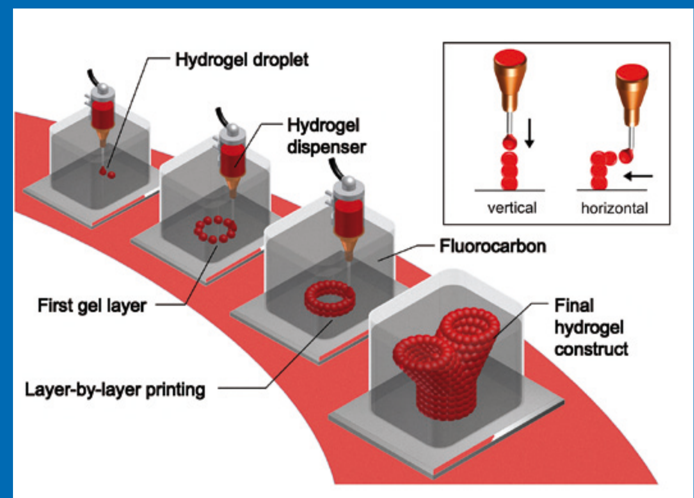
Biointerface

Faculty of Medicine

Cell-Material Interactions: Translating Basic Science Into Clinical Applications



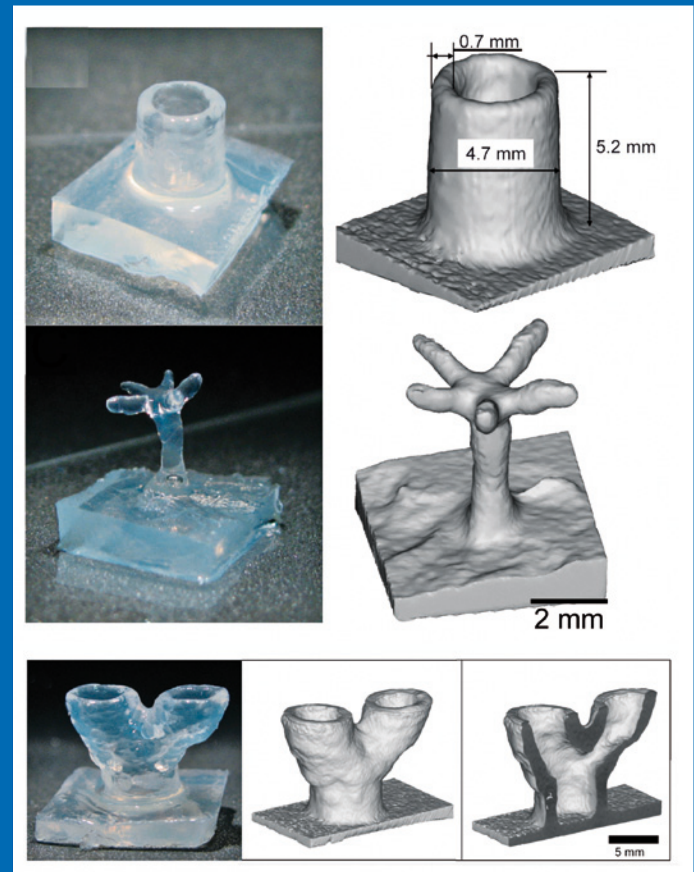


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***Fig. Title Top: Submerged bioprinting, a novel form of cast-free 3-dimensional fabrication of soft gel structures that cannot support their own weight.** Single drops of cell-laden hydrogel are dispensed layer-by-layer according to a pre-defined model to form a three-dimensional construct. The printing is performed submerged in a high-density fluorocarbon supporting liquid.*
***Center and bottom:** Hydrogel drops can be appended either vertically or laterally to an existing structure. Due to the buoyant support of the fluorocarbon, branching hydrogel structures or cantilever like constructs can be build without the need for a solid support. This patented fabrication technique was developed by Andreas Blaeser in collaboration with Prof. Horst Fischer's group at RWTH Aachen University Hospital.*[1]

Introduction

The Biointerface group continues to study two major topics – the **biology of fetuin family proteins**, and **(stem) cell-material interactions**.[1-20] Knockout mice deficient in fetuin-A, fetuin-B or histidine-rich glycoprotein are the starting point for the functional analysis of each protein. This line of research takes us wherever the results point. Over the years we have thus gained expertise in mineralization biology, calcification disease, atherosclerosis, macrophage biology, reproductive biology, cancer etc. Fetuin-A has been studied the longest with the knockout published in 1997. The role of fetuin-A in mineralized matrix metabolism has been amply discussed in previous issues of this report series.
This year we have achieved a breakthrough regarding the physiological role of fetuin-B in female fertility.[5] The fetuin-B knockout mice had become available at the end of the year 2005 as a result of Jennifer Wessling's PhD work. Already then we knew that fetuin-B deficient female mice were completely infertile – pretty puzzling considering that this protein is made in the liver and can only interact with oocytes by traveling through the bloodstream to the follicular fluid that surrounds developing oocytes. It took us until 2012 to decipher the function of fetuin-B in the prevention of oocyte zona pellucida hardening. Fetuin-B acts as a potent proteinase inhibitor of ovastacin, the proteinase that mediates definitive zona pellucida hardening. The story was published as a comprehensive paper in *Developmental Cell*, and is summarized in the next chapter by Eileen Dietzel. The fetuin-B story tells us two important things about basic science: i) Don't believe the prediction algorithms and functional networks available on the web – they can only "predict" what researchers have previously entered into the knowledge pool. Bioinformatics cannot foretell truly new knowledge, but can greatly help in formulating useful working hypotheses based on complex data sets. ii) The second lesson to be learned is: Hang in there until the job is finished and a molecular mechanism can be presented, even if it takes 8 years and 20 co-authors to finish.
Our second line of research deals with cell-material interactions in the broadest sense including toxicity studies with (nano-)materials, cell sources and cell-scaffold interactions up to implantation models in experimental tissue engineering. Two tissue engineering approaches addressing wound healing and bone tissue engineering are presented by Julia van de Kamp and Carina Adamzyk.

Work completed by former co-worker Yu Pan-Bartneck and with collaborators from RWTH Aachen Institute for Inorganic Chemistry formed the basis of a comprehensive review on the synthesis, characterization and bioactivity of molecularly stabilised ultrasmall gold nanoparticles.[15] Our work on the toxicity of ultrasmall gold nanoparticles had its grand finale in a paper published in the *Proceedings of the National Society of the USA*.[14] We figured that ultrasmall gold nanoparticles have similar size like high molecular weight ligands, e.g. proteins. Out of curiosity we asked the question if they might also interact with molecular targets present on cell membranes e.g. ion channels. One particular channel, human ether-a-go-go (hERG) is known to bind various and sundry chemicals resulting in channel blockade and cardiac arrhythmia. Indeed, ultrasmall gold nanoparticles were able to irreversibly block this channel in a patch-clamp analysis performed at Cytocentrics Bioscience.

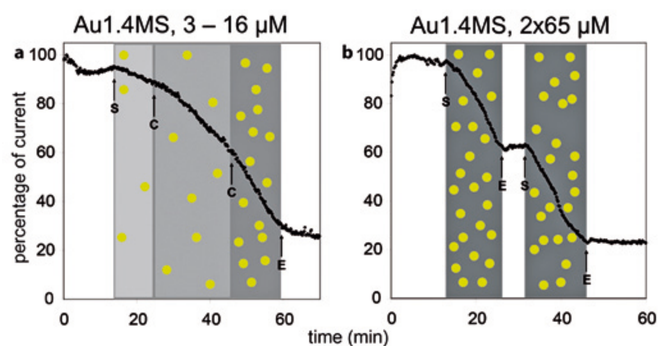


***Fig. 1: Patch clamp analysis of hERG channel**. Ultrasmall gold nanoparticles capped with triphenylphosphine dose-dependently irreversible blocked the ion conductance of the human ether-a-go-go chloride channel.*

Molecular modeling performed by Wolfgang Wenzel and his colleagues at Karlsruhe Institute for Technology (KIT) showed that the nanoparticles interacted with the chloride conductance channel of hERG.

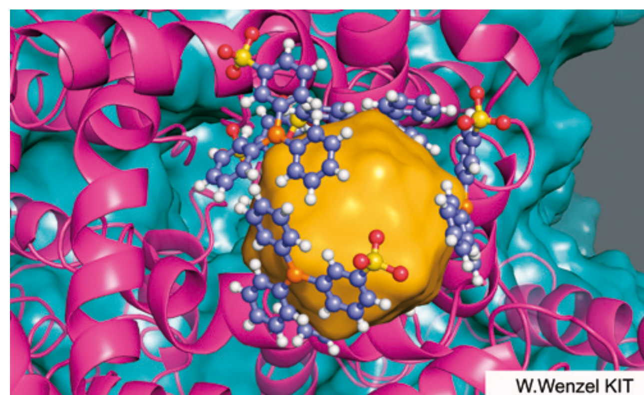


***Fig. 2: Molecular simulation of nanoparticle docking to the intracellular hERG channel.** Shown are the molecular surface of the intracellular channel entrance (green), the Au1.4 nm diameter in icosahedral form (orange), and the protein structure surrounding the entrance to the cavity on the intracellular side of the channel (pink).*

The channel blockade was studied using a potent reporter system, transfected chinese hamster ovary (CHO) cells over-expressing hERG channel. Importantly, none of

the potential ill effects were observed in protein-rich, serum-containing cell culture medium suggesting that the ultrasmall gold nanoparticles should not cause hERG-mediated cardiac arrhythmia *in vivo*.

Fetuin-B Maintains Female Fertility by Inhibiting Zona Pellucida Hardening

(Eileen Dietzel)

Human as well as other mammalian oocytes are surrounded by a glycoprotein matrix called the zona pellucida (ZP). Prior to gamete fusion, sperm are able to bind and penetrate the ZP. Fertilization however, induces rapid changes in the ZP, which transforms into a physical barrier that prevents further sperm from binding. This process is called ZP hardening, conferring resistance to proteolytic digestion and mechanical stiffening. ZP hardening is caused by proteolytic processing of ZP glycoproteins, especially the cleavage of ZP2. Cleavage is performed by proteases, which are released by the fertilized oocyte during the cortical reaction. Low level cortical granule release occurs already before fertilization during meiotic maturation and ovulation. Partial degranulation however, does not trigger ZP hardening. *In vitro*, ZP hardening occurs much faster, resulting in a decreased fertilization success.

Fetuin-B is a liver-derived plasma protein with serum concentrations of ~0.01 g/l and ~0.3 g/l in human and mouse, respectively. Fetuin-B was also detected in human ovarian follicular fluid, thus surrounding the oocyte during maturation. To study the physiological role of fetuin-B, we generated fetuin-B deficient mice (*Fetub*-/-). Female *Fetub*-/- mice were completely infertile due to an early block in fertilization.[5] In the annual report of 2011 we showed that fetuin-B deficiency leads to premature ZP hardening and that oocytes of *Fetub*-/- mice could only be fertilized after breaking the ZP. Now we were able to study the molecular mechanism between fetuin-B and ZP hardening.

Recently it was demonstrated that the cortical granula protease ovastacin is critically involved in definitive ZP hardening by cleaving ZP2. Because other astacin metalloproteases such as meprins are effectively inhibited by cystatin-like protease inhibitors including fetuin-A, we tested if recombinant mouse fetuin-B inhibited mouse ovastacin activity. Figure 3 shows that activated ovastacin was inhibited to background activity by recombinant fetuin-B (IC_{50} 76.4 nM ± 3.35 nM), but not by recombinant fetuin-A. This finding suggests that premature ZP hardening triggered by spontaneous cortical granula release of ovastacin should be entirely prevented by the micromolar concentrations of fetuin-B present in plasma and follicular fluid.

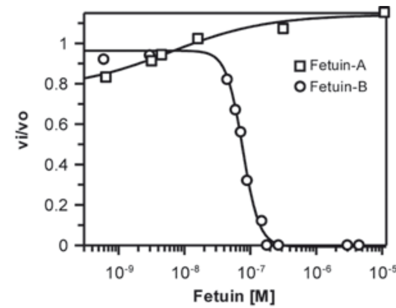


***Fig. 3: Active recombinant ovastacin** is inhibited by recombinant mouse fetuin-B (circles; concentration range: 0.6 nM - 4.5 µM) with an IC_{50} of 76.4 nM ± 3.35 nM. In contrast, recombinant fetuin-A did not inhibit ovastacin (squares; concentration range: 0.6 nM - 11 µM).*

Mechanistically, fetuin-B sustains fertility by inhibiting ovastacin, a cortical granula protease known to trigger ZP hardening, during oocyte maturation (Fig. 4). Following fertilization and degranulation of the cortical granula, the amount of ovastacin will overwhelm the inhibition capacity of fetuin-B, which is in steady state with plasma fetuin-B, but does not increase upon fertilization. Thus, plasma fetuin-B is necessary to restrain protease activity and thereby maintain ZP permeability until gamete fusion. On fetuin-B deficiency already the spontaneous release of cortical granules during maturation leads to ZP hardening and thus to female infertility.

The *Fetub* gene is well conserved in mammals. Since spontaneous ZP hardening is also reported in humans, alterations in the *FETUB* gene could also lead to human female infertility. Addition of fetuin-B to oocytes at the earliest convenience might improve IVF success in humans with mutations in the *FETUB* gene.

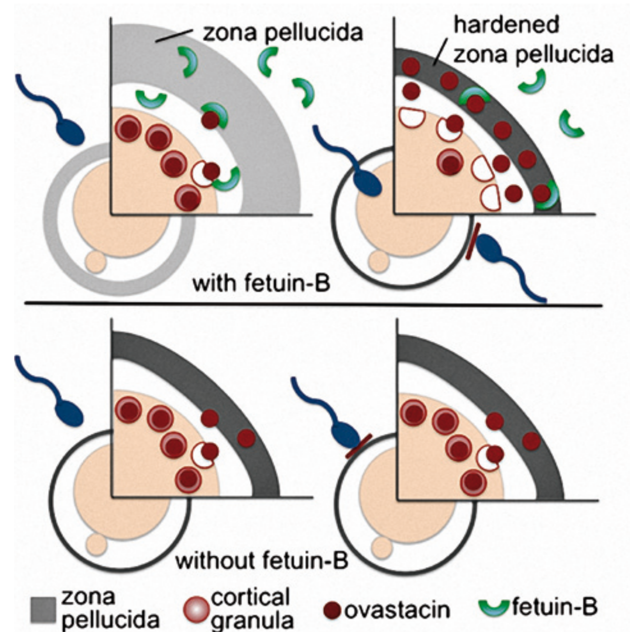


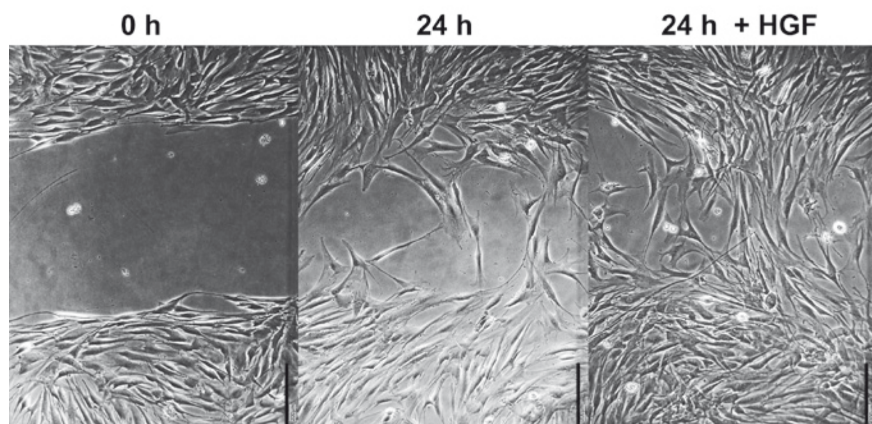
***Fig. 4: Mechanism of fetuin-B function.** During oocyte maturation fetuin-B inhibits spontaneously released ovastacin to keep the ZP permeable. After fertilization the amount of ovastacin overwhelms the inhibition capacity of fetuin-B, leading to ZP hardening and blocking polyspermy. On fetuin-B deficiency the ZP hardens already during maturation, leading to female infertility.*

Hepatocyte Growth Factor-Loaded Biomaterials for Mesenchymal Stem Cell Recruitment

(Julia van de Kamp)

Human adult mesenchymal stem cells (MSC) can be readily harvested from bone marrow through aspiration. MSC are involved in tissue regeneration and repair, particularly in wound healing. Due to their high self-renewal capacity and excellent differentiation potential *in vitro*, MSC are ideally suited for regenerative medicine. The complex interactions of MSC with their environment and their influence on the molecular and functional levels are widely studied but not completely understood. MSC secrete, for example, hepatocyte growth factor (HGF), whose concentration is enhanced in wounded areas and which is shown to act as a chemoattractant for MSC. We produced HGF-loaded biomaterials based on collagen and fibrin gels to develop a recruitment system for endogenous MSC to improve wound healing. Here, we report that HGF incorporated into collagen or fibrin gels leads to enhanced and directed MSC migration *in vitro*.[18] HGF-loaded biomaterials might be potentially used as *in vivo* wound dressings to recruit endogenous MSC from tissue-specific niches towards the wounded area. This novel approach may help to reduce costly multistep procedures of cell isolation, *in vitro* culture, and transplantation usually used in tissue engineering.

Fig. 5: ***Scratch assay showing cell migration.*** *Mesenchymal stem cells were treated with or without 75 ng/ml HGF and migration was determined by photography at 0 and 24 hours of culture. N=4, one typical view is shown exemplarily. Scale bars: 200 µm.*



Fluorescent SNAP-Tag Galectin Fusion Proteins as Novel Tools in Glycobiology

Galectins, β-galactoside binding proteins, function in several physiological and pathological processes. The further evaluation of these processes as well as possible applications of galectins in diagnosis and therapy has raised high scientific interest. Therefore, easy and reliable test systems are necessary. Here we present the simple and cost-efficient production of recombinant human galectins as fusion proteins with SNAP-tag and fluorescent proteins. These constructs show binding specificities and oligomerisation properties generally comparable to recombinant galectins.[13] Their direct fluorescence signal was utilised by ELISA type assay and flow cytometry analysis with human and ovine MSC. Flow cytometry demonstrated glycan mediated binding of His6-SNAP-YFP-Gal-3 to both MSC types, which was specifically inhibited by lactose. Moreover, directed immobilisation by SNAP-tag technology onto benzylguanine-activated sepharose was utilised to prepare galectin affinity columns for glycoprotein analysis and purification. The SNAP-tag directed coupling yielded up to three-fold higher binding capacities for the glycoprotein standard asialofetuin compared to nondirected coupled galectin suggesting improved functionality following directed coupling.

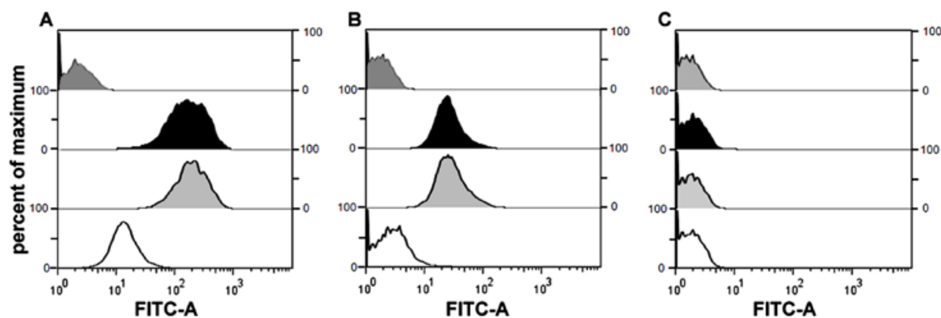


Fig. 6: ***Surface marker expression measured by flow cytometry.*** *Analysed were human MSC, ovine MSC and CHO-Lec 3.2.8.1 cells with His6-SNAP-YFP Gal-3. Fluorescent signals for bound His6-SNAP-YFP-Gal-3 after incubation of human MSC (A), ovine MSC (B) and CHO-Lec 3.2.8.1 cells (C) with the galectin probe are shown. Control signal without galectin is shown in dark grey. In black, the signal for His6-SNAP-YFP-Gal-3 binding on human and ovine MSC (A and B, respectively). In contrast, binding to CHO-Lec 3.2.8.1 did not occur (C). In light grey, no effect of 150 mM sucrose can be detected, while inhibition of binding by 150 mM lactose can be seen in white (A and B).*

Bone Tissue Engineering with Mesenchymal Stem Cells

(Carina Adamzyk)

Orthopedic implants including engineered bone tissue are commonly tested in sheep. To avoid rejection of heterologous or xenogeneic cells, autologous cells are preferably used, that is, ovine mesenchymal stem cells (oMSC).Unlike human MSC, ovine MSC are not well studied regarding isolation, expansion, and characterization. We investigated the impact of culture media composition on growth characteristics, differentiation, and surface antigen expression of oMSC.[2] The culture media varied in fetal calf serum (FCS) content and in the addition of supplements and/or additional epidermal growth factor (EGF). We found that FCS strongly influenced oMSC proliferation and that specific combinations of supplemental factors (MCDB-201, ITS-plus, dexamethasone, and L-ascorbic acid) determined the expression of surface epitopes. We compared two published protocols for oMSC differentiation towards the osteogenic, adipogenic, and chondrogenic fate and found (i) considerable donor-to-donor variations, (ii) protocol-dependent variations, and (iii) variations resulting from the preculture medium composition. Our results indicate that the isolation and culture of oMSC in different growth media are highly variable regarding oMSC phenotype and behaviour. Furthermore, variations from donor to donor critically influence growth rate, surface marker expression, and differentiation.

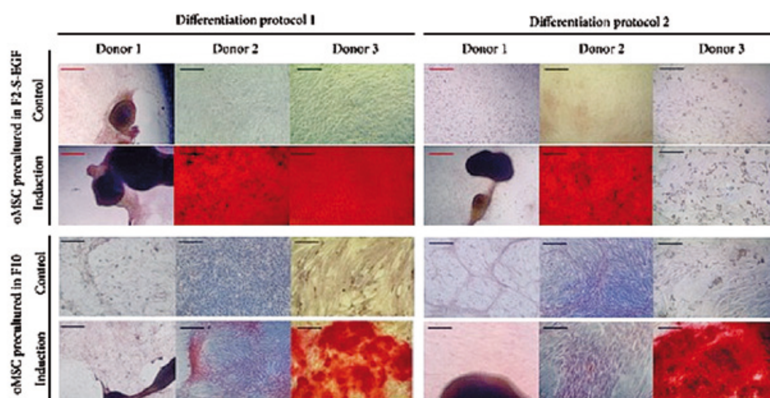


Fig. 7: ***Alizarin red staining*** *after osteogenic differentiation of oMSC precultured in medium containing 10% FCS or medium containing 2% FCS and 10 ng/ml EGF. Subsequent differentiation followed varying in induction media and cell density for 21 days. Red scale bars 500µm and black scale bars 100 µm.*

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