Biointerface
Faculty of Medicine

Biomineralization: The Hard Facts of Soft Tissue Calcification

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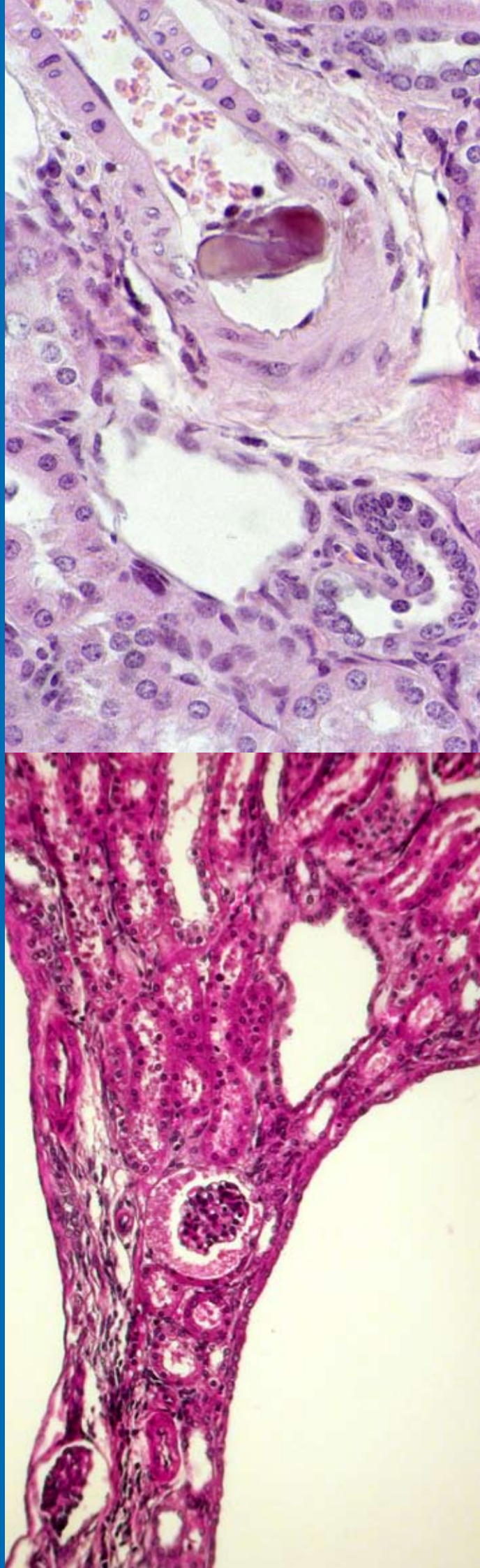


Fetuin-A, a Major Determinant of Ectopic Calcification

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. "Biomineralization" studies the natural biointerface of cells and minerals. In humans biomineralization occurs physiologically in bones and teeth. Pathological mineralization in the wrong places of the body (ectopic) is often the consequence of chronic tissue damage. Ectopic mineralization or "calcification" as it is often called, occurs when insoluble calcium salts are formed due to high concentration of the mineral ions calcium and phosphate.

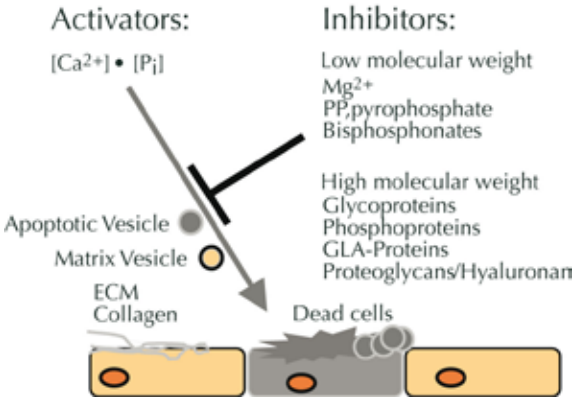


Figure 1: Activating and inhibiting principles in mammalian calcification. High calcium and phosphate serum concentration caused by metabolic disease or by kidney disease form a high Ca•P product facilitating calcification. Matrical lipidic vesicles of remodelling cells, matrix vesicles of cartilage and bone forming cells and apoptotic vesicles of dying cells strongly enhance calcification. Hence both bone osteoid and apoptotic vesicles of damaged and dying cells readily calcify. Low molecular weight inhibitors (Mg^{2+}, intracellular and extracellular pyrophosphate and their synthetic derivatives, bisphosphonates) interfere with mineral formation at the level of crystal morphology. High molecular weight inhibitors can interfere with calcification at the level of mineral formation, stability, dissolution and removal by phagocytosis (remodelling). Depending on their expression pattern in the body inhibitors can be tissue-restricted or systemic. From Jahnen-Dechent, 2004.

Thermodynamics predict that many biological fluids including blood should precipitate solid minerals because of solute supersaturation. This is however not the case. Therefore inhibitory principles are claimed to prevent the formation and build up of insoluble minerals. Genetic studies using knockout mice identified the serum protein fetuin-A / α_2-HS glycoprotein as an important inhibitor preventing pathological mineralization in soft tissues and in the extracellular fluid. The concentration of mineral solutes in mammalian blood is in fact considerably higher than that predicted by their solubility product. The plasma protein fetuin-A inhibits calcium phosphate deposition by forming colloidal calciprotein particles (CPP). Using small angle neutron scattering (SANS) in collaboration with colleagues from Forschungszentrum Jülich we have further delineated the structural dynamics of a colloidal protein - mineral complex bestowing on calcium phosphate a high solubility in biological fluids.

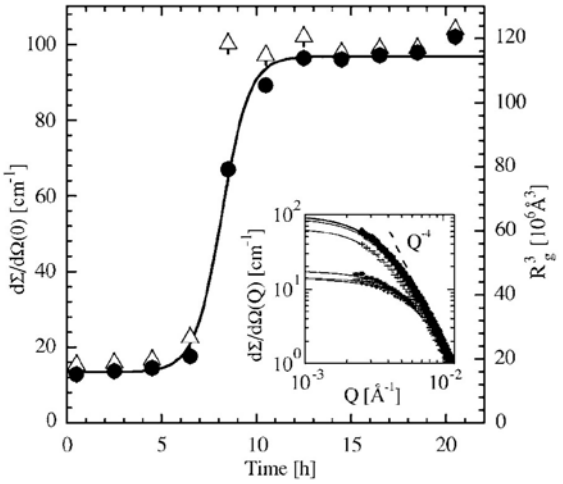


Figure 2: Time dependency of the volume (Δ) and the volume multiplied by the volume fraction (•) of the CPPs expressed as R g^3 (Δ) and the extrapolated cross section at Q = 0,vs mineralization time. The plot of the CPP sample in 100% H_2O revealed a two stage mineralization process with plateaus at 0 – 5 and 11 – 20 h. The inset shows the corresponding scattering patterns. These data were fitted, depicted by the solid lines.

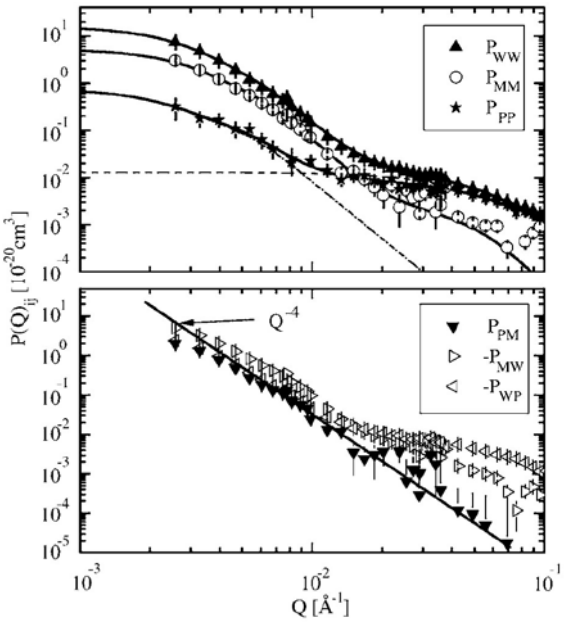


Figure 3: Partial structure functions derived from six identical CPP samples with varying H_2O / D_2O content. The self-terms of water W, mineral M, and protein P are plotted in the upper graph and the corresponding cross terms in the lower graph. The positive cross-term P_{PM} suggested a protein covered mineral core.

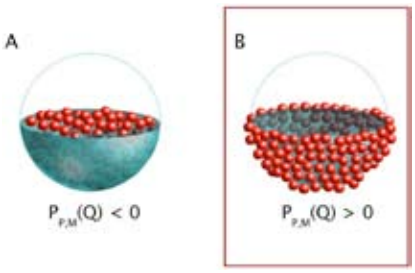


Figure 4: A graphical model depicting the two possible conformations of CPPs. The SANS measurements clearly indicated that model B is realized in nature. Thus a poorly crystalline mineral core made of octacalcium phosphate is covered by a layer of fetuin-A molecules rendering the entire complex water-soluble, stable and ready for clearing.

Calciprotein Particles in Humans

Thus calciprotein particles are calcium phosphate colloids stabilized by the plasma glycoprotein fetuin-A. Our physicochemical research concerned material generated in the test tube, *in vitro*. Apart from one study in young rats, this material has not yet been described *in vivo*. As a confirmation of our work we received a dialysis patient ascites sample with protein-mineral complexes that showed almost identical morphology and composition as the calciprotein protein particles we had prepared *in vitro* and studied in great detail. Thus the principles of mineral protein stabilization are very relevant indeed in human pathology.

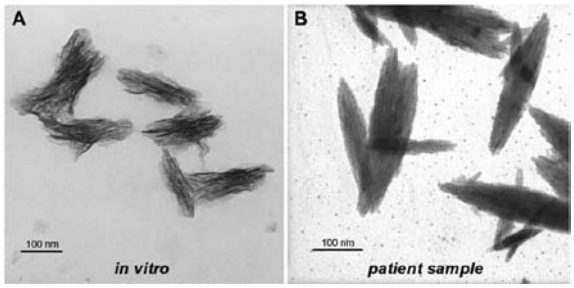


Figure 5: Transmission electron micrographs of protein-calcium phosphate mineral colloids. (A) In vitro sample of a CPP in the "second state" generated in 10 mM $CaCl_2$, 6 mM Na_2HPO_4, 50 mM Tris-HCl and 140 mM NaCl . (B) Peritoneal dialysate of a dialysis patient containing protein-calcium phosphate particles closely resembling the CPPs generated in vitro and shown in (A).

This side by side comparison revealed striking similarities between the *in vitro* synthesized second state CPPs and the patient sample. Both samples contained elongated spheroidal structures of similar size and crystallinity. This result suggested that long-term stabilization of second state CPPs may be equally important like short term inhibition of spontaneous calcium phosphate precipitation in the overall inhibition of pathological mineralization. The high density fetuin-A monolayer on the second state CPP surface is intriguing in that it suggests a putative CPP clearing mechanism. We hypothesize that the putative CPP clearing mechanism relies on the cellular recognition of clustered fetuin-A. This kind of molecular recognition by phagocytic cell receptors would efficiently discriminate against the excess monomeric fetuin-A in the extracellular fluid, which would otherwise compete with CPP uptake. Conversion of low affinity to high affinity recognition by ligand clustering is in fact common in clearing of cellular debris and particles, but has never been suggested for clearing of mineralized debris.

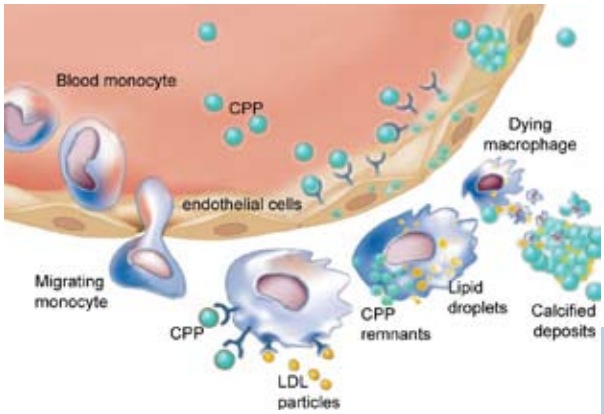
In summary, we demonstrated that SANS ideally lends itself to a study of protein - mineral interaction in biomineralization research. This method does not denature biological samples, it covers the relevant size range between 10 and 1000 Å and reveals both internal structure and composition. The SANS analysis revealed the dynamic transformation of CPPs into a layered colloid comprising an OCP core and a fetuin-A shell. Thus we provide experimental proof of a hitherto theoretical concept of a protein shielding mechanism preventing pathological mineralization. In addition the core-shell structure suggests strongly reduced diffusion of ions across the colloid mineral interface resulting in a reduced CPP growth and octacalcium phosphate (OCP) to hydroxyapatite (HAP) transformation. The structure of CPPs prepared *in vitro* closely resembled TEM pictures of CPP samples from a human patient suffering calcifying peritonitis adding strong clinical relevance to our structural analysis. In conclusion, this detailed description of CPP morphology may aid the rational design of clinical assays of pathological mineralization risk and of synthetic inhibitors alike.

Clearing of Calcified Debris

Previously we suggested a clearing pathway for CPPs largely drawn on obvious similarities with lipoprotein particle metabolism. Along these lines we suggested that CPPs like LDL particles are phagocytosed and cleared from circulation.

The next logical step will be unraveling the clearing of CPPs *in vivo*. Several reports indicate that innate immune cells like macrophages and neutrophils create an inflammatory environment in response to basic calcium phosphate (BCP) thus mediating progression of calcification. Down regulation of proinflammatory cells and enhancement of phagocytosis by opsonization by plasma proteins like fetuin-A are believed to enhance clearing of calcified remnants. In a first series of phagocytosis assays using synthetic CPPs and a macrophage cell line we observed highly efficient clearing of CPPs and comparatively less uptake of free monomeric fetuin-A.

Figure 6: Proposed clearing pathways for calciprotein particles, CPP and low density lipoprotein particles, LDL share important features. From Jahnen-Dechent, 2004.



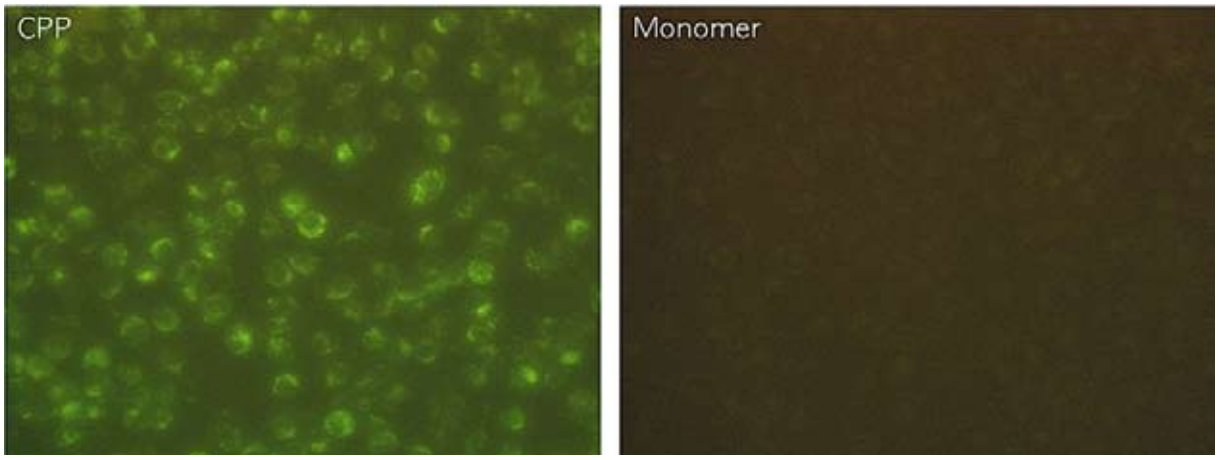


Figure 7: Uptake of calciprotein particle and fetuin-A monomer in J774 cells. Cells were fed with fluorescently labeled monomeric fetuin-A protein or with CPPs prepared thereof. Note stronger fluorescence in CPP fed cells indicating more efficient uptake than in monomer fed cells.

Studying clearing in whole animal studies is now possible. We expect that cells of the reticulo-endothelial-system, RES will be important in removing calcified remnants and CPPs very efficiently. Experiments to this effect are ongoing.

We have shown on many levels of complexity that fetuin-A stabilizes mineral and helps cells to handle excessive mineral loads. A clear picture is emerging of fetuin-A fulfilling multiple roles in the prevention of calcification. Firstly fetuin-A stabilizes calcium phosphate in solution, in the form of CPPs (see above). Next, fetuin-A allows cells to handle elevated extracellular calcium and phosphate without calcifying. Last not least fetuin-A opsonizes mineral debris facilitating its removal by the RES.

We had previously described biological effects of fetuin-A in rat osteoblasts. Virtually identical results were replicated in human smooth muscle cells. These cells are taken as a model system for arterial calcification.

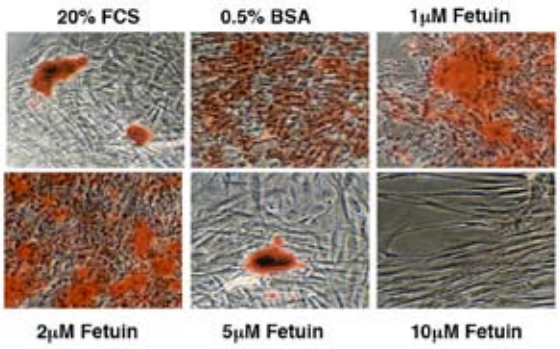


Figure 8: Fetuin-A or fetuin-A containing media (containing fetal calf serum, FCS) prevent smooth muscle cell calcification in a cell culture growing cells under elevated calcium and phosphate. Red color indicates mineral deposits. From Reynolds et al. 2005.

Furthermore it was demonstrated that calcification starts in calcium phosphate containing vesicles and that the cells undergo apoptosis before they calcify. Fetuin-A efficiently prevented both apoptosis and the ensuing calcification of the cells and the extracellular matrix.

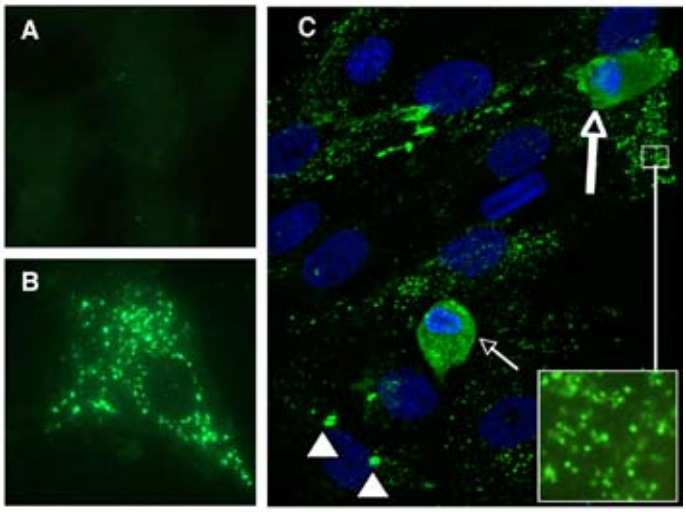


Figure 9: Fetuin-A is intracellular in VSMC and localizes to vesicles. VSMCs cultured in serume-free media contained little fetuin-A immunoreactivity (A). VSMCs cultured in the presence of serum or treated with 2.0 μM fetuin-A contained fetuin-A localized in discreet cytoplasmic vesicular structures (B). (C) Confocal microscopy confirmed this localization and also showed that in apoptotic cells fetuin-A was localized throughout the cytoplasm (arrowed in C) and was also concentrated in extracellular vesicles (arrowheads). Nuclei are stained with Hoechst. The inset shows fetuin-A loaded vesicles. From Reynolds et al. 2005.

Transcriptional Regulation of Fetuin-A

Fetuin-A is a serum protein preventing soft tissue calcification. In trauma and inflammation, fetuin-A is down-regulated and therefore considered a negative acute phase protein. Enhancement of fetuin-A expression as a protective serum protein is desirable in several diseases including tissue remodeling after trauma and infection, kidney and heart failure, and cancer. Using reporter gene

assays in hepatoma cells combined with electrophoretic mobility shift assays we determined that dexamethasone upregulates hepatic fetuin-A. A steroid response unit (SRU) within the mouse Ahsg promoter mediates the glucocorticoid-induced increase of Ahsg mRNA.

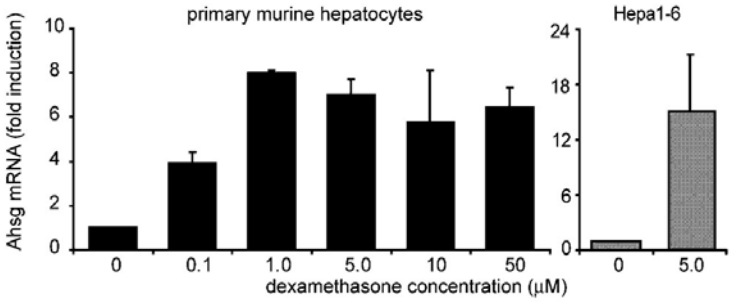


Figure 10: Effect of dexamethasone on mouse Ahsg mRNA levels. Total RNA was isolated from primary mouse hepatocytes (black columns) exposed to different concentrations of dexamethasone (0.1 to 50 µM) for 48 h. Ahsg mRNA was measured using real-time PCR. Data represent the mean of two independent primary mouse hepatocyte preparations both analysed in duplicate. Hepa1–6 cells (grey columns) were treated with 5.0 µM dexamethasone according to the treatment of the primary hepatocytes. Ahsg mRNA quantities are given as fold induction of untreated controls. For Hepa1–6 cells data represent the mean of two independent experiments. From Wöltje et al. 2006.

The SRU binds hepatocyte nuclear factor 3 and CCAAT enhancer binding protein (C/EBP). The upregulation is mediated indirectly via glucocorticoid hormone-induced transcriptional upregulation in C/EBP protein. A high degree of sequence identity in mouse, rat and human Ahsg promoters suggests that the promoter is similarly upregulated by dexamethasone in all three species. Therefore, our findings suggest that glucocorticoids may be used to enhance the level of Ahsg protein circulating in serum.

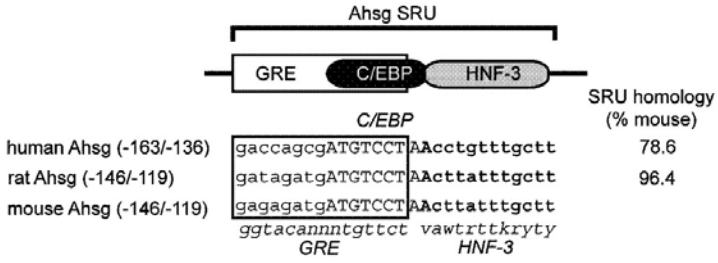


Figure 11: Schematic drawing of SRU organization and sequence homology of the Ahsg SRU. The C/EBP binding site and putative transcription factor binding sites for glucocorticoid receptor and HNF-3 within the Ahsg promoter, which might be responsible for dexamethasone-induced transcriptional upregulation, are shown. A high degree of sequence identity exists in the SRUs of mouse, rat and human Ahsg. The location of the SRUs is given with respect to the transcription initiation sites of the three Ahsg genes. From Wöltje et al. 2006

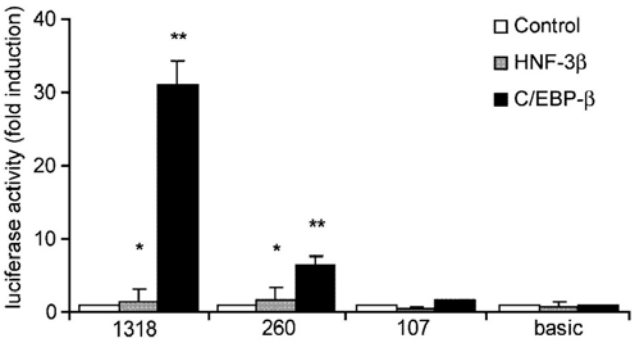


*Figure 12: Over-expression of C/EBP or HNF-3 enhances Ahsg promoter activity. Neuroblastoma × glioma cells were transiently transfected with Ahsg promoter reporter gene constructs and cotransfected with either C/EBP or HNF-3 expression plasmids (1 ng). After 24 h in culture, luciferase activity was measured. Ahsg promoter-dependent luciferase activity was expressed as fold induction for C/EBP co-transfection (black columns) and for HNF-3 cotransfection (grey columns) in comparison with unstimulated controls (white columns). Data represent the mean values ± S.E.M. derived from at least three independent experiments measured in triplicate. For statistical evaluation, Student's t-tests were performed. Significance levels are indicated as *P ≤ 0.05 or **P ≤ 0.01. From Wöltje et al. 2006.*

In conclusion, we demonstrated a positive regulation of fetuin-A gene transcription by the glucocorticoid hormone, dexamethasone and we have shown that dexamethasone strongly upregulates the mouse fetuin-A gene through a SRU comprising C/EBP and HNF-3. Moreover, binding of HNF-3β to the newly described HNF-3β binding site within the mouse fetuin-A gene promoter is important for basal promoter activity. Dexamethasone-induced fetuin-A gene transcription via C/EBP could modulate the transcriptional complex by protein–protein interactions with the bound HNF-3, and thus enhance transcription of the fetuin-A gene. This mode of gene activation may apply not only to the mouse gene but possibly also to the rat and the human fetuin-A genes, because the SRU is highly conserved in all three species. Therefore, glucocorticoid treatment may be therapeutically beneficial in situations of low serum fetuin-A. Interestingly, a recent bone scan study in calciphylaxis patients reported that steroid therapy appeared to be beneficial in some patients. It will be interesting to measure serum fetuin-A in these patients undergoing glucocorticoid treatment to determine if upregulation of serum fetuin-A may be beneficial in diseases associated with low serum fetuin-A such as soft tissue calcification for which there is currently no adequate therapy.

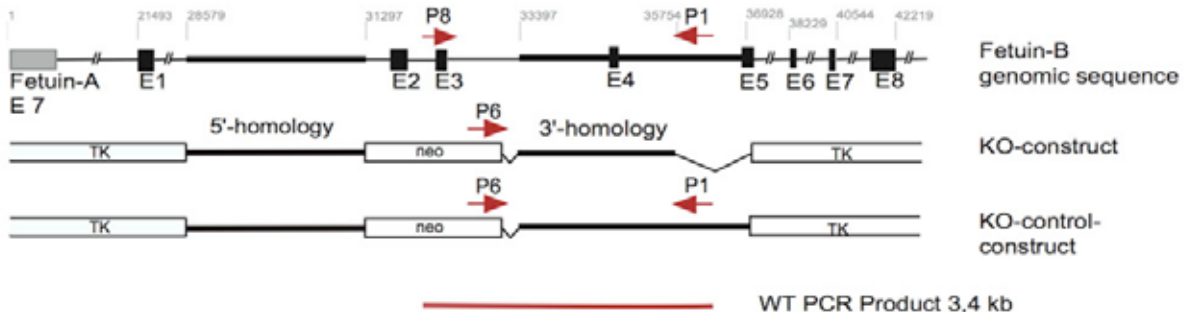


Figure 13: »Knockout strategy«. Sequences for homologous recombination are marked. The mutated Fetuin-B gene is 253 base pairs shorter that the wildtype allele and lacks the initiation codon and thus a functional mRNA. The recombination event was verified by polymerase chain reaction and Southern blotting.

News from the Latest Fetuin

In 2005 we successfully generated knockout mice for fetuin-B.

The Fetuin-B deficient mice are vital and do not show any obvious defects. We combined the fetuin-B deficiency with defined genetic backgrounds to study the physiological function of this protein. Breeding is ongoing.

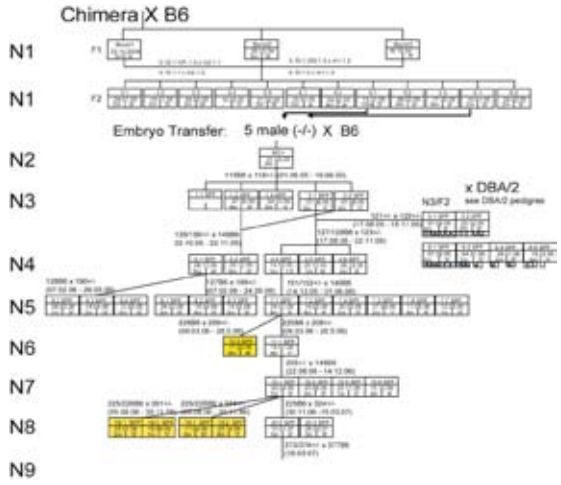


Figure 14: Pedigree of Fetuin-B deficient mouse strain during backcrossing onto pure C57BL/6 and DBA/2 genetic background. Mice are now in backcrossing generation 9 and thus over 99% pure genetic background of the respective strains.

Fetuin-B like fetuin-A is a liver derived serum protein. It is the second member of fetuin protein family within the cystatin superfamily (cystatins; histidine-rich glycoprotein HRG; kininogen, KNG). All members with a fetuin protein signature (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. Fetuin-B is a single gene protein expressing multiple splice variants. The physiological function of fetuin-B is unknown. Structural homology analysis and activity testing suggest that it is not an inhibitor of spontaneous calcium phosphate precipitation when compared to fetuin-A. Based on genetic mapping studies a function has been suggested as a putative tumor suppressor.

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Team