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Arterial calcification in bone mineralization deficient mice

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Abstract

Calcification of arterial plaques reduces the elasticity of the vessel wall, increases the risk for vessel rupture and is associated with the elevated risk of myocardial infarction and atherosclerosis. Recent studies supports the theory that arterial calcification is in fact an active process that is related with the expression of bone mineralization proteins. In this study we tested the hypothesis that the calcification in aorta is build up by the same process as in bone mineralization by using two animal models of soft tissue ossification, i.e., $Enpp1^{-/-}$ and mice deficient in the Ankylosis gene (*ank/ank*). Aortic sections from these mice were stained for calcium and mineral deposits and for bone mineralization markers as osteopontin. Smooth muscles cells from the aortas were isolated and cultured for alkaline phosphatase assays. The histology experiments showed some positive staining for calcium and mineral in *ank/ank* and severe irregularities in the vessel wall in $Enpp1^{-/-}$. Although further experiments must be performed, these data indicated that arterial calcification is associated with bone mineralization process.

Keywords
Arterial calcification, knock-out mice, immunoassay, histology

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Arterial calcification in bone mineralization deficient mice

Anna Lundquist

Sammanfattning

Under normala förhållanden är kalcificering och mineralisering begränsad till ben och tänder. Vid vissa sjukdomstillstånd kan dock mineralisering observeras i andra vävnader. Aorta är en av de vanligaste lokalisationerna för denna patologiska process. Hos människan är denna åkomma mer känd som åderförkalkning. Detta är en av vår tids största folkhälsoproblem och drabbar, enligt WHO (Världshälsoorganisationen), 80 % av populationen över 60 år. Detta tillstånd medför en ökad risk för allvarliga sjukdomar och en högre dödlighet.

För att närmare studera detta tillstånd använder man sig av musmodeller. Djurmodeller gör det möjligt att studera och modifiera deras gener för att få en ökad förståelse för humana sjukdomar. Tidigare studier har visat tecken på att kalcificeringen av aorta är en aktivt reglerad process som delar många likheter med den väl koordinerade mekanismen vid kalcificering av ben. För att vidare undersöka denna hypotes använde jag mig av tre olika musmodeller med defekt benmineralisering var av en av dem tidigare visats ha ökad kalcificering av aorta.

Examensarbete 20p i Molekylär bioteknikprogrammet
Uppsala universitet Maj 2004

Introduction

Bone mineralization is a highly regulated, cell-mediated process, which gives bone the rigidity and mechanical properties that are needed for its normal physiological functions. It is during this process that the hard tissue in bone is built up embedding the extra-cellular matrix with hydroxyapatite crystals. In contrast to this elegantly controlled mechanism, soft tissue mineralization only occurs during pathological conditions and sometimes with fatal consequences, especially when located in the walls of blood vessels and heart valves.

Calcification of arterial plaques reduces the elasticity of the vessel wall, increases the risk for plaque rupture and is associated with the elevated risks of myocardial infarction and atherosclerosis. Historically, arterial calcification has been considered to be a passive process, a nonspecific response to tissue injury or necrosis. Recently there have been several lines of evidence that support the theory that arterial calcification is an active process that is related with the expression of growth factors, matrix proteins and other bone related proteins. This new theory (Shanahan *et al.,* 2000) about the similarities between soft and hard tissue mineralization is supported by the presence of an apatitic mineral phase, matrix vesicles and the expression of proteins that are involved in both the initiation and the inhibition of mineralization, such as osteopontin and osteocalcin.

Tissue-nonspecific alkaline phosphatase (TNAP) functions to hydrolyze inorganic pyrophosphate (PPi), a potent inhibitor of mineralization, and deletion of the TNAP gene (*Akp2*) in mice results in hypophosphatasia, which is characterized by elevated levels of PPi and poorly mineralized bones (Narisawa *et al*., 1997). Mutation in the genes that are responsible for the production and transportation of PPi to the extra cellular space, i.e., NPP1 and ANK causes soft tissue ossification abnormalities. *Enpp1* knock out mice (deficient in NPP1 function), display aberrant bone mineralization and have earlier been associated with arterial calcifications and with Idiopathic Infantile arterial calcification (IIAC), a human disease characterized by hydroxyapatite deposition and smooth muscle cell proliferation in the internal elastic lamina (Rutsch *et al*., 2001). These findings suggest that vascular calcification is in fact an actively regulated process in which vascular cells may obtain osteoblast-like functions.

Aims

The aims of this project were to test the hypothesis that calcification in the aorta is build up by the same process as in bone mineralization by using two animal models of soft tissue ossification, i.e., $Enpp1^{-/-}$ and mice deficient in the Ankylosis gene *(ank/ank)*. In addition, we aimed to test new therapeutic approaches to treat arterial calcification. Specifically, we studied *ank/ank* and $Enpp^{-/-}$ mice that have been treated with tetramisole, an uncompetitive inhibitor of TNAP activity, to investigate whether tetramisole could be useful for the pharmacological treatment of arterial calcification.

Bone biology

Even though the skeleton seems like a static organ it is highly specialized and dynamic and undergoes continuous regeneration. It consists of highly specialized cells, mineralized and un-mineralized connective tissue matrix and spaces that include the bone marrow and vascular canals. During development and growth, the skeleton is formed to achieve its shape and size by its ability to remove bone from one site and deposit it at another. Bone has, besides a mechanical function in muscle attachment and a protective function for our organs and bone marrow, the crucial metabolic function as a reserve for vital ions, especially calcium and phosphate but also magnesium, sodium and carbonate (Triffitt *et al.,* 1996). During times of systematic deficiencies, the skeleton is a source of these ions and due to increased bone resorption the serum homeostasis, essential for life, can be maintained.

Bone mineralization

Bone is formed by collagen fibers with crystals of hydroxyapatite within them. Hydroxyapatite is the deposited mineral, which gives the bones its strength and is composed of phosphate and calcium $[Ca_{10}(PO_4)_6(OH)_2]$.

There are predominantly three different specialized cells in the skeleton that are responsible for controlling bone formation, i.e. osteoblasts, osteoclasts and osteocytes. Both osteoclasts and osteoblasts are derived from precursor cells that originate from the bone marrow. Osteoblasts are derived from precursors of multi-potent mesenchymal stem cells, which also give rise to bone marrow stromal cells, chondrocytes and

muscle cells (Friedenstein *et al*., 1974; Owen *et al*., 1985; Trifftt *et al*., 1996), whereas the precursors of osteoclasts are hematopoietic stem cells from the monocyte-machrophage cell lineage (Roodman *et al*., 1996; Suda *et al*., 1992). The development and differentiation of osteoblasts and osteoclasts are controlled by cytokines and growth factors that are produced in the bone marrow microenvironment as well as several systemic hormones and adhesion molecules that mediate cell-to-cell and cell to matrix interactions (Manolagas *et al*., 1995). Mature osteoclasts are large multinucleated cells whose major function is to mediate the resorption of the calcified bone matrix. Osteoblasts produce and secrete proteins that build up the bone matrix and one of the major products from a bone forming osteoblast is type 1 collagen (Robey *et al*., 1995). Bone-forming osteoblasts synthesize a number of other proteins including osteocalcin and osteonectin, which constitute the major part of non-collagenous proteins in bone. Mature osteoblasts are essential for mineralization of the matrix by deposition of hydroxyapatite (Boskey *et al*., 1996; 1998), and are thought to regulate the local concentrations of calcium and phosphate in a way that promotes hydroxyapatite formation. Osteoblasts express relatively high amounts of alkaline phosphatase, which is known to play a major role in bone mineralization (Whyte *et al*., 1994). As bone matrix becomes mineralized, some mature osteoblasts will eventually become buried in the impermeable mineralized matrix. These cells are termed osteocytes and are responsible for the maintenance of bone matrix, they also have the capacity not only to synthesize but also to resorb bone matrix to a limited extent (Buckwalter *et al*., 1995). They are also by far the most abundant cell type in bone: there are ten times more osteocytes than osteoblasts. Osteocytes are spread out through the mineralized matrix and communicate with each other and other cells on the surface via extensions from their plasma membranes. This makes them very suitable as sensor cells able to detect the need for bone augmentation or reduction during the functional re-modeling of the skeleton. Also, they can sense the need for repair of micro-damages, and then transmit signals leading to a suitable response. Osteocytes are able to sense differences in intestinal fluids and to detect changes in the hormone levels, such as estrogen, that influence their survival (Weinstein *et al*., 1998; Tomkinson *et al* 1997; 1998). Due to this, disruption of the osteocyte network could lead to increased bone fragility.

Regulation of Inorganic pyrophosphate

Inorganic pyrophosphate (PPi) is comprised of two molecules of inorganic phosphate Pi and is a potent and direct inhibitor of bone formation, interfering with the capacity of growth plate chondrocytes and osteoblast to deposit minerals, specifically hydroxyapatite crystals in the peri-cellular matrix of bone (Johnson *et al*., 1999; 2000).

At least three proteins are postulated to control the extracellular pool of PPi, i.e. TNAP (Moss *et al*., 1967; Hessle *et al*., 2002), NPP1 (Terkeltaub *et al*., 1994; Johnson *et al*., 1999) and the ankylosis protein (ANK) (Hakim *et al*., 1994).

Control of mineral deposition
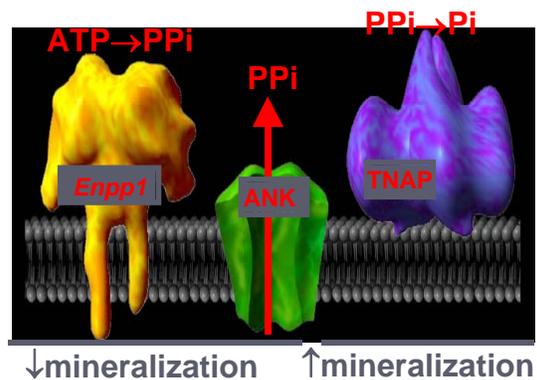
Figure 1 – Schematic model of ↑Enpp1, ↑ANK and ↓TNAP proteins responsible for the control of the extra cellular pool of PPi. Illustration used with permission from Prof. Millan.
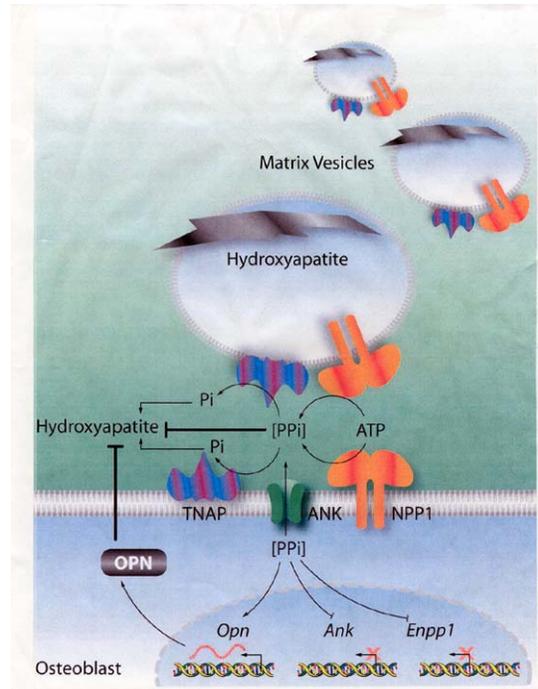
TNAP

All alkaline phosphatase isozymes are cell surface-bound proteins that are attached to the membrane via a glycosyl-phosphatidylinositol (GPI) moiety. This GPI anchor can easily be cleaved with phospholipase C and D, which generates a soluble form of the enzyme (Udenfriend and Kodukula, 1995). TNAP in bone is found on the cell surface of osteoblasts and chondrocytes, and on the matrix vesicles (MV) membrane (Bernard, 1978; Morris *et al*., 1992). TNAP may participate in generating the Pi that is needed for hydroxyapatite crystallization in bone (Majeska and Wuthier, 1975). It has also been proposed that TNAPs function in this environment is to hydrolyze the mineralization inhibitor PPi to facilitate mineral precipitation and growth (Moss *et al*., 1967). The

disease hypophosphatasia is caused by TNAP deficiency, and is characterized by poorly mineralized bones (osteomalacia) and cartilage (rickets). TNAP knockout (KO) mice ($Akp2^{-/-}$) display many characteristics similar to infantile hypophosphatasia, and are a valuable tool for further investigations of the specific functions of the enzyme (Waymire at al., 1995; Narisawa *et al*., 1997; Fedde *et al*., 1999). *Akp2^{-/-}* mice show growth reduction and suffer from epileptic seizures as well as hypomineralization of the skeleton. These mice die at approximately 2 weeks of age due to this severe phenotype. Interestingly, the skeletal phenotype of these mice cannot be detected before 4-6 days after birth, which suggests that TNAP is not essential for the initial events of bone mineral deposition, but rather plays a role in the maintenance of this process after birth (Narisawa *et al*., 1997). TNAP is present in systemic arteries, arterioles and some capillaries (Ushiki *et al*., 1998), and it is hypothesized that the enzyme is involved in arterial calcification by same mechanism as in bone (Hui *et al*., 1998).

NPP1

NPP1 was earlier called plasma cell membrane glycoprotein-1 (PC-1), is a plasma membrane-bound protein and is the primary generator of PPi (Terkeltaub *et al*., 2001). Similar to TNAP, NPP1 expression is highly abundant on the surfaces of osteoblasts and chondrocytes as well as on the membrane of their MVs (Hashimoto *et al*., 1998; Johnson *et al*., 1999). NPP1 is involved in inhibition of hydroxyapatite precipitation in or close to mineralizing areas through its PPi-generating function (Terkeltaub *et al*., 1994; Johnson *et al*., 1999). Mice deficient in NPP1 develop hypermineralization such as ankylosing spinal hyperostosis, pathological soft-tissue ossifications, including arterial calcifications (Okawa *et al*., 1998; Sali *et al*., 1999). These mice have been shown to be a good representative model for certain human diseases in which there are uncontrolled ossification of ligamentous tissue leading to spinal and peripheral joint mobility and arterial calcifications (Okawa *et al*., 1998; Johnson *et al*., 2003). These diseases include ossification of the longitudinal ligament (OPLL), diffuse idiopathic skeletal hyperostosis (DISH) and idiopathic infantile arterial calcification (IIAC) (Rutsch *et al*., 2003).

Figure 2 - Illustration of TNAP, ANK, NPP1, PPi and OPNs different roles in hydroxyapatite regulation. Both TNAP and NPP1 are functional in matrix vesicles where as ANK is not, therefore NPP1 plays a more crucial role in PPi production than ANK. A negative feedback loop exists in which PPi, produced by NPP1 and transported by the channeling protein ANK, inhibits expression of *Enpp1* and *Ank*. In addition, PPi induces expression of OPN, which further inhibits mineralization. In the absence of TNAP, high levels of PPi inhibit mineral deposition directly and also via its induction of OPN expression. Illustration used with permission from Dympna Harmey, PhD

Ankylosis protein

The ankylosis protein (ANK) has, similar to NPP1, the function of suppressing mineralization by contributing to the extracellular supply of PPi. ANK is a transmembrane PPi channeling protein that allows PPi molecules to be passed from the cytoplasm to the outside of the cell through the plasma cell membrane (Ho *et al*., 2000; Nurnberg *et al*., 2001). The ANK protein is abundant on the cell surface of osteoblasts and chondrocytes, but is not present in the membranes of MVs, in contrast to NPP1 and TNAP (Harmey *et al*., 2004).

ANK mutant mice (*ank/ank*) develop hydroxyapatite crystals in articular surfaces and synovial fluids and display pathological abnormalities similar to NPP1 deficient mice, that mimic several arthritic diseases, including ectopic calcifications, cartilage erosions, osteophyte formation seen in osteoarthritis, and vertebral fusion observed in ankylosis spondylitis patients (Sweet and Green, 1981; Hakim *et al*., 1984;

Sampson and Davis, 1988; Mahowald *et al.*, 1989). *Enpp1*^{-/-} and *ank/ank* mice share many comparable phenotypic abnormalities even though the two proteins are present in different microenvironments in the matrix and that they have two distinct ways of supplying extracellular PPi (enzymatic activity vs. a channeling function).

Arterial calcification

Physiologic calcification or mineralization of the extracellular matrix is restricted to bones, teeth and the hypertrophic zone of growth plate cartilage. Pathological calcification can in contrast be observed in any tissue of the body. The most frequently pathological affected tissue is the media of the arteries. Vascular calcification refers to the deposition of hydroxyapatite in cardiovascular tissues such as arteries and heart valves (Detrano *et al.*, 2000). This is a serious problem, since 80% of all people over 60, according to WHO have detectable calcifications in atherosclerotic plaques, which is associated with higher morbidity and mortality. The primary cells involved in atherosclerosis intimal calcification are believed to be macrophages, mast cells and vascular smooth muscle cells (VSMC) (Shanahan *et al.*, 2000; Jeziorska *et al.*, 1998). Macrophages express a variety of bone-related proteins such as matrix Gla protein, osteopontin, bone sialoprotein and alkaline phosphatase (Shanahan *et al.*, 1994; 2000). Macrophages release TNF-α in response to oxidized LDL, and TNF-α treatment of VSMC has been shown to enhance osteoblastic differentiation, alkaline phosphatase expression and mineralization (Jovinge *et al.*, 1996; Tintut *et al.*, 2000). The VSMC is almost exclusively the source from which the bone associated proteins TNAP, bone sialoprotein, bone Gla protein and other arises from in medial calcification (Shanahan *et al.*, 1999). Primary cultures of VSMCs from normal vessels express MGP, collagen, osteonectin, osteocalcin, TNAP, bone sialoprotein, osteopontin, and BMP-2, which indicate that VSMCs can express osteoblast-specific genes (Shanahan *et al.*, 1999). Genetic disorders resulting in unregulated bone mineralization has been shown to be associated with arterial calcification. As mentioned before, deficient NPP1 activity has been associated with both osteoarthritis and Idiopathic Infantile arterial calcification (IIAC) (Rutsch *et al.*, 2001). Other non-inflammatory factors such as the mineralization inhibitor PPi have also been shown to mediate arterial calcification.

Osteopontin

Osteopontin (OPN) was discovered first among all calcification-regulating proteins within calcified plaques (Jono *et al.*, 2000). OPN binds to calcium and mediate cell adhesion and migration. It can act as both a cytokine and an extra cellular matrix protein (Barry *et al.*, 2000). Macrophages in calcified atherosclerotic lesions express high levels of osteopontin, while the expression in medial VSMCs is relatively low. OPN is hypothesized to inhibit hydroxyapatite depositions by VSMC (Wada *et al.*, 1999). Osteopontin expression is increased in *Akp2*^{-/-} and decreased in *Enpp1*^{-/-} and *ank/ank* mice. Both the PPi and the OPN levels are though normalized in double knockouts of [*Akp2*^{-/-}; *Enpp1*^{-/-}] and [*Akp2*^{-/-}; *ank/ank*] (Hessle *et al.*, 2002; Harmey *et al.*, 2004).

Osteocalcin

Osteocalcin is only expressed by osteoblasts and by osteoblast-like vascular cells and maybe a potent marker of osteoblastic differentiation. It is the most abundant non-collagenous bond extra-cellular matrix protein, and is also known as bone Gla protein osteocalcin. Earlier it has been shown that at approximately two weeks after a vessel wall injury, all calcified deposits contained osteocalcin, which indicate that it is involved in calcium crystal development (Gadeau *et al.*, 2001).

Bone morphogenetic proteins

Bone morphogenetic proteins (BMPs) are a group of related growth factors including BMP 1-7 (Wozney *et al.*, 1988). Only recombinant BMP-2 and BMP-4 have been shown to induce osteoblastic differentiation when implanted in soft tissues (Riley *et al.*, 1996). BMP-2 is expressed in human atherosclerotic lesions (Bostrom *et al.*, 1993) and evidence is accumulating that it by itself has the full potential to induce ectopic bone and cartilage formation in adult vertebrates (Wozney *et al.*, 2000).

Matrix Gla protein

Matrix GLA protein (MGP) is present in cartilage (Loeser *et al.*, 1992), bone matrix (Hauschka *et al.*, 1989) and in the arterial wall (Wallin *et al.*, 1999; Shanahan *et al.*, 1994). MGP is predominantly expressed by VSMC in the media and by the macrophages, VSMC and the endothelial cells in the intima (Shanahan *et al.*, 1994). MGP knockout mice have extensive arterial calcifications (Luo *et*

al., 1997), demonstrating that MGP works as a calcification inhibitor. The mechanism of this effect is not fully understood and is still under investigation, but it has been shown that MGP binds to BMP-2 (Wallin *et al*., 2000) and data has been presented that suggests that MGP blocks the osteo-inductive properties of BMP-2 (Bostrom *et al*., 2001).

Bone Sialoprotein

Bone Sialoprotein (BSP) is a sialic acid-rich acidic ECM glycoprotein that is synthesized by both osteoblasts and osteoclasts (Fisher *et al*., 1983; Bianco *et al*., 1991). BSP binds collagen (Fujisawa *et al*., 1995) and is associated in the early phases of bone formation acting as a potent and specific nucleator of hydroxyapatite at the mineralization front of bone (Hunter *et al*., 1993; Bianco *et al*., 1993). This suggests that BSP may act as an activator of calcification. Elevated levels of serum BSP is found in patients with ankylosing spondylitis (Acebes *et al*., 1999)

My Project

In this project arterial cross sections from WT, *Enpp1*$^{-/-}$ and *ank/ank* mice were stained to investigate the presence of calcium deposits using Von Kossa and Alizarin red staining. Hematoxylin and eosin staining was also performed to probe for abnormalities in the arterial wall. Expression of bone markers, such as osteopontin and osteocalcin were also investigated by immunohistochemistry assays. In addition, conditions for aortic tissue cultures were established in order to obtain primary smooth muscle cells from WT and *Enpp1*$^{-/-}$ and *ank/ank* mice. These cells will be used for mineralization assays to investigate whether *Enpp1*$^{-/-}$ and *ank/ank* mice have an increased ability to lay down mineral *in vitro*. The phenotype of the cultured cells was confirmed by using monoclonal fluorescent anti SM-actin antibodies for immunofluorescent assays.

Materials and Methods

Tissue preparation

ank/ank and *Enpp1*$^{-/-}$ mice and their wild-type (WT) littermates were sacrificed at 1, 2 and 3 months of age. Aortic tissue from the thoracic to the abdominal aorta was harvested and then fixed in 10% Neutral Buffered Formalin for 24 hours at 4°C, followed by 12 hours in 70% alcohol. Fixed tissue was processed for paraffin embedding, and sectioned at 5.0-µm. The sections were dried and melted for 1 hour at 60°C, de-paraffinized in xylene and hydrated. The arterial cross-sections were then used for histochemical and immunohistochemical analyses.

Histology

Hematoxylin and Eosin (H&E) staining was performed on the aortas from the mutant and WT mice to observe morphological differences (Harris Hematoxylin (EM science), Eosin Y (Sigma), Xylene and Ethanol). Phosphate depositions in the *ank/ank* aortas were visualized by von Kossa staining (5% silver nitrate, 5% sodium thiosulfate) and counter stained with Nuclear fast read, to determine if there were any signs of present calcifications. Alizarin Red S (0.5%, pH 9.0 C.I. 58005) staining was implemented to reveal calcium deposits. The detailed protocols are included in the appendix.

Immunohistochemistry

Immunoperoxidase staining with the avidin-biotin peroxidase system was used to detect proteins involved in up-regulation of bone formation. OPN and OCN immunohistochemistry was performed on the *ank/ank* and *Enpp1*$^{-/-}$ mice, on both tetramisole treated and untreated animals, and on the WT mice, using a Vectastain ABC kit (Goat IgG; Cat# PK-4005) from Vector Laboratories Inc, Burlingame, CA, USA. Detailed protocol is included in the appendix.

Isolation and primary cultures of mouse artery smooth muscle cells

Enpp1$^{-/-}$ mice and WT mice were sacrificed by suffocation in a CO_2 chamber. The aorta was excised from the heart along the border of the intestine, and placed in Petri dish containing Minimum Essential Medium (MEM). Fat, adventitia and venous structures were removed by blunt dissection, and the cleaned aorta was

transferred into a 15 ml falcon tube containing 2.0 ml of Enzyme Dissociation Mixture: Minimum Essential Medium (0.2mM Ca^{2+}), 15 mM HEPES buffer (pH 7.2-7.3), 0.250 mg/ml soybean trypsin inhibitor, 1.0 mg/ml collagenase type 1 and 2.0 mg/ml crystallized bovine albumin. After incubation at 37°C for 30 minutes in a gyratory shaker bath the tissue suspension was mixed with a 10 ml plastic pipette in the tube and then past through a nylon mesh to separate dispersed cells from undigested vessel wall and fragments and debris. The suspension was centrifuged in a 15 ml falcon tube (2000 rpm, 7 min) and the cell pellet re-suspended in 1 ml of MEM. This cell suspension was then transferred into 1 cm^2 tissue culture flasks, which were incubated at 37°C in a humidified 5% CO_2-95% air atmosphere. After 18-24 h, the cultures was washed with 1% PBS to remove none adherent cells and debris, and fed with fresh medium. Medium was routinely exchanged at 48-72 h intervals thereafter. Primary cultures were examined daily using a phase-contrast microscope.

Isolation of smooth muscle cells from a single murine aorta

The mice were euthanized by suffocation in a CO_2 chamber and then placed in a supine position on a corkboard. The mice were immobilized by pinning each paw in the corkboard. Subsequently, the thorax and abdomen were rinsed with a 70% ethanol/30% water solution. The aorta was dissected out, but left attached to the left ventricle. A 3 ml syringe with a 26.5 gauge was used to puncture the left ventricle and to flush the aorta with 3 ml of sterile PBS. The aorta was then removed and put into a 100 mm Petri dish in a drop of Fungizone solution (catalog # 15290018, Life Technologies), and cleaned from adventia and fatty tissue. When it was cleaned it was transferred into a new 100 mm Petri dish and cut into 1-2mm pieces, and then put into a tube containing 200 µl of Enzyme solution (7.5 mg of Collagenase 2, code LS004174 Worthington Biochemical Corporation and 5.5ml of MEM, filter sterilized solution). The tube was placed in a tissue culture incubator at 37°C, 5% CO_2 for five hours. After the incubation the cells were re-suspended and 700 µl culture medium was added. The cells were then centrifuged for 5 minutes at 2000 rpm. The medium was aspirated and resuspended in1000 µl fresh medium and then centrifuged as before. The cells were then resuspended in 700 µl medium and

transferred into a single well of a 96-well plate and placed in the incubator.

RNA- Isolation using TRIZOL

The cells were lysed directly in the culture disks by adding 2.0 ml of TRIZOL (Sigma Cat# 15596-026), and passed through a 5 ml syringe several times. The homogenized suspension was then incubated for 5 minutes at room temperature to permit the complete dissociation of nucleoprotein complexes. 0.4 ml chloroform was added and the tubes were vigorously shaken for 15 seconds, and then incubated at room temperature for 3 minutes. The samples were then centrifuged at 12.000×g for 15 minutes at 8°C. The mixture was separated after the centrifugation and the upper aqueous phase, containing the RNA, was transferred into a new tube. The RNA was precipitated from the aqueous phase with 1 ml isopropyl alcohol and left to incubate for 10 minutes in room temperature, and then centrifuged at 12.000×g for 10 minutes at 8°C. The supernatant was removed from the precipitated RNA pellet, which was washed with 2 ml 75% ethanol. The sample was mixed by vortexing and centrifugation at 7.500×g for 5 minutes at 8°C. The RNA pellet was air-dried and re-suspended in 30 µl of RNAse free H_2O. Finally the dissolved RNA was measured by spectrophotometer at A_{260} to control that the isolation had succeeded.

Amplification of the control mouse β-actin fragment

To control if the RNA purification had succeeded, and to normalize the amounts KO and WT RNA, the 540-bp mouse β-actin fragment was amplified using the TITANIUM™ One-Step RT-PCR Kit (see protocol in appendix).

Immunnofluorescence for α-SM Actin

VSMC were cultured for five days in an 8 well plate (Lab-Tek chambered cover-glass w/cvr vcat# 155411) with DMEM. The cells were re-fed after five days and then left for 48 hours. The culture medium was removed and the cells were washed with PBS twice and fixed with 4% formaldehyde for 30 minutes. The cells were then washed with PBS and permeabilised with 0.2% triton in PBS for 30minutes. Washed with PBS and then blocked with 3% BSA in PBS for another 30 minutes. The cells were then incubated for 1-2 hours with a fluorescent antibody specific for α-SM actin

isoform (cat# F3777) in 1:100 and 1:250 dilutions. The cells were then washed and left in PBS over night. Photographs were then taken through a Nikon Eclipse TE 300 microscope with a CCD SPOT-RT (Diagnostic Instruments, Ink).

Mineralization assays

VSMCs isolated from WT cell cultures were cultured in α-MEM with 10 mmol/L β-glycerophosphate (βGP) and 50 μg/L ascorbic acid. The ascorbic acid turned out to have a toxic effect on the VSMCs and was excluded in the following experiments. β-glycerophophate treated cells and their control were stained for alkaline phosphatase activity after 7 respectively 14 days. The medium was suctioned from the wells and the cells were rinsed with PBS. The cells were fixated in 4% formaldehyde for 15 minutes and then rinsed and left in ddH2O for 15 minutes. The AP substrate (Naphtol AS MX-PO_4 5mg, DMF (N,N-Dimethylformamide) 200 μl, Tris-HCl 0.2M pH 8.3 25ml, distilled water 25 ml and Red Violet LB salt 30 mg) was prepared fresh and then filtered through a Whatman's NO.1 just before staining. Incubated the cells with substrate for 1 hour and then rinsed with and left in PBS. The stained cells were then compared and scanned for display.

Results

The aim of my work was to investigate the role of the three bone mineralization regulating proteins, TNAP, ANK and NPP1 in arterial calcifications and to explore TNAP as a target molecule for the treatment of arterial calcification inhibition. As a first step, I worked on optimizing the isolation and culturing of primary vascular smooth muscle cells. Two different protocols were used in the process of optimizing the conditions for vascular smooth muscle cell (VSMC) isolation. The first protocol did not yield enough cells to achieve adequate cell-to-cell contact in a 96-well flask, necessary for cell survival. We did succeed in isolating and culturing wt and *Enpp1$^{-/-}$* cells using this protocol, but the results were not reproducible. In order to overcome these difficulties we used another more specific protocol. The major differences between the two protocols was the enzyme dissociation mixtures used, and the time and conditions for the digestion. In the first protocol a mixture containing Minimum Essential Medium (0.2 mM Ca^{2+}), 15 mM HEPES buffer (pH 7.2-7.3), 0.250 mg/ml soybean trypsin inhibitor, 1.0 mg/ml collagenase type 1 and 2.0 mg/ml crystallized bovine albumin

was used in comparison to the enzyme solution used in the second protocol which only was made up and filtered with 7.5 mg of collagenase type 2 and 5.5 ml of DMEM. The collagenase type 2 used also had a higher activity than collagenase type 1. The aorta was left for enzyme digestion for 30 minutes in a gyratory water bath shaker in the first protocol, while for 4-6 hours in a tissue culture incubator in the second procedure. The new improved conditions for isolation, digestion and culturing increased the number of surviving cells significantly. With this approach, we obtained wt cells that survived and grew normally. The cells isolated from the *Enpp1$^{-/-}$* and *ank/ank* mice did still not grow enough. However, we proceeded to characterize the behavior of wt cells while at the same time optimizing the isolation of *ank/ank* and *Enpp1$^{-/-}$* VSMCs.

We then characterized the wt cells by immunohistochemistry. The cells were stained with a fluorescently labeled antibody directed against smooth muscle alpha-actin, a specific smooth muscle cells protein which verified that the cells were VSMC (Figure 3).
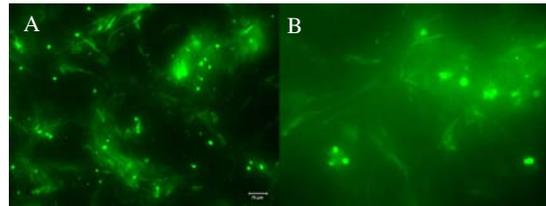
Figure 3 - WT cells stained with αSM-actin fluorescent antibody confirmed that the cells cultured were VSMCs, where picture (A) is taken with 10x magnification and (B) with 20x magnification.
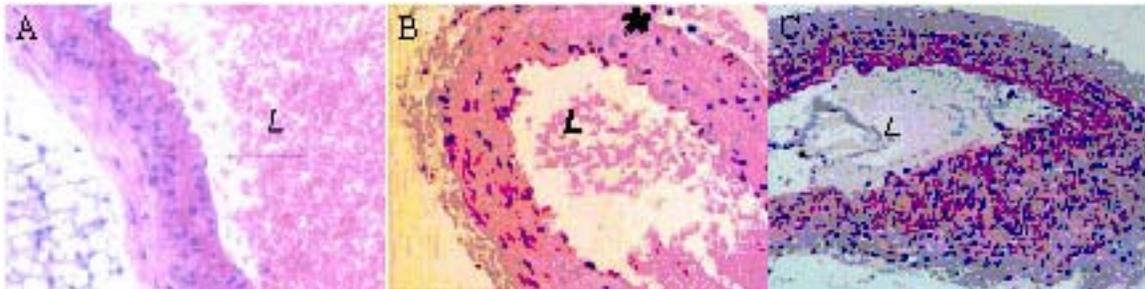
In order to assess the ability of these cells to mineralize, we cultured VSMC with 10 mmol/L of β-glycerophosphate and 5 mmol/L of ascorbic acid, according to the protocol for bone marrow stem cell. The cells were detaching from the well after one day of treatment, which made us draw the conclusion that VSMCs could not be grown under the same conditions as bone marrow stem cell. With support from earlier studies of VSMCs ability to lay down mineral *in vitro*, we excluded the ascorbic acid from the protocol. After this minor change the cells grew as expected and the cells grown in presence β-glycerophosphate developed nodules. The cells were re-fed at 48 hours intervals for 7 respectively 14 days. Alkaline phosphate activity was visualized by staining and an increase in AP activity could be identified in

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the wells treated with β-glycerophosphate, which is known to induce AP expression. As expected, the cells treated for 14 days expressed a more intense staining, indicating a higher AP activity (Figure 4).
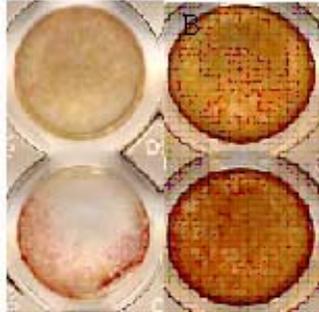

Figure 4 - Alkaline phosphatase staining of WT VSMCs grown in the presence (lower) or absence (upper) of ß-GP for 7 (A) or 14 (B) days showed an increase of AP activity with treatment of ß-GP and longer exposure time.

Hematoxylin and Eosin (H&E) staining makes it possible to visualize morphological changes in the vessel wall of aorta. Hematoxylin stain negatively charged nuclei acids (nuclei and ribosomes) blue and proteins are stained pink by the Eosin. We found alterations in the vessel walls of *ank/ank* mice compared to those of wt mice. The fibers of the wall are more loosely arranged in the *ank/ank* aorta than in the wt. The *Enpp1$^{-/-}$* aortic wall displayed even more severe alterations, not only in the rearrangement within the wall but also an increment in the thickness of the wall. We could also see a higher number of nuclei in the entire *Enpp1$^{-/-}$* aorta, specifically in the thickening of the wall, which indicate an increase in the number of cells (Figure 5).

Von Kossa silver test for calcium illustrates calcium salts as black-brown staining by silver depositions. Three months old *ank/ank* mice stained with von Kossa showed mineral deposits not visible in WT mice. *Enpp1$^{-/-}$* mice did not show any mineral deposits, but displayed irregularities on the lumen side of the wall (Figure 6).
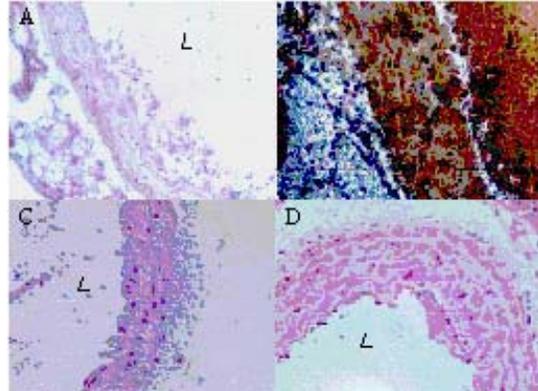
Figure 6 - *ank/ank* (B x20 magnification) sections show positive Von Kossa staining for mineral depositions not visible in WT sections (A x20 magnification). *Enpp1-/-* (D x40 magnification) mice did not show any mineral deposits, but mild alterations in the vessel wall. Tetramisole treated *Enpp1-/-* (C x40 magnification) mice did not show any signs of mineral depositions.

Alizarin red is used to demonstrate calcium depositions in tissue sections by coloring them orange-red. The alizarin red staining of three month old *ank/ank* mice shows the presence of calcium deposits in the aorta that is not visible in the aged-matched WT mice. The *Enpp1$^{-/-}$* mice did not show signs of calcification, as would have been expected, but significant alterations and thickening of the aortic wall (Figure 7).

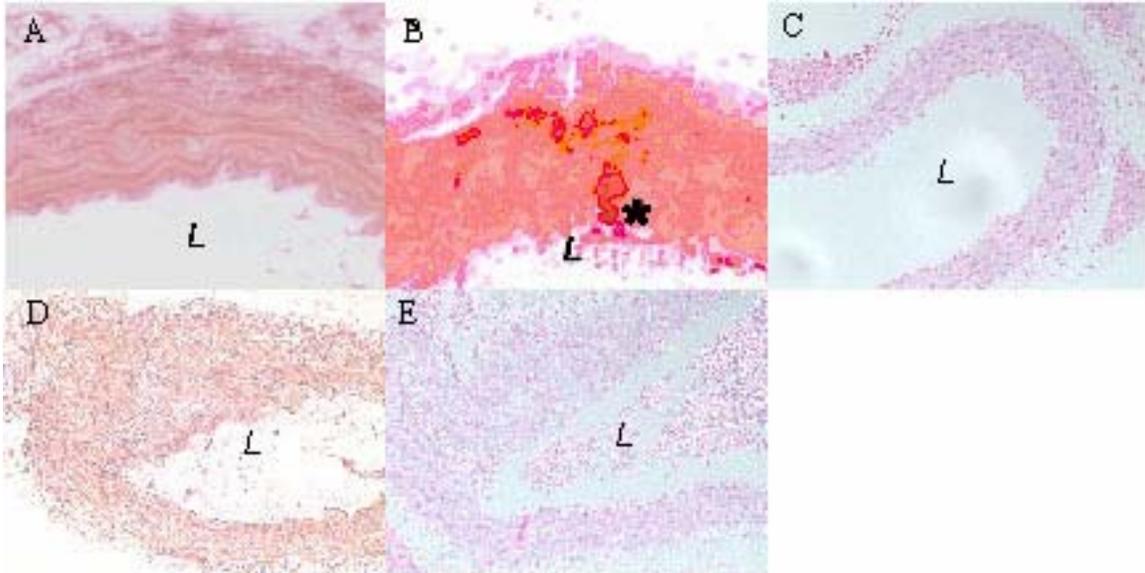

Figure 5 - Alterations in the arterial wall of *ank/ank* (A) an *Enpp1-/-* (C) mice, which was not shown in WT (A) sections was displayed with H&E staining. (All sections x20 magnification) *L*= lumen

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Figure 7 - Arterial cross sections (x20 magnification) were stained with alizarin red to detect calcium deposits. *ank/ank* (B) displayed positive staining for calcium not visible in the WT (A). The *Enpp1-/-* section (D) did not show any calcifications, but displayed significant alterations and thickening of the wall. The tetramisole treated mice, *Enpp1-/-* (C) and *ank/ank* (E) did not display any signs of calcification. (**L =** lumen *= positive staining)

To document the stepwise development of arterial calcifications, immunostaining of arterial cross-sections was performed on wt, *ank/ank* and *Enpp1$^{-/-}$* sections. The later stages of arterial calcification were assessed by detection of bone regulating proteins, such as OPN and OCN. A wt section was stained with only PBS and normal rabbit serum (NRS) as a negative control and sections of bone served as positive control for the specificity and reactivity of the OPN antibody. The wt section did not, as expected show any positive staining for OPN. OPN has earlier been shown to be expressed in injured wt vessel walls but not in normal wt vessels. The *Enpp1$^{-/-}$* did once again show severe irregularities and thickening of the wall, but no OPN staining. No positive staining was observed in the *ank/ank* aorta sections (Figure 8).

In order to verify if the antibody was specific for OCN, wt aorta sections were also used as a negative control, i.e. only incubated with PBS and normal rabbit serum (NRS). We also stained slides of spine sections, for positive control, which clearly showed OCN expression. Aorta sections from different wt and knockout mice were also stained during the same experiment. The wt sections from the staining did though show positive OCN staining, which indicated that the antibody probably reacted un-specifically with some other proteins since OCN should not be expressed in healthy wt aorta (Figure 9).
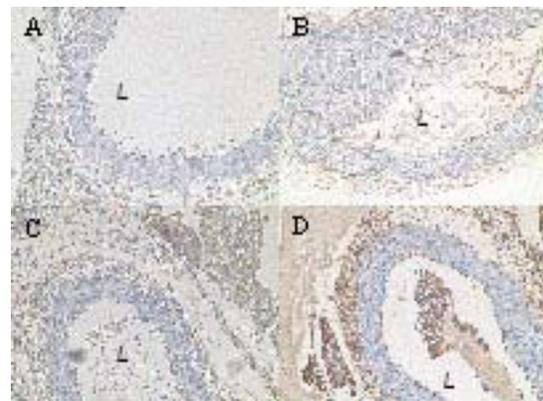

Figure 8 - OPN staining was performed on WT (A). *Enpp1-/-* (B), *ank/ank* (C) and on tetramisole treated *ank/ank* (D) sections. No positive staining could be detected in any of the samples, but the *Enpp1-/-* section displayed severe irregularities of the wall. OPN has in earlier examples only been expressed in injured WT, which is probably why it was not detectable in our study. (All sections x20 magnification)

The extraction of RNA from the *Enpp1$^{-/-}$* and WT cultures was successful and the RNA did not show any signs of degradation. The RNA was amplified with TITANIUM RT-PCR for actin, a housekeeping gene, in order to normalize the amounts of extracted RNA for further experiments with primers for OPN and other bone specific proteins. The RT-PCR from 1 µl of each RNA extraction was run on a 1% agarose gel, which showed that the RNA concentration was slightly lower in the RNA extracted from the *Enpp1$^{-/-}$*
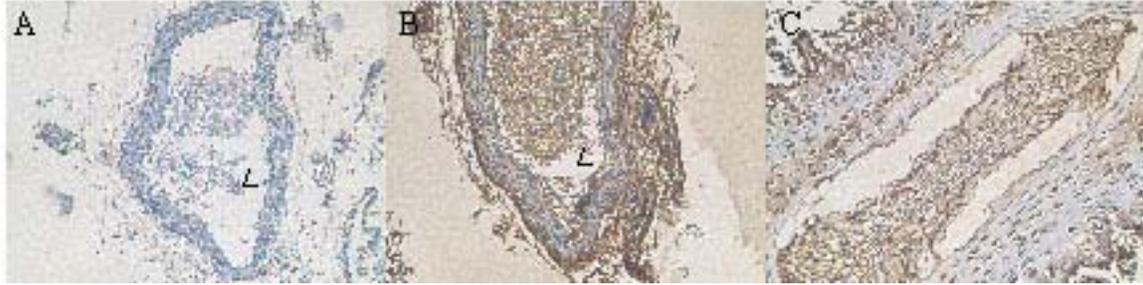
Figure 5 - We evaluated an OCN immunoassay with one untreated WT (A) aortic section as a negative control and a spine section (C) as a positive control. The OCN antibody stained the WT (B) section positive for OCN, which indicates that the antibody stains un-specifically, since OCN should not be expressed in healthy WT aortas. (All sections x20 magnification)

culture than from the wt. In order to normalize these two concentrations we used different amounts, 3 and 6 µl of *Enpp1*$^{-/-}$ RNA and ran the product on a gel together with 1 µl wt RNA product. The gel showed that 3 µl of *Enpp1*$^{-/-}$ RNA was not enough to be adequate with 1 µl of wt RNA, and unfortunate did not the RT-PCR work for 6 µl *Enpp1*$^{-/-}$ RNA (Figure 10). The next step will be to optimize the actin primers for the PCR so that the amount of RNA from the extractions can be normalized and the RNA used to measure the expression of bone specific proteins such as OPN.
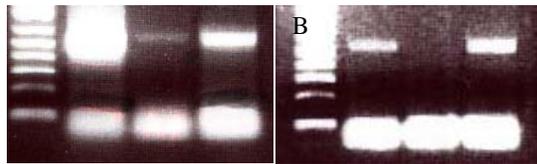
Figure 6 - Gel pictures from RT-PCR with WT and *Enpp1-/-* RNA extracted from VSMCs cultures. Picture (A) shows (first band1µl of control RNA, the second 1 µl *Enpp1-/-* RNA and the third 1 µl of WT RNA.) that the KO RNA concentration was much lower than in WT. In order to normalize these volumes, 1 µl of WT RNA (band three) was run together with 3 and 6 µl of KO RNA (Band one and two). The PCR did not work with 6 µl KO RNA, but by comparing the first and the third band we draw the conclusion that the amount of KO RNA should be between 3 and 6 µl for normalization with 1 µl of WT RNA.

Our data on the progressive development of aortic abnormalities in the *ank/ank* and *Enpp1*$^{-/-}$ mice revealed morphological abnormalities in the aortic wall and positive staining for mineral and calcium in the *ank/ank*, which supports our hypothesis that *ank/ank* mice display an aortic hyper-mineralized phenotype as well as a hyper-mineralized bone phenotype as shown earlier (Harmey *et al*., 2004).

We then proceeded to assess if the pharmacological inhibition of TNAP activity could

ameliorate these abnormalities. Thus we used *ank/ank* and *Enpp1*$^{-/-}$ mice, which had been treated with tetramisole for 3 months and examined the aortas by the same procedures as described above. In these experiments, we found that tetramisole treated *ank/ank* and *Enpp1*$^{-/-}$ mice did not stain positive for calcium nor for any other mineral in the alizarin red and von Kossa experiments. Treated *ank/ank* sections stained for OPN showed unspecific staining in the tissue surrounding the aorta but not in the aortic wall. These findings support earlier studies which indicate that tetramisole functions as an inhibitor of TNAP activity, not only in bone mineralization as shown before, but also in arterial calcification, as hypothesized.

Discussion

Calcification of vascular tissue is a common finding in atherosclerosis, diabetes, renal failure and aging. These arterial calcifications have in earlier studies been shown to share many common features with the actors in mineralization of bone, which suggests that arterial calcification is a controlled and highly regulated process. In this study we have investigated the roles of three bone mineralization regulating proteins, TNAP, ANK and NPP1 in arterial calcifications in order to highlight TNAP as a potent target molecule for arterial calcification inhibition. In earlier studies, Rutsch *et al* have investigated *Enpp1*$^{-/-}$ mice ability to develop arterial calcification in early life and the sharing of phenotypic features with human patients with Idiopathic Infantile Arterial Calcification IIAC (Rutsch *et al*., 2001). *Enpp1*$^{-/-}$ mice have also been shown (Harmey *et al*., 2004) to have a more severe hypermineralized phenotype than *ank/ank* mice and that NPP1 but not ANK is localized in the matrix vesicles. The hypomineralized phenotype in *Akp2*$^{-/-}$ has been shown to be normalized in double knock out mice [Akp2$^{-/-}$;

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Enpp1$^{-/-}$] (Hessle *et al*., 2002) but only partly normalized in the [Akp2$^{-/-}$; ank/ank] mice, which suggests that ANK and NPP1 are corrected in different ways due their different localizations within the cell (Harmey *et al*., 2004). This could maybe also explain some of the differences between the *ank/ank* and *Enpp1*$^{-/-}$ aorta sections that we have studied. We have observed positive staining for calcium and mineral in *ank/ank* aorta, which could not be detected in the *Enpp1*$^{-/-}$ sections. The *Enpp1*$^{-/-}$ sections did though show severe alterations in the aortic wall not seen in the *ank/ank* sections nor in the tetramisole treated *ank/ank* and *Enpp1*$^{-/-}$ sections which indicates that *Enpp1*$^{-/-}$ mice, that show abnormal bone mineralization, also have an altered aortic phenotype. *Enpp1*$^{-/-}$ sections stained with H&E for morphological changes showed an increase in cell density within the thickening, which also showed signs of a severe rearrangement of the fibers within the wall. *ank/ank* mice also displayed an alteration in the vessel wall fibers but not nearly as severe as in *Enpp1*$^{-/-}$ mice. OPN expression in arterial calcification has earlier been described (Speer *et al*., 2002) in other models of bone deficient mice, such as *Mgp*$^{-/-}$, *Opn*$^{-/-}$ and in [*Mgp*$^{-/-}$; *Opn*$^{-/-}$] knockouts. *Opn*$^{-/-}$ mice does not display any mineralization defects which might be explained by that OPN is not expressed in normal blood vessels, but only appears during pathological conditions. This initiated a crossing of *Mgp*$^{-/-}$ mice, which spontaneously develop vascular calcifications, and *Opn*$^{-/-}$ mice. These mice showed enhanced vascular calcifications, which indicate that OPN might act during an adaptive response as an inducible inhibitor (Speer *et al*., 2002). These findings together with the published data that *ank/ank* and *Enpp1*$^{-/-}$ mice display decreased levels of OPN (Harmey *et al*., 2004), could explain why we can not observe any positive OPN staining in the knock out mice sections.

The conditions for development and optimization of smooth muscle cell isolation for primary cultures was worked out by changing the enzyme digestion solution and the incubation time and conditions as described to increase and improve the number and the specificity of cells isolated. We succeeded to isolate and culture VSMCs from aortic tissue by prolonging the incubation time to four and a half hours, which in earlier studies have been optimized to be between four and six hours for isolation of essentially SMC, and to change the enzyme solution from collagenase type 1 to type 2 with a higher activity, which also has been proved to increase the purity and amount of cells (Benson *et al*., 2002). When we changed the protocol we were no longer able to isolate and culture VSMCs from the *Enpp1*$^{-/-}$ mice, which we actually succeeded with one time when using the first protocol. This might be due to the change of collagenase from type 1 to type 2, since *Enpp1*$^{-/-}$ mice are hypothesized to have an increase in arterial calcification, hypothesized to be build up by the same mechanisms as in calcification of bone, which predominantly consist of collagen type 1, which would explain why collagenase type 1 would be more suitable in this particular case.

There is also a high risk of contamination when working with cell isolation, especially fungal contamination, which was prevented by sterilizing all the instruments, changing the instruments after removal of the skin and then dip the instruments in 70% Ethanol and sterile DMEM during the dissection. With all these improvements of the protocol we managed to successfully isolate and culture primary vascular smooth muscle cells.

The mineralization assay was optimized for VSMCs by exclusion of the ascorbic acid, which appeared to be toxic for the cells. The cells treated with β-glycerophosphate showed an increase in alkaline phosphate activity, which increased even more after a longer period of treatment. β-glycerophosphate has the ability to increase the amount of AP expressed, but also the expression of OPN and OCN as described in Steitz *et al*. This could be explained by the theory that VSMCs loses their smooth muscle cell markers when treated with β-glycerophosphate to induce calcification.

Taken together, our findings suggest that TNAP is in fact an interesting target molecule for arterial calcification inhibition that needs to be further evaluated.

Appendix

OPN Immunohistochemistry

Material:

Vectastain ABC kit (Goat anti IgG; cat#PK-4005) form Vector Laboratories Inc, Burlingame, CA, USA. Kit includes Blocking Serum, Biotinylated Secondary Antibody, Avidin DH (A) and Biotinylated Horseradish Peroxidase H (B).

Primary Goat-Antibody (OPN P-18, cat# sc-10593, Santa Cruz Biotechnology)

Diaminobenzidine Tetrahydrochloride (DAB), 40mg/ml brown stock (1/200) solution.

0.05M Tris-HCl, pH 7.6

2M Sodium Azide solution

PBS, Ethanol, Xylene, H2O

31% H2O2 (concentrated)

Mayers Hematoxylin solution from Sigma Diagnostics, Cat# MHS-16

Permount from Fisher Scientific, Cat# SP15-100

Method:

1. Dehydrate sections in Xylene 3x5', 100% EtOH 2x5', 70% EtOH 5 minutes and then in running water for 5 minutes.
2. Wash sections with PBS, 3x5 minutes.
3. Inactivate endogenous peroxidase activity: Mix 0.5 ml H_2O_2 (31% concentrated) in 50ml Methanol. Incubate slides for 30 minutes.
4. Wash sections with PBS 3x5 minutes.
5. Block with Normal Rabbit Serum (~2%). Put 150μl stock into 10ml PBS. Incubate slides in blocking solution for 30 minutes.
6. Add primary antibody into blocking solution, making a 1/100 dilution. Drop the solution onto the slides and put them into a humidity chamber over night. Negative control: Normal Goat Serum and PBS.
7. Wash sections with PBS 3x5 minutes.
8. Incubate slides with Biotinylated Anti-goat Antibody (secondary). Put 50μl stock into 10ml of PBS. Incubate for 30 minutes. Premix solution in # 10.
9. Wash sections with PBS, 3x5 minutes.
10. Mix 100μl of A and 100μl of B into 10ml PBS. Incubate slides for 10 minutes.
11. Wash sections with PBS 3x5 minutes.
12. Dilute 250μl DAB stock and 250μl of Sodium azide stock into 50ml of 0.02M Tris. Add 10μl concentrated (31%) H_2O_2 into the 50ml mixture and incubate slides in the solution for 15 minutes in the dark at room temperature.
13. Wash sections with H_2O, 10 times.
14. Counterstain with Hematoxylin for 5-10 minutes.
15. Wash sections with H_2O twice.
16. Make Hematoxylin staining blue by incubating slides into PBS for at least 5 minutes.
17. Wash sections with H_2O twice
18. Dehydrate sections with serial concentrations of Ethanol (50%, 70%, 80%, 90%, 95%, 100%x3) and Xylene (100%x3), under ventilation hood.
19. Slightly dry slides and mount slides with Permount and cover slips.

Hematoxylin and Eosin staining:

Reagents:

Harris Hematoxylin (EM science)

Eosin Y (Sigma)

Ethanol

Xylene

Procedure:

1. Dehydrate sections in Xylene 3x5', 100% EtOH 2x5', 70% EtOH 5 minutes and then in running water for 5 minutes.
2. Put in Hematoxylin for 2 min.
3. Wash slides in water 2x1 min, or until water looks clear.
4. Put slides in 50% EtOH, 1min, 80% EtOH 1 min, 95% EtOH 1 min, 100% EtOH 2x1min.
5. Soak slides in Eosin-Y (10dips or 45 seconds).
6. Wash in 100% EtOH 2x1min each or until the EtOH looks clear.
7. Re-hydrate slides with 50% EtOH 1min, 70% EtOH 1 min, 90% EtOH I min, 100% EtOH 2x1 min, Xylene 2x1 min.
8. Slightly dry slides and mount slides with Permount and cover slips.

Von Kossa silver test for calcium:

Solutions:

5% aqueous silver nitrate solution: Dissolve 5g of Silvernitrate per 100ml ddw. Keep in RT.

5% sodium thiosulfate solution: Dissolve 5g of Sodiumthiosulfate (($Na_2S_2O_3$ x 5 H_2O); Sigma S-8503) per 100ml ddw. Keep in RT.

Nuclear fast red solution: 0.1g Nuclear fast red (Kernechtrot) in 100ml 5%Aluminiumsulfate. Heat the solution until it dissolves. Chill down and filter solution. Keep in RT.

Procedure:

1. Dehydrate sections in Xylene 3x5', 100% EtOH 2x5', 70% EtOH 5 minutes and then in running water for 5 minutes.

2.Immerse or flood slides with the 5% silver nitrate solution.

3.Expose the immersed or flooded slides to bright sunlight or ultraviolet light for 10 to 20 minutes or to a 60-watt electric bulb at a range of 4-5 inches for 60 minutes. Stop exposure when calcium salts are black-brown.

4.Wash slides in several changes of distilled water.

5.Remove un-reacted silver with 5% sodium thiosulfate for 2 minutes.

6.Counterstain with nuclear fast red for 3-5 minutes. Filter back.

7.Rinse slides in several changes of distilled water.

8.Re-hydrate slides with 50% EtOH 1min, 70% EtOH 1 min, 90% EtOH I min, 100% EtOH 2x1 min, Xylene 2x1 min.

9.Slightly dry slides and mount slides with Permount and cover slips.

Results:
Calcium salts- black to brown black
Nuclei-red
Cytoplasm-pink

Alizarin red staining:

Solutions:
Staining solution
Alizarin red S (C.I. 58005) 2mg
Distilled water 100ml
Mix and adjust the pH of the solution to a range of 4.1-4.3 with dilute ammonium hydroxide.

Technique:
1.Dehydrate sections in Xylene 3x5', 100% EtOH 2x5', 70% EtOH 5 minutes and then in running water for 5 minutes.

2.Cover the sections with alizarin red solution.

3.Observe the reaction under a microscope and re-move when a red-orange lake forms (30 seconds to 5 minutes). The lake should be heavy but not too diffuse.

4.Shake of excess dye and blot carefully.

5.Dehydrate in aceton for 10-20 seconds and aceton-xylene (50:50) for 10-20 seconds.

6.Clear in Xylene, 2x1 minutes

7.Slightly dry slides and mount slides with Permount and cover slips.

TITANIUM™ One-Step RT-PCR Kit

A. Preparing an RT-PCR Master Mix

5μl	10x One-Step Buffer
1μl	50X dNTP Mix
0.5μl	Recombinatnt Rnase inhibitor (40 units/μl)
0.25μl	Thermostabilizing Reagent
10μl	GC-Melt™
1μl	Oligo(dT) Primer
1μl	50x RT-TITANIUM™ Taq

Enzyme Mix

Total volume 43.5μl

B. Setting up the reactions

1-5.5μl	RNA sample (1ng-1μg)
1 μl	PCR primer mix (45μM each)
43.5 μl	Master Mix
	RNase-Free H$_2$O (add to 50 μl final volume)

C. Running the reactions
- ○ 50°C for 1hr
- ○ 94°C for 5 minutes
- ○ 25-35 cycles of
  94°C 30sec
  65°C 30sec
  68°C 1 minute
- ○ 68°C for 2 minutes

DNA isolation for genotyping:
Solutions:
1.Lysis buffer
50mM Tris HCl (pH 8)
100mM EDTA (pH 8)
1% SDS
100mM NaCl
2.Protinase K
Protinase K dissolved in ddH2O, stock solution 10mg/ml
3.TE
10mM Tris
0.1mM EDTA

Procedure:
1.Incubate tails in 700μl lysis buffer + 20μl proteinase K o/n at 56°.

2.Transfer the solution to SST tubes (with polymer on the bottom of tube), add 700μl phenol : chloroform : iso-amyl alcohol (25:24:1)

3.Mix well and centrifuge at 2000 rpm for 10 minutes (rt)

4.Add another 700μl phenol : chloroform : iso-amyl alcohol (25:24:1)

5. Mix well and centrifuge at 2000 rpm for 10 minutes (rt)

6.Add 700μl of chloroform

7. Mix well and centrifuge at 2000 rpm for 10 minutes (rt)

8.Transfer aqueous phase to eppendorf tube and add 700μl of isopropanol

9.Mix and centrifuge at max speed for 5 minutes (rt)

10.Remove supernatant and add 700μl of 70% EtOH

11.Centrifuge 7000-10000 rpm for 5 minutes

12.Remove supernatant and let pellet air-dry

13.Dissolve in 200μl TE. Store at 4°C

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