF $\alpha$ , 80 $\pm$ 7% inhibition for

IL10 and 73 $\pm$ 10% inhibition for IL-8 (Figure 2). Similar but less pronounced inhibitions were observed when the macrophages were activated with LPS. Interestingly, ectosomes released from erythrocytes by a calcium ionophore (Figure 2) or after ATP depletion *in vitro* (not shown) had similar inhibitory activities on zymosan-activated macrophages, although less pronounced.

We have previously shown that ectosomes produced by polymorphonuclear leukocytes also inhibited the release of TNF $\alpha$ , IL-10 and IL-8 by zymosan- and LPS-activated macrophages. However, these effects were probably related to the capacity of polymorphonuclear leukocyte-derived ectosomes to induce the release of TGF $\beta$  by macrophages (TGF $\beta$  is known to inhibit the production of pro-inflammatory cytokines such as TNF $\alpha$  and IL-8)<sup>1</sup>. Interestingly erythrocyte-derived ectosomes did not induce the release of TGF $\beta$  from macrophages, indicating that other mechanisms may be responsible for their capacity to downmodulate macrophages (Figure 2).

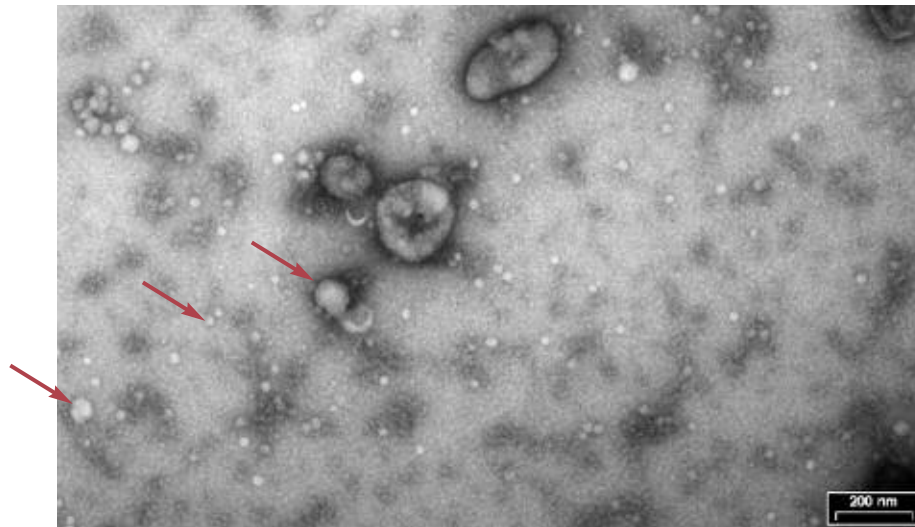
More research needs to be performed to better define these immunomodulatory properties of erythrocyte-derived ectosomes. However, the modulation of inflammation/innate immunity by erythrocyte-derived ectosomes may account for some of the immunosuppressive properties attributed to blood transfusions.

#### **Reference**

Gasser O, *et al.* Activated polymorphonuclear neutrophils disseminate anti-inflammatory microparticles by ectocytosis. *Blood* 2004; 104:2543-2548.

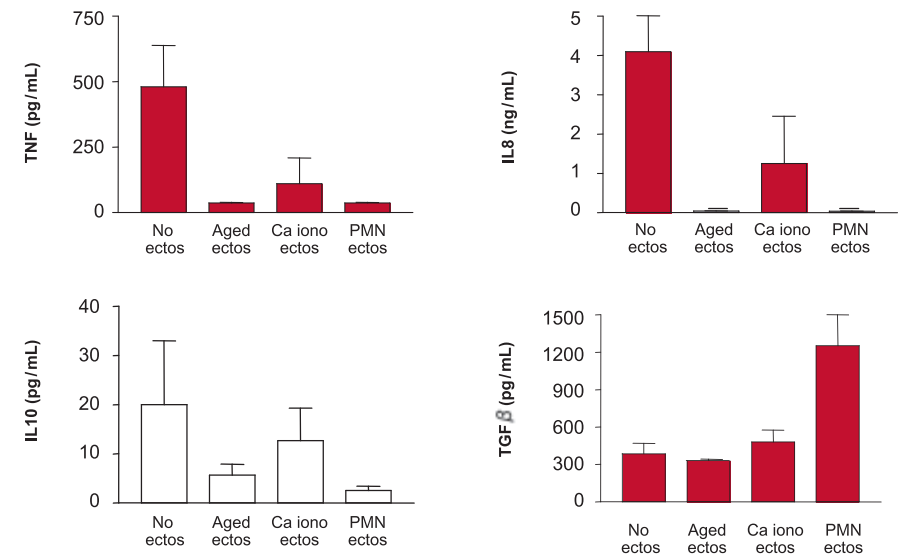
## 6 Progress reports of RoFAR award winners

### Erythrocyte-derived ectosomes released during storage



**Figure 1.** Ectosomes purified from the supernatant of leukodepleted packed RBCs. Size bar: 200nm. The ectosomes are heterogeneous with sizes of 20 to 200 nm.

### Erythrocyte-derived ectosomes inhibit the release of cytokines by zymosan-activated macrophages



**Figure 2.** No Ectos = the control release of cytokines by zymosan-activated human macrophages  
 Aged ectos = ectosomes released by erythrocytes during storage in the blood bank  
 Ca iono ectos = ectosomes derived from erythrocytes exposed to a calcium ionophore  
 PMN ectos = ectosomes released by polymorphonuclear leukocytes activated with FMLP

## 6 Progress reports of RoFAR award winners

### Dr Marcela Votruba (principal applicant)

Prof. Mike Boulton (co-applicant)

Dr Paul Cornes (co-applicant)

(Cycle III)



### Cardiff University, UK

#### Erythropoietin neuroprotection in retinal neurodegeneration

We aim to characterise the effect of erythropoietin (EPO) on retinal ganglion cells (RGCs) in *in vitro* and *in vivo* models of *opa1* mitochondrial GTPase knockdown. *Opa1* is linked to RGC loss in autosomal dominant optic atrophy, a retinal neurodegenerative disease. We aim to determine whether EPO can protect RGCs in models of this disorder.

**Aim 1:** Initial work included characterising a range of RGC cell models, namely R28, RGC-5 and primary RGCs (Figure 1). R28 growth characteristics for transfection and viability assay proved incompatible with the desired treatment time courses. EPO receptor (EPO-R) expression was successfully assessed by Western blotting in RGC-5 cells, which were used for initial EPO viability assays (MTT assay). See Figure 2. Lack of apparent protective effect in the simple MTT assay has led to further tests, including astrocyte co-culture and pre-treatment approaches. Isolation of mixed retinal cell populations from the *opa1* mutant mouse model has been successful (B in Figure 1) and refinements of mouse immuno-panning studies are ongoing. Rat immuno-panning provided much initial data; application of anti-Opa1 siRNA knockdown in both RGC-5 and rat primary RGC cells showed significant alteration in mitochondrial morphology.

A gene trap clone containing a deletion of the *opa1* gene was cultured in a mouse embryonic stem cell (ES) line (clone ID: CF0520). It was found to be non-viable due to the *opa1* deletion, though potentially also due to the a neuploid status of the ES cells.

**Aim 2:** We are currently progressing into the *in vivo* phase of the study, bringing together the *in vitro* knowledge and tools of Aim 1 and the *opa1* mutant mouse model to elucidate the molecular, cellular and phenotypic responses to EPO treatments.

In the second year, we will further investigate the effects of dose and regimen in light of recent glaucoma-based EPO studies<sup>2,3</sup>.

**Aim 3:** Building on the information from viability studies (Aim 1), we are now probing these conditions with a combination of mitochondrial morphology and apoptotic assays and a real-time PCR “mini-array” of primers developed in-house (using probes for genes of interest: *opa1*, *EPO*, *EPO-R*, RGC cell type-specific markers, signalling, apoptotic, oxidative stress, and house-keeper genes). Many of the mediators of mitochondrial fusion/fission recently reviewed by Gazaryan & Brown<sup>1</sup> are incorporated into the array.

**Aim 4:** Towards the end of the second year, we plan to build upon behavioural work recently published by our research group<sup>4</sup> by examining the potential benefits of EPO treatment in a disease model system using retinal and optic nerve imaging and functional visual phenotyping.

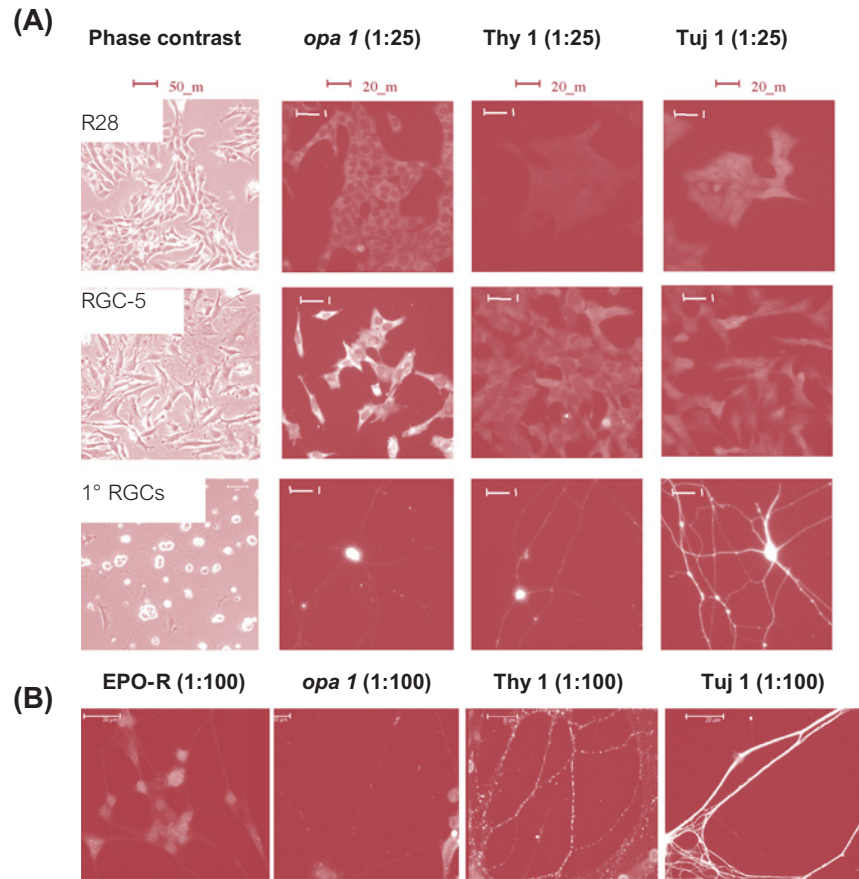
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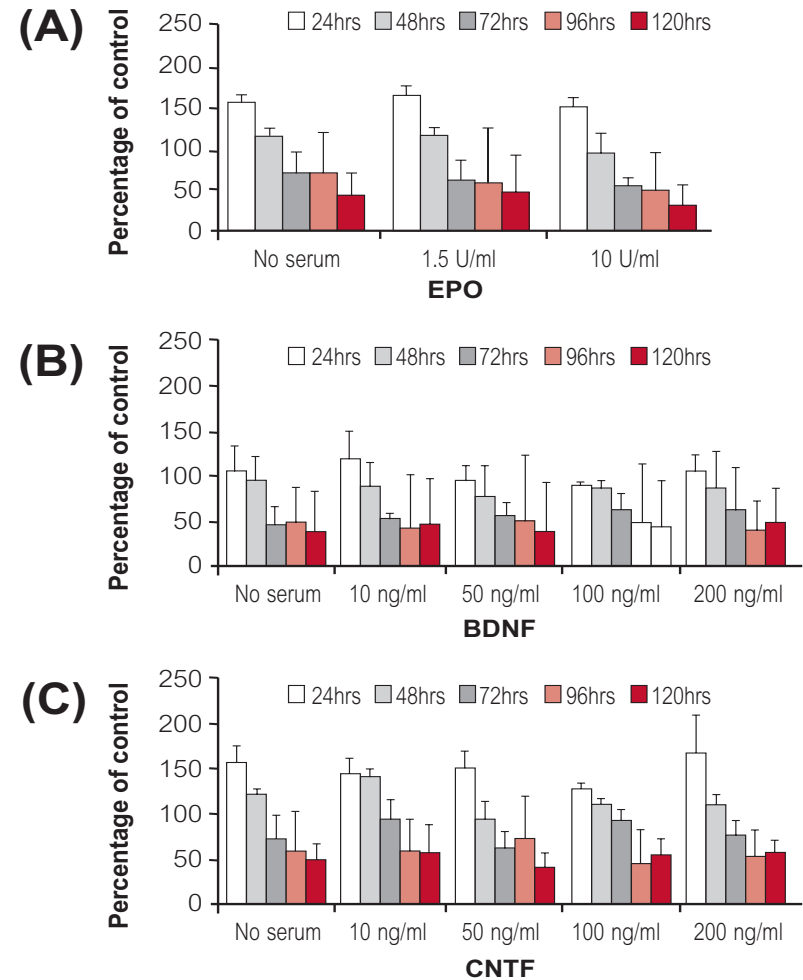
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7. Davies VJ, *et al.* Phenotypic assessment of a novel murine ENU-induced mutant line expressing a nonsense mutation in the *opa1* gene. *Acta Ophthalmologica Scandinavica* 2006; 84(s239).

## 6 Progress reports of RoFAR award winners



**Figure 1.**

Immunofluorescence microscopy of RGC models stained with a range of neural markers. **(A)** RGC-5 cells, R28 cells and primary rat RGCs stained for *opa1*, Thy1, and Tuj1 (the latter two being RGC-specific marker proteins for the retinal cell population). Positive staining was noted for these markers in RGC-5 cells and primary RGCs but not in the precursor cell line R28 **(B)** A mixed retinal cell population from *opa1* mutant mouse model stained for EPO-R, *opa1*, Thy1 and Tuj1. The stain shows RGCs in a mixed cell population. Cells appeared morphologically as expected. For all images primary antibody dilutions are shown. All Alexa-488 conjugated secondary antibodies were used at a 1:1000 dilution.



**Figure 2.**

MTT assay carried out upon serum-deprived RGC-5 cells over a period of 120 hrs: treated with **(A)** EPO, **(B)** Brain-derived neurotrophic factor (BDNF), and **(C)** Ciliary neurotrophic factor (CNTF). Across the treatments used, there was no apparent rescue of cell numbers compared with the serum-deprived cells without EPO ( $n = 6$ ). Pre-treatment may be required to show an observable difference. Cells grown in the presence of 10% heat-inactivated foetal calf serum were used as controls.

## 6 Progress reports of RoFAR award winners

**Prof. Tomas Ganz**  
(Cycle IV)



### University of California, Los Angeles, USA

#### *Pathogenesis of anaemia of chronic infection*

The original aims of the proposal were:

Aim 1: Develop and characterise a simple non-surgical model of anaemia of chronic infection

Aim 2: Determine the role of hepcidin in anaemia of chronic infection

Aim 3: Characterise the role of IL-6 in anaemia of chronic infection

**Aim 1** is nearly completed. We tested and validated a chronic model of anaemia of infection in mice that uses a single intraperitoneal injection of a mycobacterial preparation that is safe for laboratory personnel. Table 1 summarises the findings 3 weeks after injection.

mice	haemoglobin	MCV	Fe	hepcidin/ $\beta$ -actin mRNA	serum amyloid A mRNA	RBC half-life
control	12.8 $\pm$ 0.7	44.4 $\pm$ 1.0	13.5 $\pm$ 5.4	1.0 (3.6 $\times$ 10 <sup>-2</sup> – 54)	1.0 (0.31 – 52)	to be done
bacteria	10.3 $\pm$ 0.6	41.8 $\pm$ 1.3	11.2 $\pm$ 3.0	13 (2.4 – 150)	77 (7.3 – 190)	to be done
<i>P</i>	<0.001	0.001	NS	0.012	0.007	

**Table 1.** A new model of chronic anaemia of inflammation

Results: Mice treated with mycobacteria developed inflammation-related increased hepcidin expression and iron-restricted (low MCV) anaemia.

Conclusion: We established a model of anaemia of chronic inflammation in mice.

Discussion: A more detailed timecourse will be necessary to determine whether the mice also develop hypoferraemia during earlier phases of the inflammatory response. A publication is being prepared. The model has already been useful in Aim 3 in establishing the redundancy of IL-6 during chronic inflammation. The model should also prove useful in studying potential interventions that can reverse the anaemia of inflammation.

**Aim 2** is proceeding well. Using accelerated marker assisted-breeding, hepcidin KO mice, originally on mixed background, have been bred to 97.5% C57Bl6 background and a colony of these mice is ready for the proposed studies.

**Aim 3** is partially completed. We compared the haematological and iron parameters and hepcidin mRNA in IL-6 KO mice and normal mice treated with the mycobacterial preparation described in Aim 1. The results are summarised in Table 2.

mice	haemoglobin	MCV	Fe	hepcidin/ $\beta$ -actin mRNA	serum amyloid A	RBC half-life
IL-6 KO	8.3 $\pm$ 1.8	45.6 $\pm$ 1.3	15.6 $\pm$ 12.2	1.0 (0.22 – 140)	1.7 (0.69 – 9.3)	to be done
WT	10.3 $\pm$ 0.6	41.8 $\pm$ 1.3	11.2 $\pm$ 3.0	13 (2.4 – 150)	77 (7.3 – 190)	to be done
<i>P</i>	0.014	<0.001	NS	NS	0.001	

**Table 2.** Comparison of chronic anaemia of inflammation in IL-6 KO vs. wt mice

Results: IL-6 KO mice developed inflammation-related anaemia without evidence of hepcidin increase or iron restriction.

Conclusion: The mechanism of anaemia in IL-6 KO mice may be different than in WT mice and remains to be characterised.

Discussion: We showed previously that IL-6 was required for the development of acute hypoferraemia after turpentine-induced inflammation in the same strain of mice. It appears that IL-6 is not required for the development of anaemia in this model, but the specific mechanism involved in anaemia in IL-6 KO mice still needs to be characterised in more detail. RBC half-life studies are pending and should be informative.

## 6 Progress reports of RoFAR award winners

**Dr Dirk Hermann<sup>1</sup> (principal applicant)**

Prof. Markus Rudin<sup>2</sup> (co-applicant)

(Cycle IV)



**University Hospital Zurich, Switzerland<sup>1</sup>  
and Swiss Federal Institute of Technology (ETH),  
Zurich, Switzerland<sup>2</sup>**

*Effects of human erythropoietin on brain plasticity and functional recovery following stroke*

In view of its survival-promoting effects on brain neurons, human erythropoietin (EPO) has attracted strong interest in the treatment of brain diseases in which neurons are lost. Neuroprotective effects of human EPO were previously described in experimental models of focal cerebral ischaemia as well as in a clinical phase II proof-of-concept trial in human stroke patients.

A major limitation of neuroprotection therapies after stroke is the limited time-window, considering that ischaemic brain injury mostly develops within a few hours after stroke onset. Thus, only a small number of patients are able to benefit from neuroprotection treatment. There is a clear need for treatments which can also be applied in the sub-acute stroke phase.

In the present project, we have been evaluating the therapeutic potential of human EPO delivered in the post-acute stroke phase. Ischaemic animals subjected to 30 minutes MCA occlusions were treated with human EPO starting at 3 days after stroke onset, i.e. at a time point at which brain injury has already occurred.

In the first year of this project, we performed a detailed behavioural analysis of the effects of EPO in the stroke brain, evaluating its effects on motor recovery (assessed by grip strength and rotarod tests) as well as on neuropsychological abnormalities (i.e. animal hyperactivity, anxiety and depression).

In our studies, we observed a gradual improvement in the animals' motor function, which unlike the neuroprotective effect in the acute stroke phase, developed with a delay of approximately 2-4 weeks after stroke. As such, the animals' motor force in

the paretic right forelimb as well as limb coordination significantly improved compared with vehicle-treated control mice. The enhanced motor recovery was associated with an increased hyperactivity of the stroke animals, which in contrast to the consistent improvement in motor function, disappeared, when the EPO treatment was finished.

In the meantime, we injected (i) anterograde tract tracer into the left- and right-sided motor cortex and (ii) BrdU in ischaemic mice, evaluating the effect of EPO on motor cortex plasticity both ipsi- and contra-lateral to the stroke, as well as on endogenous neurogenesis. We also started dose-response studies, evaluating concentration thresholds of the restorative effects of EPO we found. Studies investigating molecular readouts underlying brain plasticity will follow shortly.

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<b>Prof. Patrick Maxwell</b>	<i>Professor of Nephrology</i> Imperial College London, UK
<b>Dr William McClellan</b>	<i>Assistant Professor</i> Department of Epidemiology Emory University Atlanta, USA
<b>Dr Jerry Spivak</b>	<i>Professor of Medicine and Oncology</i> Johns Hopkins University Baltimore, USA
<b>Prof. Gary Striker</b>	<i>Professor of Medicine and Nephrology</i> Mount Sinai School of Medicine New York, USA

### 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

The screenshot shows the RoFAR website homepage. At the top left is the URL 'www.rofar.org'. To the right is the RoFAR logo, which consists of a red circle with a white 'R' and the text 'RoFAR Foundation for Anaemia Research'. Below the logo is a navigation menu with links: Home, Information, Regulations, Regular grants, Special grant, Grant awards, News/Books, Secretariat, and Q & A. The main content area features several news items:

- Our Mission:** The RoFAR Foundation for Anaemia Research is a registered medical research charity with the mission of "encouraging research that will open new avenues of exploration in the study of anaemia, its mechanisms, erythropoietin agents and outcomes." These eligible for grants are members of academic staff in universities, dialysis centres and research institutes.
- RoFAR announces a new special competition cycle:** In addition to its regular research grant awards, RoFAR now invites scientists and institutions to submit applications for a special grant of up to 1.6 million CHF. RoFAR is particularly interested in innovative proposals, involving established researchers, which provide proof of principle and/or translational research, particularly studies which have the potential to be transferred into clinical practice. Letter of Intent submission will start on 30 November 2006 and end on 30 June 2007. Applications must be submitted via the RoFAR website. For more information, please download the PDF leaflet.
- How to apply for regular grants?** Letters of Intent to RoFAR for Cycle VI has closed. The deadline for submission in Cycle VII is 31 June 2007. Submissions will be possible soon from the submission page.
- RoFAR successful reports available for download:** Please access the award winners, final projects and progress in the Archived section.
- Upcoming congress activities:** Visit RoFAR's exhibition booth at following congresses:
  - ABN 2006, December 9-11, Orlando FL, USA (Booth Number 2411)
  - WON 2007, April 31-25, Rio de Janeiro, Brazil (Booth number not assigned yet)
- For more information and if you have any questions, please contact the RoFAR Secretariat.**
- Status of ongoing cycles:**
  - Cycle VI - LOI are being evaluated. Letters of Intent are being evaluated. Applicants will be invited to submit a full application within a 6 weeks by deadline will be provided.

### 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.