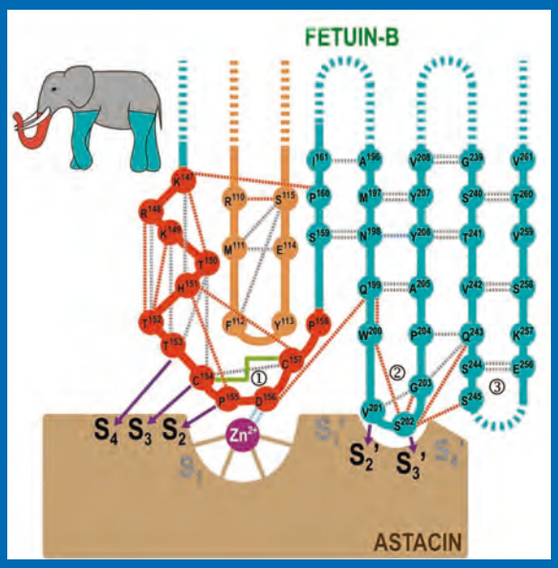
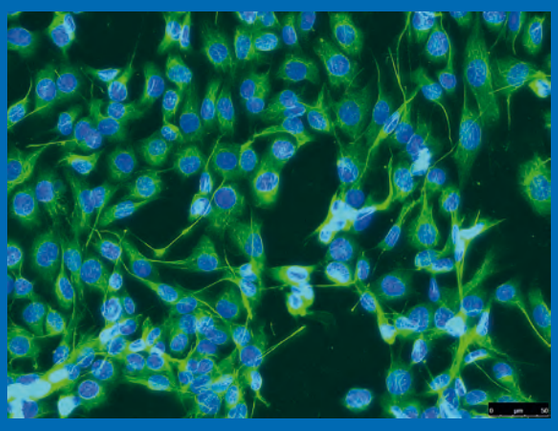
Biointerface Laboratory at Helmholtz-Institute for Biomedical Engineering

RWTH AACHEN UNIVERSITY

Faculty of Medicine

Cell-Material Interactions: Translating Basic Science Into Clinical Applications





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***Cover Figures: Top**, mouse oocyte or "egg". **Center,** "raised-elephant-trunk" mechanism for astacin (light brown) inhibition by fetuin-B. The CPDCP amino acid motif in the linker region (red) of fetuin-B forms the raised trunk, while hairpin I and hairpin II of cystatin-like domain 2 (teal) form the front and back limbs of the elephant, respectively. **Bottom,** synthetic phenotype of vascular smooth muscle cells. Cytoskeleton myosin stained green and nuclei stained blue.*

Introduction

In the year 2019 we finally gained some insight into the "holy grail of protein science" of fetuin-B, a liver-derived blood protein involved in female fertility. As among the leading groups on fetuin researchers worldwide this constitutes a major collaborative achievement. With Xavier Gomis-Rüth's group from Barcelona in the lead, and Walter Stöcker's (Mainz) and Luca Jovine's groups (Stockholm) at our side, we co-published the three-dimensional structure of fetuin-B bound to its target proteinase, astacin. To a molecular biologist the three-dimensional structure and a mechanistic view to the detail of atom-atom interaction is as good as it gets in understanding how "nature works". Two post-Docs in the group, Carlo Schmitz and Julia Floehr, explain the structure of fetuin-B in the first topic of this research report. The second contribution by PhD students Andrea Büscher and Sina Köppert and Master student Aaron Morgan describe ongoing work on the role of protein-mineral complexes called calciprotein particles, an entity which we have first described in 2003. Aaron, an engineer by training adds a strong technical spin to this line of work by designing and fabricating 3D-printed microincubators hosting organs-on-a-chip. This kind of device enables complex interaction studies that go well beyond conventional single cell type culture, and complement our line of mouse experimentation. Finally, Sabine Neuss-Stein reports on progress made in her group towards cardiovascular and bone tissue engineering solutions. This highly interdisciplinary work improves biomaterials either by preventing adverse reactions, or by adding biological activity to direct cell behavior.

As a University-based researcher team we combine research and teaching to qualify young colleagues for their future jobs – on the job. It is therefore a great joy to see students successfully graduate at various levels of their academic careers. We congratulate Franziska Wahl for her BSc in Biology, Patrick Schmitz for his BSc in Chemistry, Hanna Malyaran for her MSc in Biomedical Engineering and Carlo Schmitz for his PhD in Natural Sciences. Sina Köppert received a 1000 $ Young Investigator Award at the International Conference on the Chemistry and Biology of Mineralized Tissues in Quebec, Canada. Congratulations to all of them!

Mouse Eggs and Elephant Trunks: the Structure of Mammalian Plasma Fetuin-B and its Inhibition Mechanism of Ovastacin

Carlo Schmitz

Julia Floehr

Mammalian fetuin-B is a circulating hepatic glycoprotein of the cystatin-superfamily of cysteine proteinase inhibitors. The cystatin superfamiliy is subgrouped into type I to III cystatins. Fetuin-B belongs to type III cystatins comprising glycosylated proteins with two or three cystatin-like repeats. Unlike the related single domain cystatins, fetuin-B is not a cysteine proteinase inhibitor. Instead, fetuin-B is a potent inhibitor of zinc-dependent metalloproteinases of the astacin family, which includes the oocyte-specific enzyme ovastacin in mammals. By inhibiting ovastacin activity in mouse eggs, fetuin-B prevents premature zona pellucida hardening and thus maintains female fertility.

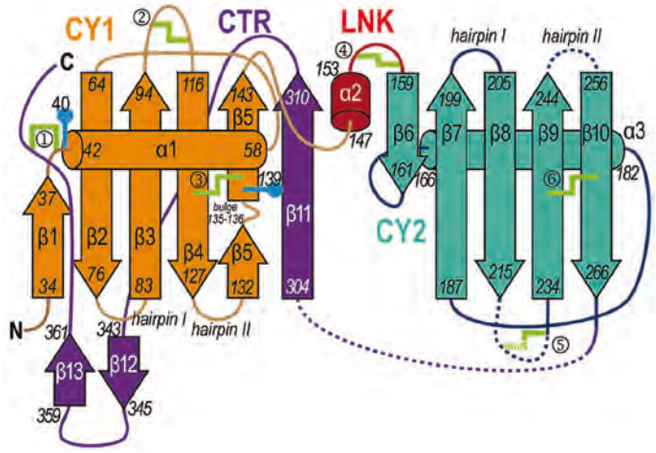

Fig. 1: Protein structure of fetuin-B.
Cartoon of murine fetuin-B. Fetuin-B consists of two cystatin-like domains (CY1, orange and CY2, teal) followed by the C-terminal region (CTR, purple). CY1 and CY2 are connected by an exposed linker (LNK, red), which mediates the inhibition of the proteinase astacin blocking access to the active center.

To understand this function at the molecular level, we solved the structure of mouse as well as human fetuin-B in complex with astacin that was used as a model for the closely related physiological target ovastacin [1,2] (Fig. 1). The 3D-structure revealed that fetuin-B consists of three domains, two N-terminal cystatin-like domains (CY1 andCY2, illustrated in orange and teal, respectively), followed by a proline-rich C-terminal region (CTR in purple). CY1 and CY2 adopt the typical cystatin folding and form the fundamental scaffold for the inhibitory potential of fetuin-B. We showed that the essential inhibitory segment is an exposed linker (LNK, depicted in red, Fig. 1) with a rigid, disulfide-linked CPDCP motif, located between CY1 and CY2. This linker region binds to the active site of astacin like a wedge into a cleft and thus blocks the catalytic activity of the proteinase. The mode of astacin inhibition by fetuin-B was termed 'raised-elephant-trunk' mechanism. The linker represents the raised trunk, while the hairpin structures I and II of CY2 form the front and the back limbs of the elephant, respectively (title figure, center).

To verify this structure-based mechanism we tested the inhibition of several fetuin-B mutants against astacin and ovastacin. It was striking that by a single point mutation at amino acid position 156 (D156A) the wildtype protein fetuin-B loses it inhibitory potential (Fig. 2). In comparison, the fetuin-B wildtype protein inhibited both astacin and ovastacin very potent with similar low K_i values (constants of inhibition) in the picomolar range. However, due to a point mutation affecting the CPDCP motif a distinct loss of inhibitory power, indicated by increased K_i values in the micromolar range, was observed. This result confirms the inhibitory mechanism that was proposed by solving the 3-D structure of the fetuin-B-astacin complex. The structural understanding of mouse and additional human fetuin-B shows that investigations using the mouse model can provide useful information for the study of female infertility.

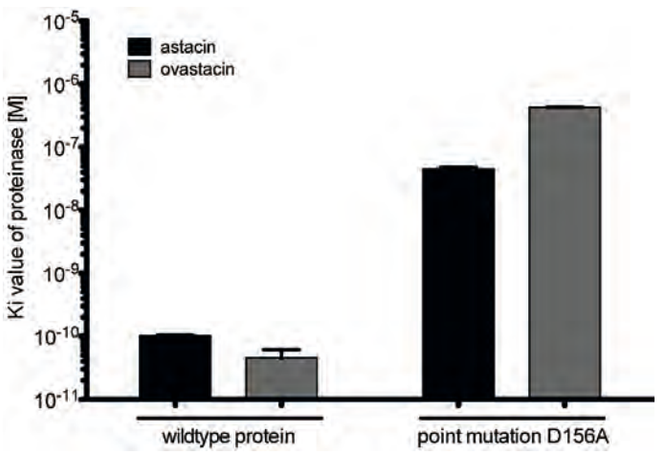

***Fig. 2:* In vitro *validation of the inhibitory mechanism*.** *Constants of inhibition (K_i values in M, logarithmic scale) of murine fetuin-B wildtype protein and fetuin-B point mutant D156A for both astacin (black) and ovastacin (gray), respectively.*

Calciprotein Particles CPP Regulate Calcification

Andrea Büscher

Sina Köppert

Aaron Morgan

Calcium and phosphate are indispensable for the cellular metabolism of all living beings. Both ions typically occur in millimolar concentrations in biological fluids. This causes a solubility and transport problem, because calcium phosphates precipitate easily from supersaturated solutions. Nature has found a way to handle water-insoluble minerals in circulation by forming colloids with proteins. This is highly reminiscent of cholesterol transport, which is mediated by lipoprotein particles, colloidal complexes of lipids and proteins. We proposed the concept of calciprotein particles (CPP) as carriers of otherwise insoluble calcium phosphates. These particles form with fetuin-A in supersaturated solutions. The particles start out as roundish, amorphous primary CPP. Primary CPP spontaneously convert into secondary CPP, which are larger, oblongate, more crystalline and less soluble. Thus, CPP mediate excess mineral transport and clearance from circulation.

We study CPP synthesis, metabolism and role in physiological mineralization and in pathological calcification. To this end we developed cell and tissue-based calcification assays. *In vitro*, vascular smooth muscle cell (vSMC)-based calcification assays showed calcification patterns deviating from *ex vivo* aortic ring cultures. vSMC were easily calcified by both calcium and phosphate, and CPP, while aortic rings only calcified with calcium and phosphate. The fact that CPP did not calcify aortic rings suggests that the endothelial cells (EC) formed a tight barrier against particles (Fig. 3).

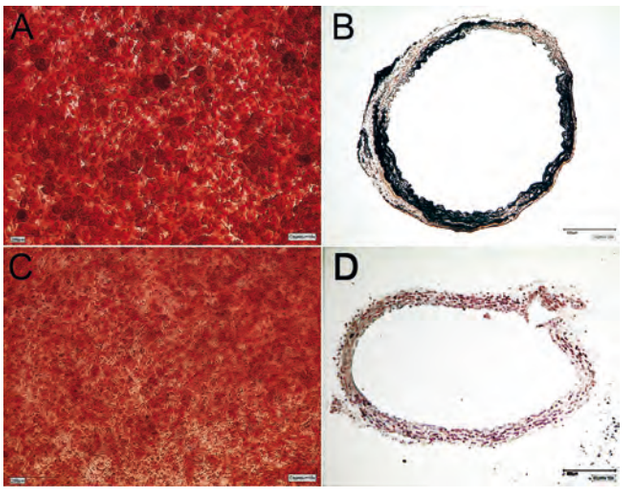

Fig. 3: Calcification assays of smooth muscle cells and aortic rings.
*Smooth muscle cells (**A, C**) and mouse aortic rings (**B, D**) were treated for four days with calcium and phosphate (final concentration 4.2 mM and 3.0 mM; **A, B**) or secondary calciprotein particles (sCPP; **C, D**) containing equal amounts of calcium and phosphate. Calcium was visualized by alizarin red (left) or van Kossa staining (right, both bars 200 µm). In SMC treated with calcium and phosphate or sCPP formation of calcium phosphate crystals on the cell-surface is observed. Mouse aortic rings treated with the same amount of calcium and phosphate showed media calcification in the calcium phosphate treated vessels, but not in the particle treated samples.*

We established a co-culture model of vSMC and EC using a microfluidic chip. The chip permits culture of both cell types in two opposing chambers separated by a porous membrane. The EC side is exposed to laminar flow of culture medium mimicking the blood stream (Fig. 4). The chip is produced in two parts, which are assembled with a cell-laden porous membrane in between. Computational fluid dynamics simulations show near laminar flow behavior with acceptable shear rates between 10-12 dyn/cm^2 in around 80% of the active test region (red square in Fig. 4C and Fig. 4G).

In summary, CPP stabilize and transport calcium phosphates. An intact endothelial layer prevents the transfer of CPP from the circulation into interstitial spaces. Endothelial damage, however, permits access of CPP to SMC and thus drives vascular media calcification.

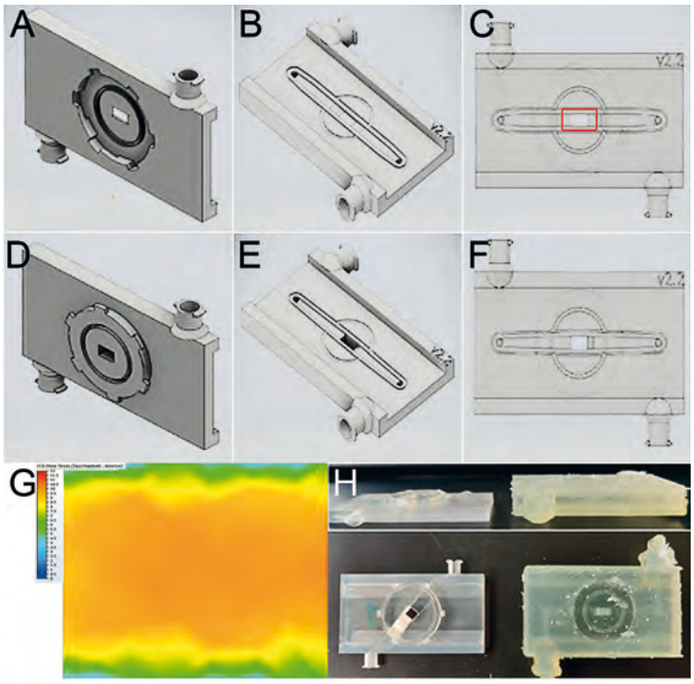

Fig. 4: 3D-printed microfluidic device for vessel-on-a-chip culture.
***(A)** Bottom view of endothelial cell side, **(B)**, top view of endothelial cell side, **(C)**, top view of endothelial cell side illustrating microfluidic flow, **(D)**, bottom view of smooth muscle cell side, **(E)**, top view of smooth muscle cell side, **(F)**, top view of smooth muscle cell side illustrating microfluidic flow **(G)**, computational fluid dynamics of active test region (area marked with red square in **(C)** at flow rate 10 ml/min. **(H)**, photographs of 3D-printed chip after (left) and before final cleaning (right).*

Stem Cells and Tissue Engineering

Sabine Neuß-Stein

In 2019, the group of Prof. Sabine Neuss-Stein on "Stem Cells and Tissue Engineering" further developed their research areas in bone and cardiovascular tissue engineering as well as in the biology of mesenchymal stem cells (MSC) and their use in tissue regeneration strategies. Over the last 15 years, an *in vivo* recruitment system was generated to guide endogenous MSC via a growth-factor loaded biomaterial towards a defect area and thus improve wound healing. We produced a tethered recom-

binant chemoattractant for MSC comprizing hepatocyte growth factor, HGF, which is readily released by protease activity present in wound fluid (Fig. 5).

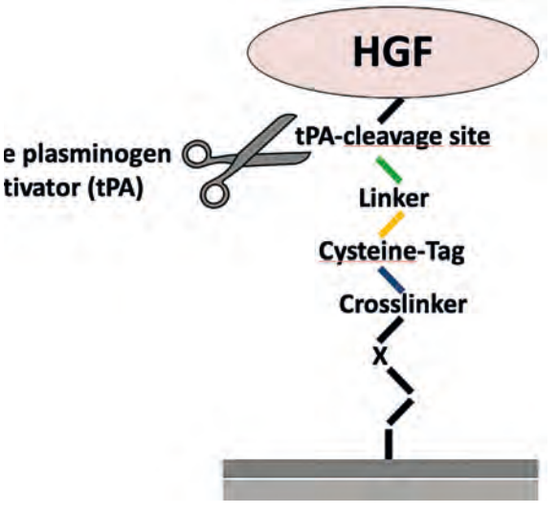

Fig. 5: Engineered hepatocyte growth factor with a cleavage site to be released from biomaterials in wound fluids.
We showed that modified HGF can be covalently bound to ceramic substrates and can be released by a specific serine protease. Released HGF is functionally active.

The project CeramActive with Prof. H. Fischer and Prof. M. Tingart (RWTH Aachen University Clinics) studies triggered release of HGF with future medical applications in mind.

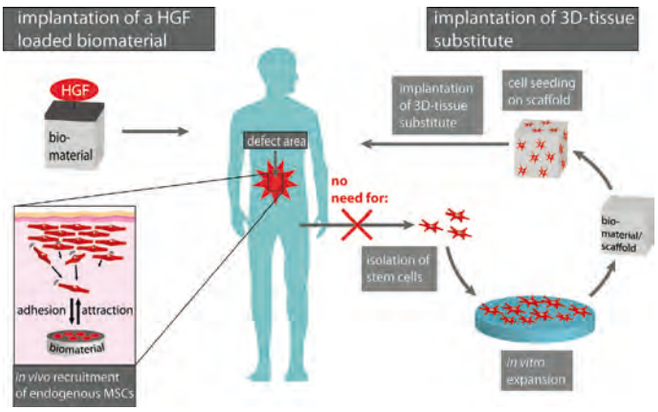

Fig. 6:* In vivo *recruitment system to guide endogenous MSC out of their niche towards a defect area for improved wound healing.

Regarding bone tissue engineering, we explored growth factor-loaded silk fibers[3] and graphene-based substrates as efficient supporting materials for the osteogenic differentiation of MSC. Fig. 7 shows scanning electron microscopy proof of osteoblastic differentiation of MSC. Alizarin red staining and transcriptome analyses together with Dr.-Ing. K. Schickle (Department of Ceramics and Refractory Materials, RWTH Aachen University) indicated more robust osteogenic induction and mineral formation of osteoblasts on graphene surfaces compared to plastic cell culture dishes.

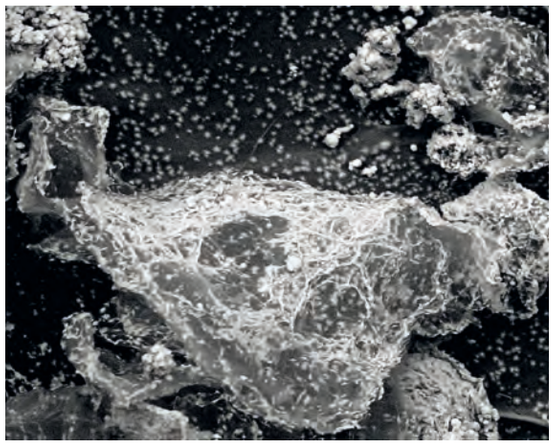

Fig. 7: MSC-derived osteoblasts on a graphene-based substrate.
Scanning electron microscopy after 21 days of incubation in osteogenic induction medium.

The group participates in the newly founded consortium "organ crosstalk" funded by "IZKF" the local Interdisciplinary Center for Clinical Research. The consortium will analyze the potential for alveolar bone regeneration of mesenchymal stem cells derived from the upper vs. lower jaws.

Regarding cardiovascular tissue engineering, we develop hemocompatible cardiovascular implants preventing restenosis and allowing for proper integration into the surrounding tissue as well endothelialization on the implant. Together with Prof. Andrij Pich (Institute of Textile and Macromolecular Chemistry, RWTH Aachen University) we evaluate fibrin-based hydrogels, which regulate cell differentiation. With Dr.-Ing. Karolina Schickle, we test ceramic nanoparticles for cardiovascular stent coating. A patent application was filed covering this work. Fig. 8 illustrates complex testing of hemocompatibility, hemolysis and thrombogenic activity under static and dynamic culture conditions [4].

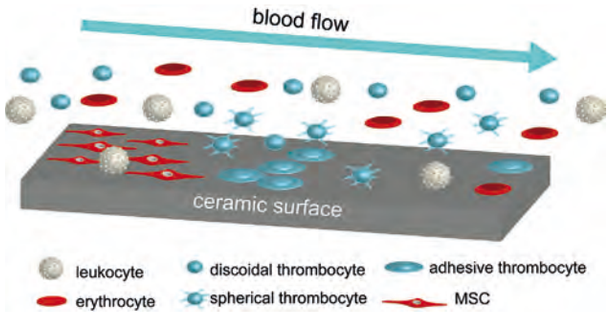

Fig. 8: Schematic representation of distribution and cell-activation during blood flow over an implant surface.

Fig. 9: UniStemDay 2019

As part of our public outreach we organized the first UniStemDay at RWTH Aachen. This meeting took place on 15th of March all over Europe. Scientists invited high school students to learn more about stem cell research and therapy. We hosted 70 high school students lecturing in the lecture hall and experimenting hands-on in stem cell laboratories.

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Team in February 2020