License Number: MA58/004306/2012/5 – Amendment 1 (April 2015)

Project Title: "Expression of Genes regulating growth of mouse blood cells during development (Model A) "

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Number of Mice: 10,000 within 5 years

License valid until: 15. August 2017

Project proposal and description:

Introduction:

The purpose of the animal experiment is to examine the function of various regulatory genes during blood cell development in the mouse. In this respect focus will be on analysis of cellular oncogenes, growth factors and transcription factors in differentiation and division of blood cells as well as development of leukaemia. Factors leading to the development of cancer are still not precisely understood, and this animal experiment should allow better analysis of such. This animal experiment should additionally help to clarify the function of important transcription and epigenetic factors in the control of blood cell differentiation.

In the course of this experiment haematopoietic progenitor cells will be injected in sub-lethally radiated mice to observe their differentiation into mature cells. Haematopoietic progenitor cells have – like haematopoietic stem cells – the potential of unlimited cell division; however differ from stem cells through already limited differentiation potential. These progenitor cells can be grown ex vivo and modified using retroviral gene transfer. Genetic modifications of the haematopoietic progenitor cells allow studying of certain genes during the growth and differentiation processes of haematopoietic cells. These genetic modifications involve either insertion and expression of a gene using retroviral gene transfer or deactivation of particular genes through transgenic knock-out or RNA interference (RNAi). or CRISPR-Cas9 induced mutagenesis. In the case of retroviral gene transfer the coded gene (cDNA) respectively RNAi trigger (so-called shRNA) are induced along with fluorescent or bio-luminescent reporter genes serving monitoring and identification of genetically modified cells in the course of the project.

The transplantation of genetically modified progenitor cells serves studying the function of transcription factors (such as Myb, Ebf1, Runx1, Ikaros and Pax5) as well as epigenetic regulators (including Brd4 Dnmt3a, Tet2, Ezh2 and Eed) in the development of normal and leukemic blood cells. The number of transcription factors and epigenetic regulators to be

newly identified is constantly growing. New genome-wide analysis in human leukemic cells and initial functional studies suggest that many of these factors play a central role in both creation of normal blood cells and development of leukaemia, lymphomas and immune disorders. The animal experiments covered hereby serve the purpose of more precise definition of the function of these factors in normal haematopoiesis and leukaemia development which is of underlying importance for developing new therapy concepts for the aforesaid diseases.

For the purpose of carrying out research in the defined leukaemia relevant contexts, in addition to the new genes, function of which is to be researched and which belong to the class of transcription factors and epigenetic regulators (see above), the known leukaemia associated alleles will also be introduced through retroviral gene transfer (e.g. MLL/AF9, AML1/ETO, AML1/EVI1, BCR/ABL, FLT3-ITD, c-Myc, $Nras^{G12D}$, $Kras^{G12D}$, Notch1-IC) or by using progenitor cells from transgenic knock-out mice. The established transgenic models applied hereby include amongst others knock-out mice lacking the *Pax5*, *Ebf1*, *Ikaros*, *Rag2*, *Pten*, *Trp53* or *Cdkn2a* gene as well as knock-out mice with a conditioned oncologic allele (e.g. *.lox-STOP-lox-*$Kras^{G12D}$*, lox-STOP-lox* $Nras^{G12D}$).

To study the influence of regulative genes on the function of the differentiated haematopoietic cells the differentiated cells which have been genetically modified (as described above) are isolated from lymphoid organs such as spleen and lymphatic glands and injected into non-irradiated mice. The injected cells normally integrate with the normal cells in a secondary lymphoid organ where their function can be studied in comparison to the normal cells.

These studies serve prevention, recognition and curing of illness in humans and were approved with the decision MA58-4721 in December 1989 for the very first time. Our present work based on this animal experiment has been very successful and was published in internationally acknowledged journals. Enclosed publications of the last three years in which the function of major genes in development of the blood system and leukaemia through transplantation was examined (please refer to the annex for more details): Malin et al. (2010, Nat. Immunol. *11*, 171-179); Zuber et al. (2011a; Genes Dev. *25*, 1628-1640); Zuber et al. (2011b, Nature *478*, 524-528); Vilagos et al. (2012; J. Exp. Med. *209*, 775-792).

Since the transplantation technique in this animal experiment also remains indispensable for researching important regulatory genes in the blood system differentiation and the development of leukaemia and lymphomas in the future we herewith submit an application for a permit for this animal experiment.

Type of Procedure and/or Treatment:

Haematopoietic progenitor cells are retrieved either from foetal liver cells (ED13.5-15.5) or bone marrow of adult mice. Prior to bone marrow extraction the animals receive often but not always intraperitoneal 5-fluororacil injections (150 mg/kg in 250 µl per mouse).

5-fluororacil is used to mobilise the haemocytoblasts so that haematopoietic stem and progenitor cells are present in the bone marrow during its extraction on day five. The retroviral infection of many stem and progenitor cells is decisive for the expression of the implanted genes in as many blood cells as possible after the transfer of the infected bone marrow into the recipient mice. Antibiotics are often added to the drinking water to prevent bacterial infections in the injected mice.

The animals are euthanized five days later through cervical dislocation exposure to CO_2. The marrow is washed out from femur and tibia.
For the retroviral gene transfer the cells are cultivated in a virus-containing medium for 48 to 72 hours and then injected (minimum of 0.5 x 10^6 cells/mouse) into a tail vein of sub-lethally radiated, genetically related mice (single dose of 9.5 Gy or dual dose of 2x 5 Gy). All transplanted mice should survive this experiment.

In certain transplantation experiments injected progenitor cells are used which after the retroviral gene transfer contain a gene controlled by a doxycycline dependent promoter element as well as a second gene for a doxycycline regulated activator (Gossen et al., Curr. Opin. Biotechnol. 5, 516-520). By adding the doxycycline antibiotic to the feed (625 mg/kg, Harlan Laboratories) and/or drinking water (2 mg/ml in a 2% sucrose solution) the doxycycline dependent gene can be specifically activated in the transplanted mice.

In the first days after the transplantation the animals will be intensively monitored and euthanized should radiation-based clinical symptoms occur. This will be carried out either through cervical dislocation or CO_2. Approximately four to six weeks after transplantation the animals will be examined regarding the reconstitution of their haematopoietic system through the donor progenitor and – if applicable – expression of the injected genes. For this purpose, a few drops of blood will be taken from the tail vein and analysed using cytology, differential haemogram and flow cytometry. In the case of implanting of bio-luminescent reporter genes (e.g. luciferase) the transplanted cells are additionally depicted using full-body bio-luminescence imaging (IVIS Spectrum, Caliper); initially two weeks after the transplantation and then at one to two week intervals. For this purpose the animals receive an intraperitoneal injection of D-luciferin (150 mg/kg in 200 µl) and are then analysed in the imager chamber under isofluran short anaesthesia (anaesthesia duration of approx. 5 min.; anaesthetic unit integrated in the IVIS Spectrum equipment; use in compliance with the manufacture's instructions.)

After euthanasia a comprehensive analysis of the haematopoietic system is carried out in vitro. Haematopoietic organs such as bone marrow, spleen, thymus and lymph nodes will be

removed. The cells of these organs are then molecularly and cytologically examined. When applicable, bone marrow cells from these animals will be reused for transplantation into secondary recipients.

End Point:

Generally, such experiments are deemed completed after all animals have been examined, i.e. normally after six months. Should such experiments deliver pathological clinical results (such as development of leukaemia and lymphomas) the animals will be immediately euthanized, and a post mortem examination performed. Documentation and analysis will be carried out in the laboratory journals of the respective scientists and serve as a basis for future publications.