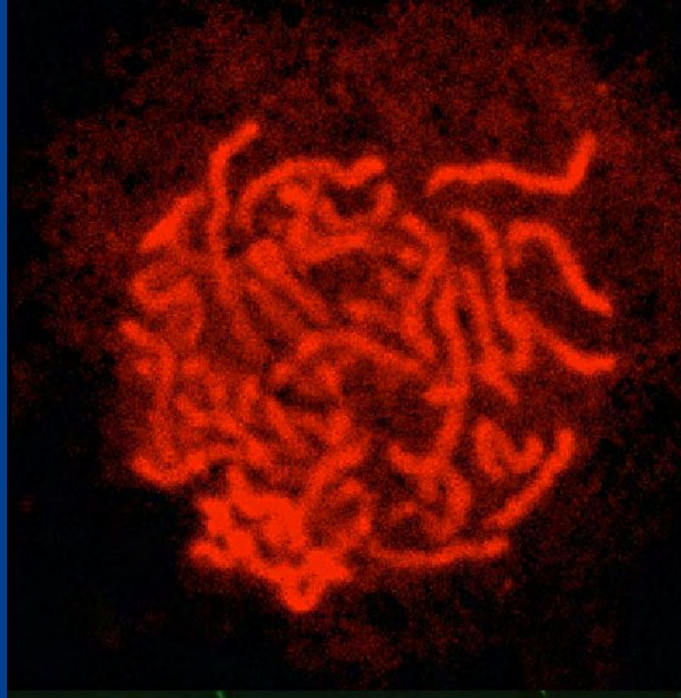
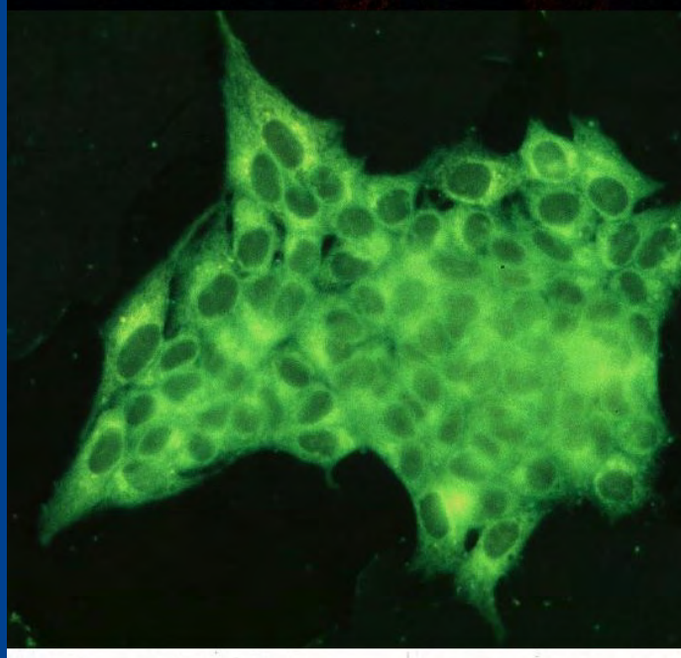
Biointerface

Faculty of Medicine

Role of Serum Proteins in Cell-Material Interactions





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Introduction

Cells naturally contact non-living materials and form an interface - typically a layer of biomolecules bridging the gap between the material surface and the cell surface. For example in the "Biomineralization" field the natural biointerface of cells and minerals is studied. Shell forming molluscs have cells that contact calcium carbonate containing shell structures. In the skeleton and in teeth bone forming cells, osteoblasts grow in direct contact with the mineral hydroxyapatite. Pathological mineralization in the wrong places of the body (ectopic) is often the consequence of chronic tissue damage. This is especially true for vascular tissues including heart valves and the aorta. Ectopic mineralization or "calcification" as it is often called, is highly increased in certain metabolic disorders disrupting the body's mineral homeostasis, i.e. the mechanisms maintaining constant blood levels of mineral ions in particular calcium and phosphate.

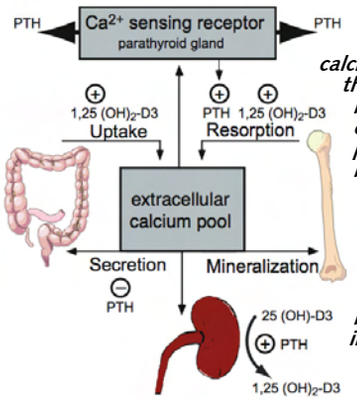


Figure 1: Simplified scheme of mammalian extracellular calcium homeostasis. In the gut calcium intake is effected by calcium binding proteins, calbindins, mediating epithelial transport to the blood. Excess calcium is secreted into faeces (gut) or urine (kidney). Parathyroid hormone (PTH) inhibits calcium secretion. In healthy subjects high serum calcium (and phosphate) enhances physiological bone mineralization. The extracellular calcium pool (blood and extracellular fluids) calcium concentration is monitored by a calcium sensing receptor in the parathyroid gland. Low serum calcium triggers the release of PTH, which stimulates bone resorption, renal calcium re-uptake in the kidney tubules and the synthesis of calcitriol (1, 25 (OH)$_2$ vitamin D3) in the kidney. Calcitriol stimulates both bone resorption and synthesis of calbindins and hence calcium uptake in the gut. Note that the kidney is involved at almost every step of this regulatory loop. Therefore patients with renal failure (no functioning kidneys or no kidneys at all) run an extremely high risk of vascular and soft tissue calcification. In addition their bone tissue becomes unresponsive to bone forming stimuli (adynamic bone disease) associated with excess soft tissue calcification.

In fact there is so much calcium and phosphate even in the blood of healthy individuals that according to chemical equilibrium insoluble calcium phosphate should precipitate spontaneously. This is however, and thankfully not the case. Efficient inhibitors of ectopic calcification must exist preventing the spontaneous mineralization of all tissues not meant to mineralize.

Our main line of research is concerned with the exact mechanism of mineralization prevention in the general circulation. We study this important phenomenon in a systemic approach: principally this includes all levels of complexity ranging from molecular interactions determined by high-resolution physicochemical methods, cell-based assays determining synthesis and transport of critical molecules to whole animal studies illustrating the impact of defined genes. To this end we employ gene knockout technology in mice.

The protein we know best is fetuin-A, a liver-derived protein secreted into the blood stream. Related proteins also under study are fetuin-B and the histidine-rich glycoprotein, HRG. An in depth study of fetuin-A synthesis requires a thorough understanding of liver cell physiology. Therefore we have adopted methods to directly study liver cells outside the body.

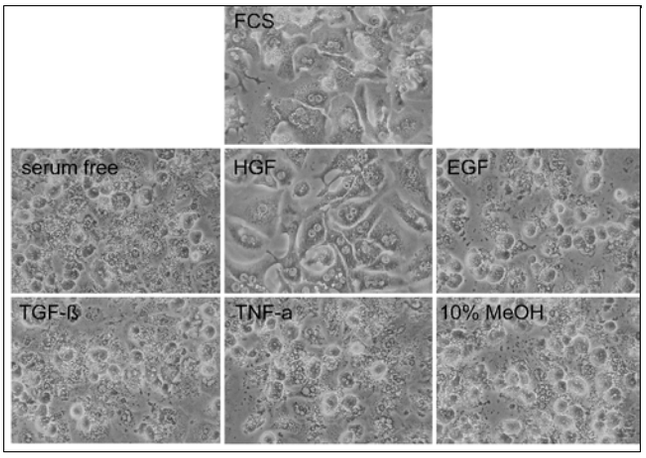


Figure 2: Hepatocytes from mouse liver freshly isolated and cultured with medium supplements as indicated. Fetal calf serum and serum-free medium containing hepatocyte growth factor sustained hepatocyte growth. All other supplements resulted in poor cell growth. When kept under optimal growth conditions HepG2 hepatoma cells actively make and secrete large amounts of fetuin-A protein.

Using fetuin-A deficient mice we firmly established that fetuin-A is a mineral binding protein preventing ectopic calcification in the body. Fetuin-A knockout mice spontaneously develop widespread soft tissue calcification, including significant myocardial calcification, whereas larger arteries are spared.

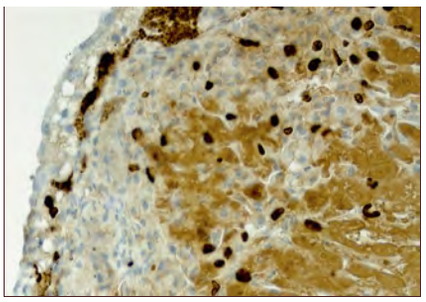


Figure 3: DNA-staining with bromodesoxyuridine, BrdU indicates vigorous cell growth of primary hepatocytes cultured under appropriate conditions.

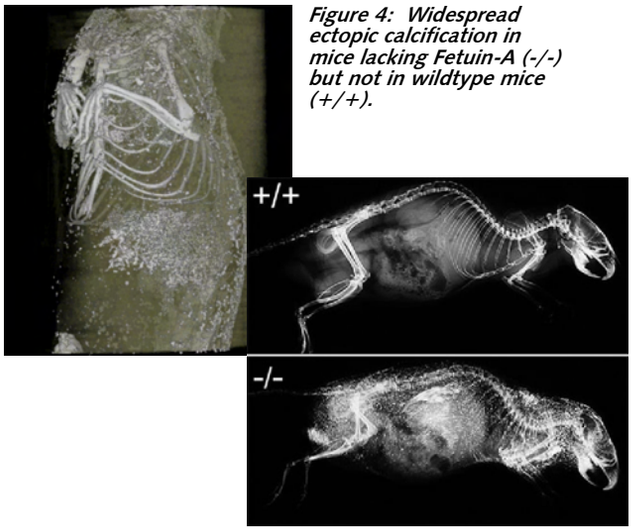
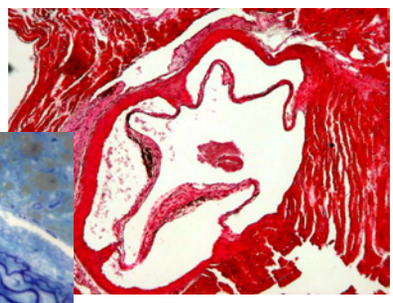


Figure 4: Widespread ectopic calcification in mice lacking Fetuin-A (-/-) but not in wildtype mice (+/+).

Fetuin-A, a major determinant of ectopic calcification

Unwanted mineralization namely vascular, valvular and soft-tissue calcifications increasingly afflict our aging and dysmetabolic population. Several studies documented that an increased calcification burden is associated with increased morbidity and mortality especially in uremia. In vitro and in vivo research has demonstrated that tissue calcification is based on passive calcium and phosphate precipitation as well as actively regulated principles resembling the mineralization of endochondral and membranous bone. A number of local and systemic calcium-regulatory factors control and prevent unwanted extraosseous calcification. Various extracellular calcification inhibitors such as fetuin-A, matrix Gla protein (MGP) and pyrophosphate were identified by genetic manipulation of mice. Of these, so far only fetuin-A deficiency has been found to be associated with increased mortality, cardiovascular calcification and calciphylaxis in uraemic patients.

Figure 5: Heart valve calcification in mice lacking Fetuin-A.



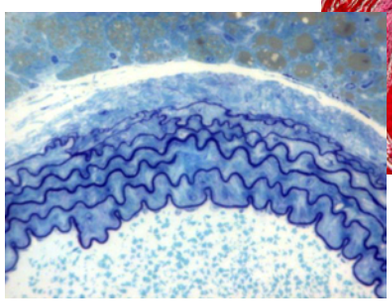


Figure 6: No calcification in arterial wall in untreated Fetuin-A deficient mice.

Fetuin-A is a systemic inhibitor of calcification

Fetuin-A is a liver-derived acidic glycoprotein that circulates in the blood and therefore works as a systemic inhibitor of ectopic calcification. Fetuin-A knockout mice spontaneously develop widespread soft tissue calcification, including significant calcification in major organs like lung, kidney and myocardium.

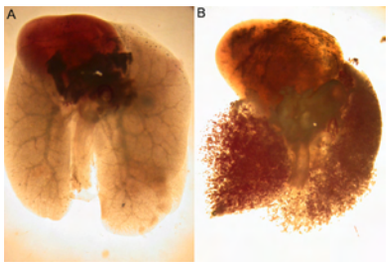


Figure 7: Macroscopic view of heart und lung of 10 month old wildtype (A) and fetuin-A deficient mice (B). The calcium phosphate precipitates in the fetuin-A knockout tissue are stained with Alizarin Red.

Recently, we studied the influence of calcification on the compliance of the cardiovascular system in the fetuin-KO mice in detail. In contrast to end-stage renal disease patients where the vascular calcification is predominant and the arterial stiffness, augmenting the aortic pulse wave velocity, results in augmentation of the central systolic blood pressure and in increasing left ventricular workload and left ventricular hypertrophy we found a different situation in the mouse model.

The fetuin-deficient mouse shows myocardial calcification, whereas larger arteries are spared. The calcification-associated myocardial stiffness was characterized by cardiac fibrosis, diastolic dysfunction and impaired tolerance to ischemia. Therefore this mouse model is suitable to study the functional role of isolated myocardial calcification on hemodynamics independent of arterial stiffness.

Figure 8: Aortic plaque typical of atherosclerosis. Representative macroscopic findings of oil red O- stained aortal preparations of 50-week-old female mice fed a normal chow diet, showing red lipid-rich atherosclerotic lesions. Left: wildtype control mouse, right: Apolipoprotein E-deficient mouse.

One important difference in vascular calcification of dialysis patients and the calcification-prone fetuin-A knockout mice may be that endothelium is intact in the mice contrasting the human situation, where atherosclerosis serves as a nidus for calcification. To test this hypothesis we crossed the fetuin-deficient mice with apolipoprotein E knockout mice, which spontaneously develop atherosclerosis. The phenotypic analysis of these mice under phosphate and uremic challenge is ongoing and will provide a model of human metabolic disease.

Studying calcified globules in the skin of fetuin-A-deficient mice we are analysing the composition of the globules (mineral and protein content) as well as cellular reactions to the buildup of calcifying remnants.

Our unique collection of fetuin-A deficient mouse strains will enable us to identify additional genes involved in ectopic calcification. To this end we perform a gene expression analysis of fetuin-deficient mice on the C57BL/6 genetic background (no spontaneous calcification) and the DBA/2 genetic background (highly prevalent systemic calcification) with Affymetrix DNA Chips.

How Fetuin-A works - Discovery of Calciprotein Particles

In mammals all body fluids are supersaturated with regard to calcium and phosphate necessitating potent inhibitors of mineral deposition. One of the chief inhibitors is fetuin-A that buffers the high ion load in serum by formation of soluble colloidal mineral containing particles. We termed these high molecular weight complexes "colloidal calciprotein particles", CPP in analogy to lipoprotein particles. Dynamic light scattering suggested a spontaneous nucleation but a drastically reduced growth rate in the presence of fetuin-A at supersaturated conditions.

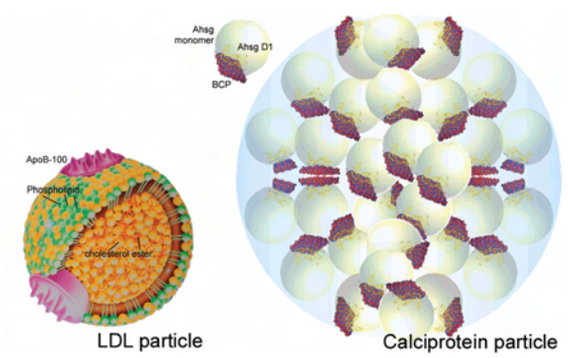


Figure 9: Molecular topology of low density lipoprotein particles, LDL and calciprotein particles, CPP.

CPPs in vitro at 37°C are stable for up to 24h. Within this time they have to be cleared from circulation in the body. The clearing process is equally important like the colloid formation in preventing unwanted calcification. Therefore we generated in vitro biotinylated calciprotein particles and injected them in comparison to biotinylated fetuin monomer into mice to study the clearance of both (kinetic and target tissues). Using this approach, we want to identify the receptor(s) and accessory molecules which are involved.

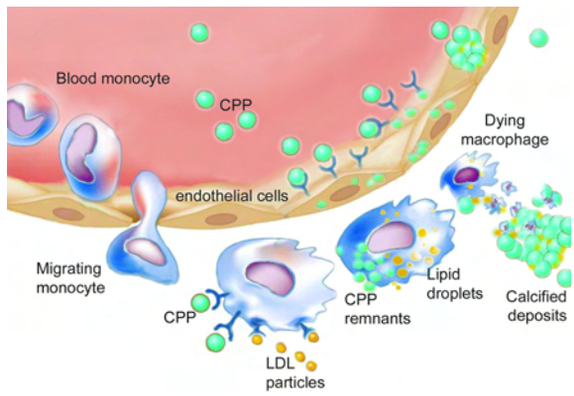


Figure 10: Proposed clearing pathways for Calciprotein particles, CPP and low density lipoprotein particles, LDL share important features.

More detailed information about size, shape, density and internal topology of colloids is obtained from small angle neutron scattering (SANS). In cooperation with D. Schwahn from the FZ-Jülich, we began to study the CPPs employing this sophisticated technique.

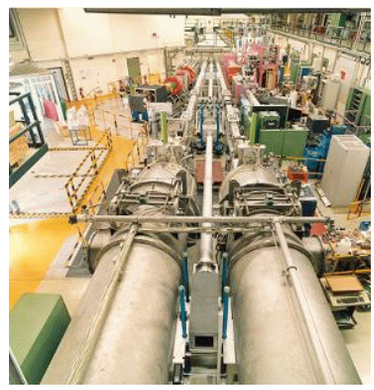


Figure 11: Big machines for small phenomena: experimental hall at the neutron scattering facility of the Forschungszentrum Jülich. The long collimator and detector tubes of the SANS instrument (20 m each) can be seen at the center.

The Fetuin-A-mineral interface

The effective inhibition of calcium phosphate precipitation indicates a strong interaction at the protein-mineral interface. However, as no experimental X-ray or NMR data is available we generated models of all three fetuin-A domains by comparative structure modelling using the MODELLER software package. A comparison of experimental data from deletion mutants with the modelled structures led to the conclusion that the highly acidic extended ß-sheet of the first fetuin-A domain mediates the attachment to the mineral surface.

To explore the quaternary structure of fetuin-A, the computer generated models (i.e. the pdb coordinate files) were used to assemble three dimensional models of these fetuin-A domains by rapid prototyping. This model helps us to evaluate computer generated domain assemblies, map antibody epitopes and putative carbohydrate binding sites.

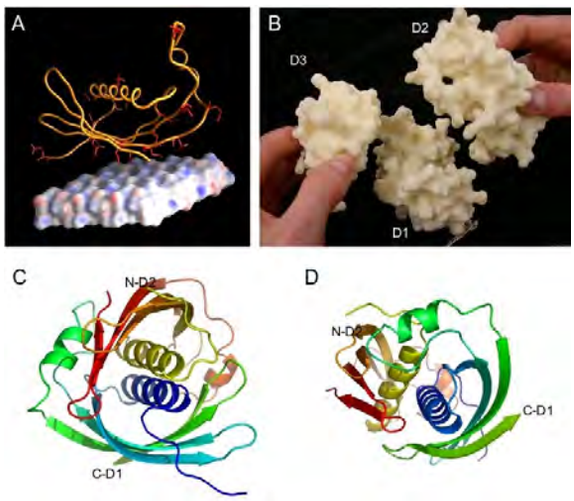


Figure 12: (A) acidic residues of the fetuin-A domain 1 interacting with the (001) plane of hydroxyapatite. (B) Fetuin-A domains 1-3 made by rapid prototyping. (C&D) Quaternary structure models of the cystatin-like domains D1 and D2.

HRG knockout - another entry in our mouse zoo

Histidine-rich glycoprotein (HRG) is a single-chain plasma protein that circulates at relatively high amounts of 100 to 150 mg per liter in the blood of many vertebrates. It is found in human, mice, rats, rabbits, chicken and cows.

HRG is a multi domain glycoprotein with a molecular weight of 75 kDa consisting of two cystatin-like domains at the N-terminus, a histidine/proline-rich domain and a C-terminal domain. Due to its N-terminal cystatin-like domains HRG belongs to the cystatin super family of proteins like fetuin-A, fetuin-B and kininogen.

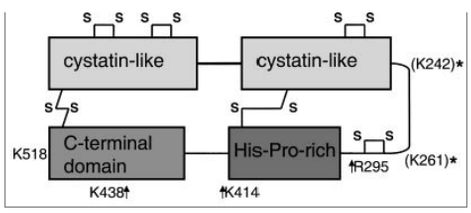


Figure 13: Domain structure of human HRG (taken from Juarez et al. Cancer Res. 2002).

Limited plasmin proteolysis cleaves HRG at the sites indicated by arrows, followed by sites indicated by * on further incubation. Subsequent treatment with DTT after controlled digestion releases the H/P domain. The remaining molecule is the N/C fragment, which is held together by a buried disulfide bond between the NH_2- and COOH-terminal domains.

HRG is synthesized exclusively in the liver but it is also found in megakaryocytes and platelets, which can release it after thrombin stimulation. HRG is known to interact with various ligands in vitro. Molecular binding of HRG to different ligands is mediated through specific binding domains, which act independent of each other. Binding to heparin, plasminogen and fibrinogen suggests a role in hemostasis and fibrinolysis. Indeed, several individuals with a congenital deficiency of HRG were discovered in three families with thrombophilia corroborating such a role.

HRG also binds divalent metal ions of which Zn^{2+} seems to be one of the most important. Binding of Zn^{2+} via the histidine-proline rich domain strongly enhances the affinity of HRG to heparan sulfate proteoglycans on cell surfaces. Such a mechanism was also experimentally verified for HRG binding of plasminogen. The authors proposed that under conditions of low pH and elevated free Zn^{2+}, HRG acts as a high-affinity receptor for plasminogen thus enhancing the migratory potential of cells. This binding behaviour can greatly influence HRG bioactivity at sites of local changes in pH value and Zn^{2+} concentration, suggesting that HRG may be a tunable adaptor protein for several ligands involved in inflammation and tissue remodelling.

Furthermore HRG interacts with several components belonging to the immune system, such as IgG, FcγR and C1q. HRG is reported to bind to macrophages and supports the phagocytosis of apoptotic as well as necrotic cells and thus may play a key role in maintaining the clearance of apoptotic cells and necrotic cell debris from the circulation. Recently, it was reported that HRG has potent antiangiogenic activity. Despite these abundant biological properties reported for HRG, an unequivocal physiological function has yet to be established. To explore the function of HRG in vivo, we cloned the mouse HRG gene and produced, by homologous recombination, mice lacking HRG.

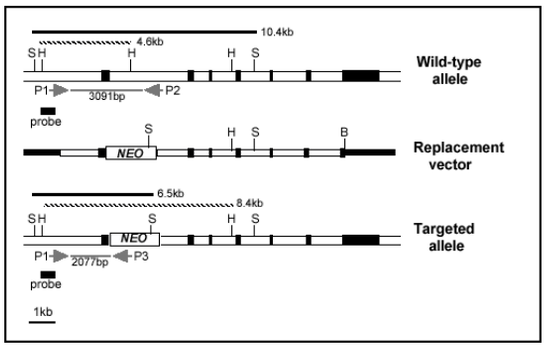


Figure 14: Structure of the mouse Hrg gene. The mouse Hrg gene and its partial restriction map are schematically drawn as the wildtype allele (Top). Exons are represented by black boxes. Restriction enzymes are BamHI (B), HindIII (H), and ScaI (S). The targeting vector is drawn in the middle. The 3'-portion of exon I and the 5'-half of intron A were replaced by the neomycin cassette in the targeted Hrg gene (bottom) effectively removing the translation start. The position of PCR primers (P1, P2, and P3) and the size of the expected PCR products are shown below the genes. Restriction fragments digested by ScaI or HindIII restriction enzymes are indicated with their respective sizes in kb probed by a 0.6 kb SmaI fragment.

For this purpose a targeting vector for homologous recombination was constructed and transfected into embryonic stem cells by electroporation.

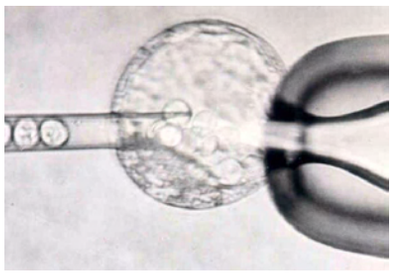


Figure 15: Injection into blastocysts of genetically modified embryonic stem cells carrying the targeted HRG locus to create partially (chimeric) HRG-deficient embryos for transfer into foster mother mice.

Two independent correctly targeted ES cell clones were successfully used to derive chimeric mice after blastocyst injection and embryo transfer.

Both clones were derived into chimeric mice that transmitted the targeted HRG locus through the germline. Mice of all three Hrg genotypes were born from crosses of heterozygous F1 offspring.

Figure 16: Chimeric HRG deficient mice

The chimeric mice were bred into the F1 generation thus creating heterozygous HRG-deficient mice.

Further breeding onto defined background created a new mouse strain, 129/B6-*Hrg*^{tm1Wja}.

Figure 17: F1-offspring homozygous HRG deficient mice

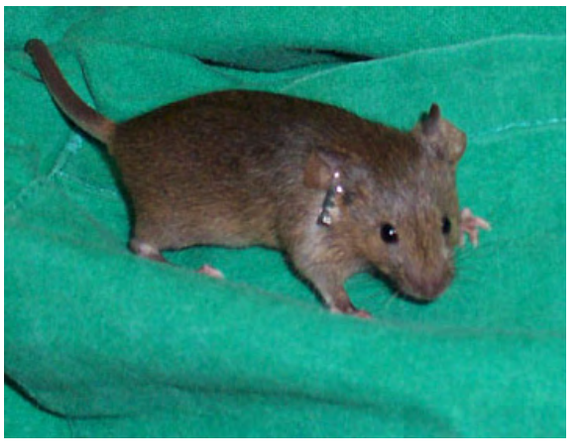
Analysis of HRG deficient mice

Homozygous HRG-deficient mice were phenotypically normal. They were viable and produced healthy offspring. Spontaneous mortality during the first three months was normal at 2.6%.We performed routine histology staining and could not find any abnormalities in HRG-deficient mice compared to wild type.

Previous work suggested that HRG plays a regulatory role in hemostasis and fibrinolysis and to explore this we performed coagulation assays. We determined modest but statistically significant differences between HRG knockout and wildtype mice suggesting that HRG plays a role both as an anticoagulant and antifibrinolytic modifier, and may regulate platelet function in vivo.

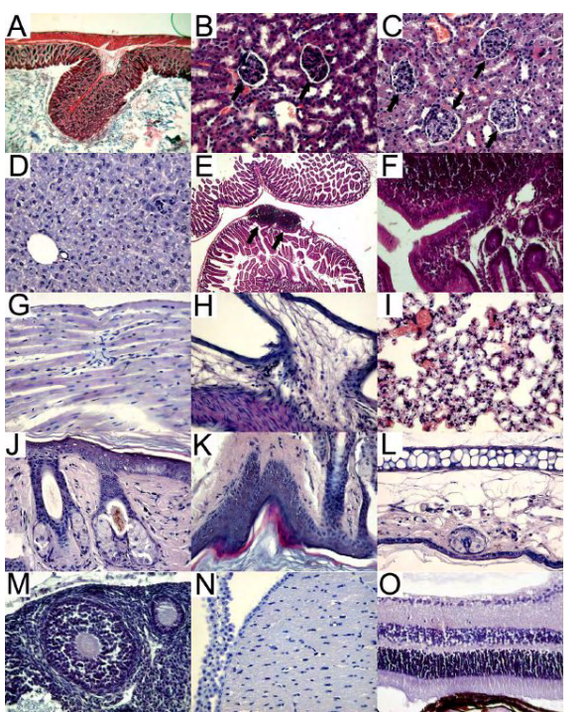
Figure 18: Histological analysis of paraffin sections of tissues from HRG-deficient mice showing no gross abnormalities in A)skin, B)C)kidney, D) liver, E) F) intestine, G) skeletial muscle, H) fat. I) lung, J) skin hair follicles, K) tongue, L) ear, M) ovary, N) eye lens or O) retina.

It's a knockout!



As of 2005 we have also successfully generated knockout mice for the second member of the fetuin family of proteins, fetuin-B. Up until the year 2000 fetuin-A has been the only known member of this family. Fetuin-B was discovered as a negatively regulated acute phase protein in the liver, which is one feature it has in common with fetuin-A. Fetuins belong to the cystatin superfamily. All its members (fetuins, histidine-rich-glycoprotein and kininogens) are located within a tight gene cluster on mouse chromosome 16, region B1. This arrangement is mirrored within the human genome on chromosome three.

The first homozygous fetuin-B deficient mice are vital and do not show an obvious phenotype. The physiological function of the fetuin- B protein remains to be determined. Plenty of work ahead for 2006!



Acknowledgements

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Further reading

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Team

Our Team with colleagues from the Interdisciplinary Centre for Clinical Research on Biomaterials and Implants (IZKF BioMAT) in December 2005

Facts

Third-party funding

	Number of Projects	Total Expense of Projects
German Research Foundation (DFG)	17	764.375 €
German Federal Ministry of Education and Research (BMBF)	26	851.670 €
EU	1	42.082 €
Industry	20	501.692 €
other	48	845.219 €
Sum	112	3.005.038 €

Theses

	Number
Bachelor	2
Diploma / Master	25
Doctoral	11

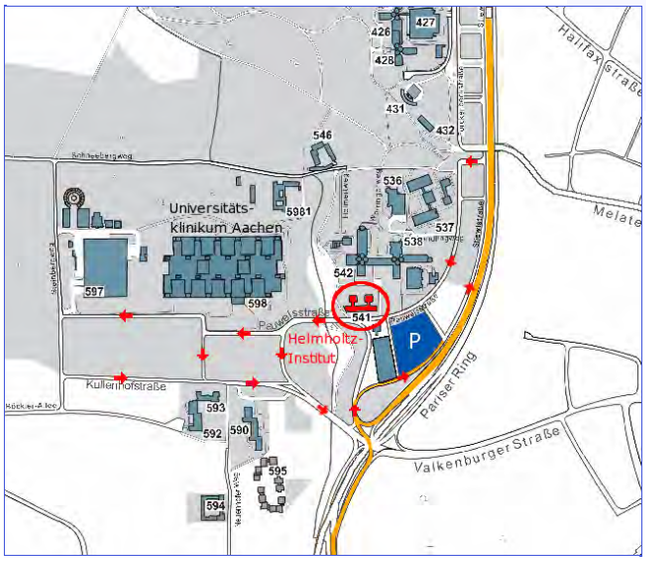
Personnel

	scientific	non-scientific
established posts	26	21,5
project-based posts	74,5	53

in full-time equivalents (FTE)

How to reach us

At the Aachen motorway intersection hold on the Holland route toward Antwerpen / Heerlen. You leave the motorway with exit no. 2, Aachen-Laurensberg, drive off right, then farther direction Maastricht and follow the shuttle with 4 lanes with the signpost "Universitätsklinikum".



After leaving the Pariser Ring turn right on the Stiwistraße. Park your car after 200 meters on the parking site to your left, behind the Medizintechnisches Zentrum (MTZ). The Helmholtz Institute is house number 20 (RWTH building number 541), you find it on the opposite side.