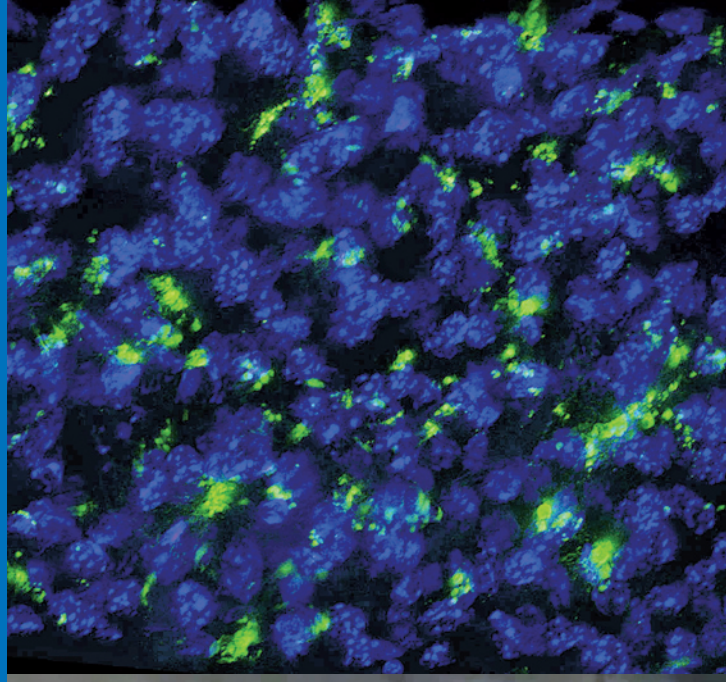
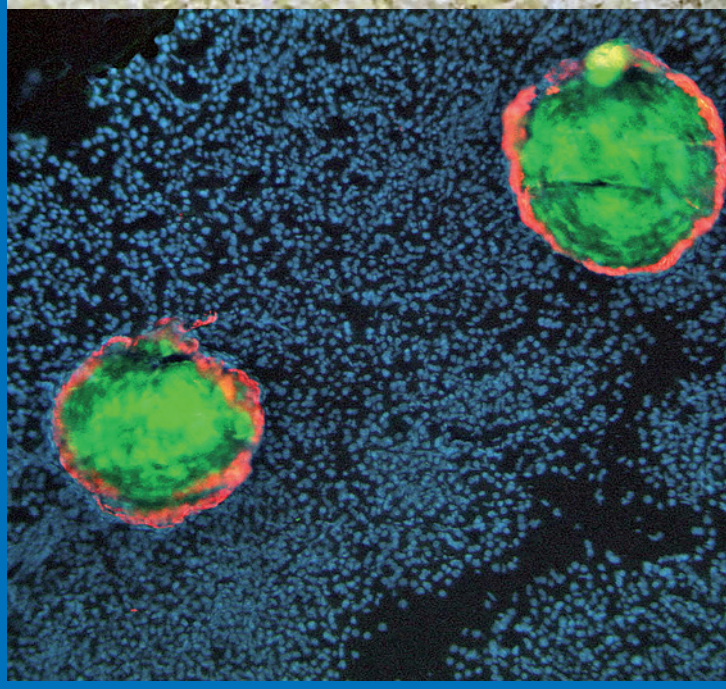
Biointerface

Faculty of Medicine

Cell-Material Interactions: Translating Basic Science Into Clinical Applications





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Title Figure Top: *Fetuin-A (green) containing calciprotein particle clearance by liver Kupffer cells; Center, chimeric mouse babies with their mother; Bottom, calcified lesions stained in vivo by fetuin-A (green) and osteopontin (red).*

Introduction

The Biointerface Group has celebrated six Master graduations, two Bachelors and one Friedrich-Wilhelm-Prize in the past year. Four students will continue their projects into PhD work two more are soon to follow. Like the people the projects have matured approaching the publication stage. Time to wrap up - with the help of Alexander Heiss and Cora Schäfer who left in late 2010 and with our longtime clinical partner Markus Ketteler we have published an extensive review on the role of fetuin-A in mineralized matrix metabolism.[1] The review puts our own two decades of work on the role of fetuin-A in mineralization biology into a larger context of what other people have found. We always thought that the "functions" of fetuin-A *in vitro* is virtually boundless because there is so many ways this protein can interact.[1-8] Some of the findings *in vitro* clearly went down the wrong track and ended up in a red herring chase.[9] We have learned however, that more than one facet of fetuin-A binding might also apply to living animals. Fetuin-A is involved in mineralization biology, lipid metabolism and other important biological pathways including inflammation and cancer.[4, 7] Even though we do not currently understand all the details we can begin to harness the interesting binding properties of fetuin-A. Anne Kinkeldey introduces the potential use of fetuin-A as a molecular imaging probe for calcifying atherosclerotic lesions.

Fetuin-A Based *In Vivo* Imaging of Calcified Lesions

(Anne Kinkeldey)

Fetuin-A is a liver-derived serum protein involved in mineral homeostasis. Fetuin-A deficient mice show widespread soft tissue calcifications supporting the notion that fetuin-A is a mineral chaperone mediating the stabilization of protein-mineral complexes and their clearance in the body. Fetuin-A solubilizes basic calcium phosphate by forming colloids. It also has a strong affinity for apatite in mineralized bone. We hypothesized that fluorescent fetuin-A could likewise label calcified lesions and thus could be used for imaging. To this end we synthesized fluorescent fetuin-A derivatives for histology and Fluorescence Molecular Tomography (FMT).

Labeled fetuin-A strongly bound calcified lesions when incubated on histological sections of the organs known to calcify in fetuin-A deficient mice – kidney, heart, lung, brown adipose tissue – and thus enabled their detection via fluorescence microscopy (Fig. 1).

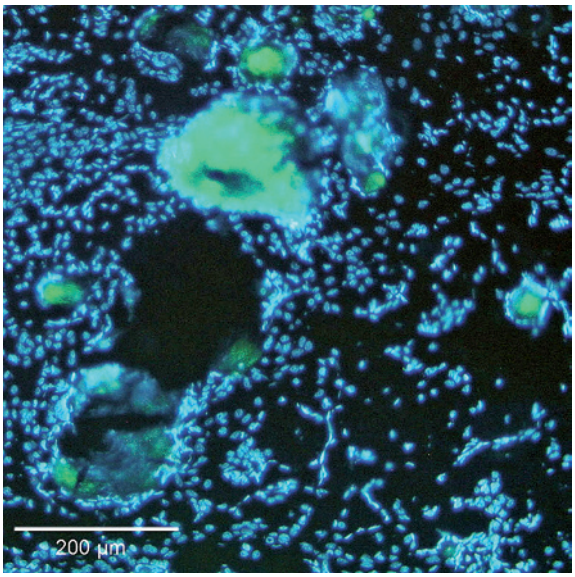

Fig. 1: Staining of calcified lesions with fetuin-A Alexa Fluor® 488. *Shown is a cryosection from brown adipose tissue of a DBA/2 fetuin-A -/- mouse. The section was stained with Alexa Fluor® 488-labeled fetuin-A (green) strongly binding to calcified lesions. Cell nuclei were stained with DAPI (blue).*

Intraperitoneal injection of labeled fetuin-A into living mice likewise demarcated calcified lesions and facilitated their microscopic observation (Fig. 2). Note that in contrast to the method mentioned above, fluorescent signal is detectable only around the lesions and not on the core of the lesions. This different distribution is due to the fact that the binding *in vivo* is limited to the surface of the lesions.

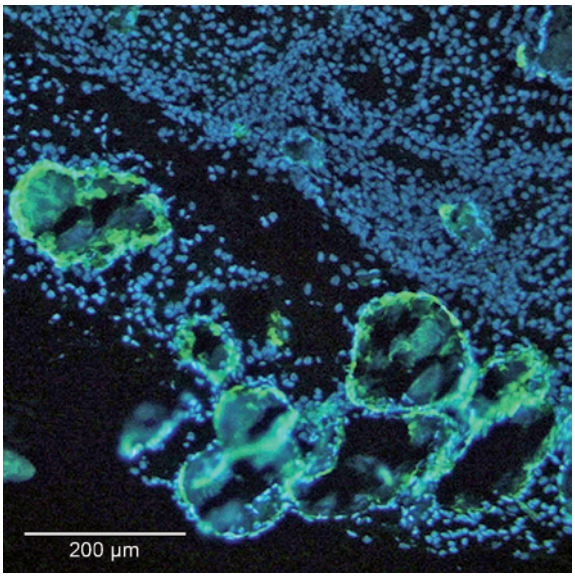

Fig. 2: Staining of calcified lesions in DBA/2 fetuin-A -/- mice with i.p. injected fetuin-A Alexa Fluor® 488. *Shown is a cryosection from brown adipose tissue from a mouse injected with a single bolus of Alexa Fluor® 488-labeled fetuin-A. Cell nuclei were stained with DAPI (blue).*

There are many possible applications for imaging with fetuin-A - one is the visualization of calcifying atherosclerosis. To show labeling of atherosclerotic lesions we employed a mouse model prone to develop atherosclerotic plaque, the

apolipoprotein E / fetuin-A double deficient mouse. The bifurcation of carotid arteries in these animals is known to be an area where plaque formation occurs frequently. Thus carotid arteries of apolipoprotein E / fetuin-A double deficient animals were analyzed 24 h post i.p. injection of fluorescent fetuin-A by Two-Photon Laser Scanning Microscopy (TPLSM) to detect calcified structures (Fig. 3).

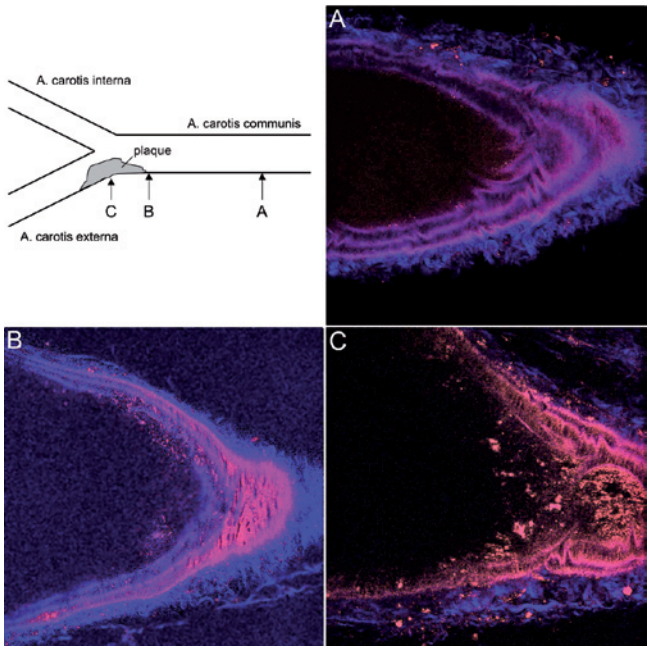

Fig. 3: Staining of atherosclerotic carotid arteries with fetuin-A Alexa Fluor® 546. *An apolipoprotein E / fetuin-A double deficient mouse was injected with fetuin-A Alexa Fluor® 546. After 24 h the carotid artery was excised and mounted on a perfusion chamber for imaging with 2-photon microscopy. A: Plaque free area. Collagen fibres show blue autofluorescence. B: Border region of the plaque. Red fluorescence derived from injected fetuin-A Alexa Fluor® 546 was detected in the media of the carotid artery, suggesting vascular smooth muscle cell calcification. C: Atherosclerotic plaque in the bifurcation zone of the carotid artery. Fetuin-A Alexa Fluor® 546 signal in the media suggests remodeling of vascular layers in plaque regions. Staining in the lumen of the vessel depicts the calcified plaque core (Collaboration with Martin Schmitt, IMCAR).*

Another promising approach is the *in vivo* whole animal imaging achieved by Fluorescence Molecular Tomography (FMT). Fetuin-A coupled to a near-infrared fluorophore offers the possibility to detect calcified lesions deep in the body with the help of laser-based tomography (Fig. 4). To receive meaningful data concerning the distribution of signal in the body, the FMT pictures were co-registered with CT data of the same animal. This experimental setting enables the observation of different calcification related diseases.

In conclusion, using labeled fetuin-A as an imaging tool offers great possibilities for the detection and observation of calcified atherosclerotic lesions. The method has been patented by RWTH Aachen University.

Immediate neighbors of the gene for fetuin-A (AHSG or FETUA) in the genome are the genes for fetuin-B (FETUB) and histidine-rich glycoprotein (HRG).

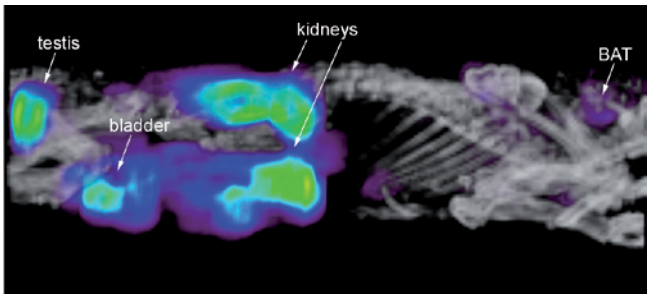

Fig. 4: Fetuin-A Alexa Fluor® 680 fluorescence molecular tomography in a DBA/2 fetuin-A –/– mouse. *The animal was injected i.p. with Alexa Fluor® 680-labeled fetuin-A 24 h before measurement. In vivo whole body fluorescence was recorded in 3D tomography mode and fused with the corresponding CT scan. The fluorescence signal was detected in the kidneys, testis and brown adipose tissue. Furthermore, fluorescence visible in the bladder was due to the excretion pathway (Collaboration with EXMI).*

Collectively these genes form a sub-family of secreted cystatin-domain containing proteins within the cystatin superfamily. We reported on this relationship in a review article that was concatenated in the 2009 HIA Report. Our collaborators in Sweden and the United States have used our HRG-deficient mice and reagents to further our knowledge on the biological role of HRG especially in cancer biology.[10]

Eileen Dietzel and Julia Floehr have been working on the immediate next neighbor of Fetuin-A in the genome and they will tell anyone that fetuin-B »has nothing to do with calcification«, but much more with fertilization biology. Through their BSc and MSc theses Eileen and Julia have steadfastly continued the work started by Jenny Wessling in her PhD thesis. Together they have made a major step in unraveling the biological role of fetuin-B.

A Role for Fetuin-B in Reproduction

(Eileen Dietzel)

Fetuin-B is a liver-derived plasma protein like fetuin-A. Despite structure similarity on both the amino acid sequence level and the domain structural level, these two proteins probably have completely different functions in mammals. While fetuin-A is mainly involved in mineralized matrix metabolism,[1] fetuin-B is involved in female fertility. Fetuin-B deficient mice are anatomically and metabolically inconspicous, yet 100% female infertile.

Hypothesis about the Female Infertility of Fetuin-B Deficient Mice

Female fertility is influenced by many factors. Firstly oocytes must develop properly. Many hormones and cytokines especially members of the TGFβ family must reach the oocyte at the right time in the right amount. The time of ovulation

is equally critical. If oocytes are released prematurely they are usually not ready for fertilization. If they leave the ovary too late they may start to degrade. Following ovulation it is essential for the sperm to reach the oocyte by swimming up the female reproductive tract. On their journey towards the oocyte sperm cells are guided by mechanical and chemical cues provided by the linings of the female reproductive tract. On this way the sperms are changed by female factors. This process is called capacitation, and only if this happened the spermatozoen is able to fertilize the oocyte. If all the previous requirements were met the sperm will reach the oocyte. Next it has to penetrate a layer of extracellular matrix that surrounds the oocyte, the *Zona pellucida* (ZP). After the two gametes have fused the ZP changes its structure, rendering it more resistant against enzyme digestion and thus »hardened«. This process is called ZP hardening, which sometimes happens spontaneously, often causing a problem in *in vitro* fertilizations (IVF). Next the fertilized oocyte has to be implanted into the uterus for the embryo to start development. Work dating from the 1990ies suggested that fetuin-A is an inhibitor of ZP hardening. Given the fact that our fetuin-A deficient mice are fully fertile we hypothesized that fetuin-A and fetuin-B may have been intermingles. This is easily excused, because only one fetuin - fetuin-A - was known at the time. Fetuin-B only entered stage in 2000-2003 also through our own work described in the HIA Report 2005.

Oocytes of Fetuin-B Deficient Females Develop Normally and Ovulate

To check the development of the oocytes we performed routine histology of ovaries. Thus we could show that all follicular developmental stages were present (Fig. 5). Further we determined the different developmental stages and established that the fetuin-B deficient follicles developed in numbers comparable to the wildtype. We could further demonstrate that the follicles can be ovulated and that the fetuin-B deficient females do not show any effects in the four most important hormones for female reproduction (estrogen, progesterone, luteinizing hormone, follicle stimulating hormone). This indicated that the oocytes should be ready for fertilization, but that there was a defect in oocyte-sperm interaction.

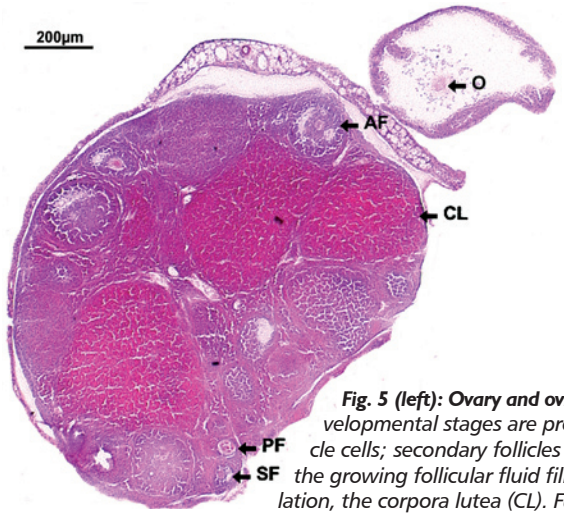

Fig. 5 (left): Ovary and oviduct of a fetuin-B deficient mouse after H&E staining. *All follicular developmental stages are present: primary follicles (PF), with only one surrounding layer of follicle cells; secondary follicles (SF), showing two or more follicle cell layers; antral follicles (AF), with the growing follicular fluid filled space (the antrum) and the remained follicular cells after the ovulation, the corpora lutea (CL). Further an oocyte (O) after ovulation is visible in the oviduct.*

Oocytes of Female Fetuin-B Deficient Mice Can only Be Fertilized after Breaking the ZP

We analyzed oocytes with the surrounding cumulus cells for signs of fertilization. While the wildtype oocytes showed two polar bodies, a bigger perivitelline space and thus appeared fertilized, the fetuin-B deficient oocytes appeared unfertilized (Fig. 6 A, B). Next we performed *in vitro* fertilization (IVF) (Fig. 6 C, D) (Collaboration with Tanja Tropartz, Experimental Animal Science). Again the oocytes of the fetuin-B deficient mice could not be fertilized. Therefore we performed laser-assisted IVF (LA-IVF) to penetrate the ZP using a short laser pulse. Indeed following LA-IVF we could observe two-cell embryos derived from the fetuin-B deficient oocytes (Fig. 6 E, F). This shows on the one hand, that the oocytes were mature and in principle could be fertilized. On the other hand the laser experiment also showed that the prime reason for infertility of fetuin-B deficient females was a hardened ZP.

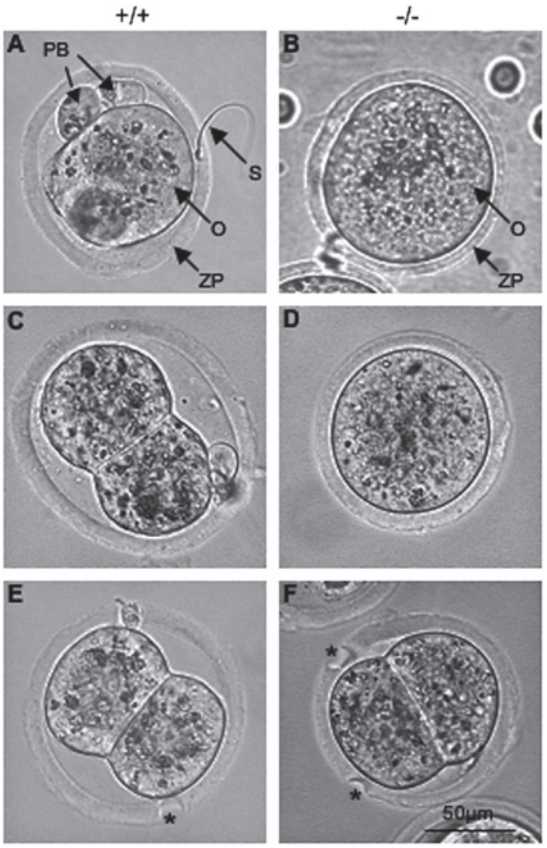

Fig. 6: Fertilized wildtype (+/+) and fetuin-B deficient (-/-) oocytes. *0.5 days after natural mating only the wildtype (A) oocytes (O) showed signs of fertilization like two polar bodies (PB). The Zona pellucida (ZP) and a sperm (S) adhering to the ZP are visible. In fetuin-B deficient oocytes the ZP appears tight (B). 1.5 days after in vitro fertilization (IVF) only wildtype oocytes (C) were fertilized, while the fetuin-B deficient oocytes (D) remained unfertilized. 1.5 days after laser assisted IVF also the fetuin-B deficient oocytes developed into 2-cell-stages (F). The laser perforations are marked with asteriks *.*

Fetuin-B is Essential for Inhibiting ZP Hardening

Premature ZP hardening was confirmed by an enzyme assay demonstrating that the ZP of the fetuin-B deficient oocytes were digested more slowly than the ZP of the unfertilized wildtype oocytes, but comparable to the 2-cell-stages (Fig. 7). This finding strongly suggests that the ZP of the fetuin-B deficient oocytes had hardened before the oocytes were fertilized.

Thus fetuin-B is an essential non-hormonal factor in female reproduction in mice. We are currently testing fetuin-B as supplement for IVF to keep the oocytes soft and fertilizable. Last but not least fetuin-B might be a target for non-hormonal birth control.

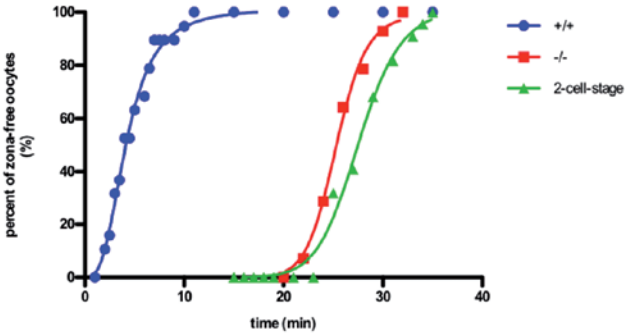

Fig. 7: Digestion of the ZP of wildtype (+/+) and fetuin-B deficient (-/-) mice, as well as 2-cell-stages. *The ZPs of fetuin-B deficient oocytes need much longer to be digested compared to the wildtypes, but the digestion almost takes the same time compared to the 2-cell-stages with known harded ZP.*

Apart from the basic science shown above on fetuin-A and fetuin-B a more applied line of research concerns ways of combining cells and materials for regenerative medicine.

Stem Cells and Tissue Engineering

(Sabine Neuß-Stein)

Our research on stem cells and tissue engineering explores a variety of human, murine and ovine stem cells. These stem cell types can be divided into (i) pluripotent stem cells, such as embryonic stem cells (ES cells), induced pluripotent stem cells (iPS cells) and germline-derived pluripotent stem cells (gPS cells), all possessing the potential to generate a whole organism and (ii) multipotent adult stem cells including e.g. mesenchymal stem cells (MSC) and the well-known hematopoietic stem cells (HSC). Projects are focusing on stem cell-based tissue engineering as depicted in Fig. 8. We modify biomaterials to support stem cell adhesion via specific receptors, stem cell growth and proliferation [11, 12] or stem cell differentiation, in particular osteogenic

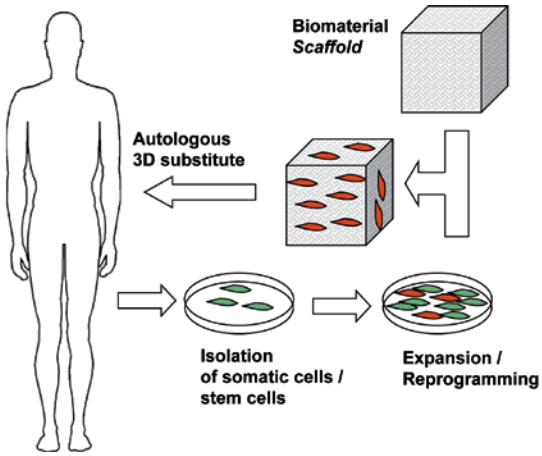

Fig. 8: Control of cell-biomaterial interaction critically determines the successful tissue engineering.

differentiation [13] (van de Kamp et al., Differentiation, in press). Further, an established biomaterial test platform is used to analyze in a standardized and automatic manner up to 20 biomaterials in parallel to identify suitable candidates for specific tissue engineering applications [14].
In addition, we aim at mimicking *in vitro* the highly specialized microenvironment of stem cells *in vivo*, referred to as stem cell niche. Here we are in particular interested in mimicking the HSC niche, by combining three-dimensional scaffolds (e.g. collagen gels) with stromal cells and biochemical factors [11].

Awards in 2011

Sabine Neuss-Stein received the Friedrich-Wilhelm-Preis 2011 of the RWTH Aachen for her habilitation entitled "Mesenchymal stem cells and their interactions with biomaterials for tissue engineering applications". Her habilitation led up to the papers published in 2011 fully justifying this prestigous award [11-19].

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Team

Fig. 9: The Biointerface Group at the Rursee Labout in June 2011.

Fig. 10: Little Carlotta, Lotta and Phil. *Three perfect reasons why Sabine Neuß-Stein and Jenny Wessling are missing on the group photo.*