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Cellular signaling in pseudoxanthoma elasticum: an update

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ABSTRACT

Pseudoxanthoma elasticum is an autosomal recessive genodermatosis with variable expression, due to mutations in the *ABCC6* or *ENPP1* gene. It is characterized by elastic fiber mineralization and fragmentation, resulting in skin, eye and cardiovascular symptoms. Significant advances have been made in the last 20 years with respect to the phenotypic characterization and pathophysiological mechanisms leading to elastic fiber mineralization. Nonetheless, the substrates of the ABCC6 transporter - the main cause of PXE - remain currently unknown. Though the precise mechanisms linking the ABCC6 transporter to mineralization of the extracellular matrix are unclear, several studies have looked into the cellular consequences of ABCC6 deficiency in PXE patients and/or animal models. In this paper, we compile the evidence on cellular signaling in PXE, which seems to revolve mainly around TGF-βs, BMPs and inorganic pyrophosphate signaling cascades. Where conflicting results or fragmented data are present, we address these with novel signaling data. This way, we aim to better understand the up- and down-stream signaling of TGF-βs and BMPs in PXE and we demonstrate that ANKH deficiency can be an additional mechanism contributing to decreased serum PPi levels in PXE patients.

1. Introduction

Pseudoxanthoma elasticum (PXE, OMIM# 264800) is an autosomal recessive genodermatosis characterized by ectopic mineralization and fragmentation of the elastic fibers [1]. In patients, these extracellular matrix (ECM) changes will lead to skin (papular lesions and increased skin laxity in flexural areas), ophthalmological (peau d'orange, angioid streaks and subchoroidal neovascularisation with hemorrhage) and cardiovascular symptoms (peripheral occlusive disease, coronary and cerebrovascular artery disease), though the severity of these manifestations can be quite variable between different patients [1].

In most patients, PXE is caused by biallelic mutations in the *ABCC6* (ATP-binding cassette, subfamily C, member 6; OMIM*603234) gene [2]. *ABCC6* encodes an ABC efflux transporter which is most abundant in the liver and kidney, hence the paradigm that PXE should be considered a metabolic disease [3]. The putative substrates of this transporter remain however unknown as do the exact pathophysiological mechanisms that link ABCC6 to the ECM changes in PXE. More recently, mutations in *ENPP1*, encoding an ectonucleotide pyrophosphatase/phosphodiesterase 1 (OMIM*173335) were found in a small subset of patients [4]. In the past decade, several additional PXE-like diseases have been described, which show clinical resemblance with (part of) the PXE phenotype and are due to homozygous or compound heterozygous mutations in the *GGCX* gene, encoding a gamma-glutamyl carboxylase (OMIM*137167). These include the PXE-like disorder with multiple coagulation factor deficiency (OMIM# 610842) and the PXE-like phenotype with pigmented retinopathy [5,6]. Following the description and molecular elucidation of these PXE-like phenotypes, it was noted that also digenic inheritance of a heterozygous *ABCC6* mutation with a *GGCX* mutation can cause PXE in few patients [7].

PXE holds a unique position among soft tissue mineralization disorders and elastinopathies because of its multisystemic phenotype. Though in the last decade important advances have been made in understanding its pathophysiology, the unresolved question about the ABCC6 substrate(s) and its exact role in ECM mineralization remains an important disadvantage, hampering among others the development of an efficient treatment for PXE patients. One of the approaches that could be envisaged to gain more insights into the enigmatic role of ABCC6, is to study the molecular pathways which are influenced by ABCC6 deficiency in PXE patients and animal models. As often in this intriguing but complex disorder, this proves to be more challenging than initially thought. Nonetheless, it has become clear that the main signaling cascades involved are the TGF-β superfamily (mainly TGF-β and BMPs) and inorganic pyrophosphate (PPi). In the first part of this paper, we provide an overview of the current knowledge on these signaling cascades in PXE and highlight discrepancies and gaps in

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knowledge about these signaling pathways in PXE. In the second part, we address some of these discrepancies and gaps by presenting novel signaling data.

2. Materials and methods

2.1. Review of the literature

The focus of this paper being the dysregulation of TGF-β, BMP and PPI related signaling cascades, we obtained a state-of-the-art overview via a literature review using the following keywords: pseudoxanthoma elasticum, PXE, ABCC6, MRP6, BMP, TGF-β, inorganic pyrophosphate, PPi, ANKH and signaling. Both original articles and reviews were considered. Only papers in English were reviewed. In total, 41 papers were found on these topics and included in the review.

2.2. Skin biopsies and cell culture conditions

Dermal tissues and fibroblast cultures were obtained through full thickness skin biopsies in macroscopic skin lesions from clinically and molecularly confirmed PXE patients followed in the PXE Clinic of the Ghent Center for Medical Genetics. All patients had a PXE phenodex score between 4 and 5. All patients had bi-allelic *ABCC6* mutations, with at least one allele being the recurrent p.(R1141X) (c.3421C > T) mutation. The study was approved by the Ethical Committee of the Ghent University Hospital. Informed consent was obtained from all patients and the Declaration of Helsinki protocols were followed.

Fibroblasts of age- and sex-matched controls were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% Fetal Calf Serum, 1% penicillin/Streptomycin, 1% Kanamycin, 1% non-essential amino acid and 0.1% fungizone. Cultures were maintained by refreshing media twice a week and incubated at 37 °C (5% CO2). First, fibroblasts were grown in T25 cm^2 flask; when 100% confluent, fibroblasts were spliced and transferred in T75 cm2 flask. When 100% confluent, cells were spliced and grown in 60 mm petri-dishes. Fully confluent 60 mm petri-dishes were scraped and centrifuged to extract the tissues and stored at −80 °C.

2.3. Gene expression quantification via qPCR

qPCR analysis was performed for genes involved in the TGFβ signaling pathway (*TGFβ-1, TGFβ-2, TGFβ-3, ITG6, ITG8, BMP1, MT-MPP1, TSP1, SMAD2, SMAD3, ERK1, HRAS, RAF1, MEK1, PI3K, TAK1, p38, TRAF4, RHOA, NFKB, TRIP1* and *CTGF*), BMP signaling (*BMP1, SMAD4*) and *ANKH*. RNA was isolated from fully confluent fibroblasts using the RNeasy® kit (Qiagen, GmbH, Germany) according to manufacturer's recommendation. To purify the RNA from any DNA that may be present, it was incubated with DNase (15 min. at RT). Concentration of total RNA was measured via the DropSense-96 multichannel spectrophotometer (Micronic North America, USA). cDNA was prepared from 2 μg of RNA using the iScript cDNA synthesis kit (Bio- Rad Laboratories, CA) and diluted 10-fold. qPCR was performed on control ($n = 5$) and PXE fibroblasts ($n = 8$) using *HPRT1* (hypoxanthine phosphoribosyl transferase 1) and *YWHAZ* (tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta isoform) as reference genes and the FastStart Universal Probe Maser Mix (Roche Applied Science, GmbH, Germany) in the Roche-LightCycler®480 real-time PCR system (Roche Applied Science, Belgium). qPCR primers are available upon request to the author. Real time PCR data were analyzed via the qbasePLUS software (Biogazelle, Belgium).

2.4. Immunofluorescence staining

To detect ANKH, fluorescent immunohistochemistry was performed on DMEM-cultured fibroblasts of two PXE patients and two matched controls. Cells were seeded per 30.000 in 500 μl medium in chambers on glass slides. After incubation at 37 °C (5% CO2) for 3 nights, fixation was started. Slides were subsequently incubated with 4% paraformaldehyde for 10 min, 0.05 M NH4Cl (NH4Cl 0669 g/ 250 ml PBS) for 5 min. and 0.2% Triton X-100 (Triton X-100500 μl (Sigma)/250 ml PBS) for 5 min. Before every incubation and after the last one, slides were washed twice with PBS. Wells were blocked with 5% BSA (1 h), incubated with an anti-rabbit polyclonal ANKH antibody (1:100; Santa Cruz Biotechnology Inc., Germany) over night. After removal of the primary antibody, the sections were incubated with the secondary antibody IgG (anti-rabbit, 1:100; Thermo Fisher Scientific, USA) for 2–4 h.

After washing, all tissues were mounted with vectashield with DAPI (Vectastain kit, Labconsult, CA) and images were taken under the fluorescent microscope (Axiovision Reflected Light 4.6, Zeiss, Germany). Background signals were determined from the negative controls and microscope settings were subsequently applied to the IF samples.

3. Dysregulated TGF-β, BMP and PPi signaling in PXE: state-of-the-art

3.1. TGF-β signaling influences mineralization and ABCC6 expression in PXE

TGF-β, a growth factor belonging to the 'TGF-β superfamily' of regulatory proteins, plays a key role in cell division and differentiation, the immune system, the homeostasis of the ECM and was found to be an important mediator of vascular calcification [8–11]. In this hallmark example of soft tissue mineralization, presence of TGF-βs in calcified cardiac valves and its regulatory activity on vascular calcification and osteoblastic differentiation of VSMCs has been demonstrated [12,13]. Three isoforms of TGF-β -TGF-β1, TGF-β2 and TGF-β3 - exist, encoded by respectively the *TGFB1* (OMIM* 190180), *TGFB2* (OMIM* 190220) and *TGFB3* gene (OMIM* 190230).

The mechanisms that can lead to TGF-β dysregulation are numerous, due to the complex TGF-β homeostasis which includes secretion, TGF-β latency and activation of TGF-β (Fig. 1). Following transcription and translation, two TGF-β precursor proteins bind to each other in the endoplasmic reticulum (ER) [10]. They consist of the actual TGF-β precursor (C-terminal), covalently linked to the Latency Associated Peptide (LAP; N-terminal region), and named according to the associated TGF-β isoform i.e. LAP1, LAP2 and LAP3 [14,15]. Also in the ER, Latent TGF-β Binding Protein (LTBP) binds the pro-TGF-β complex via disulfide bonds with LAP. In the trans-golgi network, further processing and cleavage of the precursor proteins takes place forming the mature TGF-β and LAP dimer. The TGF-β dimer is then non-covalently bound by its LAP dimer with high affinity, creating the Small Latent Complex (SLC). The association of SLC and LTBP is called the Large Latent Complex (LLC). After cell secretion of the LLC, the complex binds to ECM components such as fibrillin-1 and fibronectin via the LTBP hinge domain (Fig. 1) [15].

In neither the SLC nor LLC, TGF-β is able to bind its receptor. To ensure receptor binding, TGF-β has to be 'activated' i.e. released from its latent complex [9]. This activation is the rate limiting step in TGF-β bioavailability [8]. TGF-β release may occur through various mechanisms such as mechanical deformation of LAP by cell surface-bound integrins, independent of proteolysis (Fig. 1) [9,16,17]. This reaction occurs as the arginyl-glycyl-aspartic acid motif (RGD-motif) in LAP can be bound by a cytoskeleton-anchored integrin (integrin αvβ6 (ITG6)) from nearby cells. Traction produced by the actin cytoskeleton of the nearby cell causes a deformation of LAP, which can then no longer bind TGF-β, resulting in the release of the cytokine [14]. This mechanism of TGF-β activation allows strict spatial regulation of TGF-β, which can only bind to receptors of adjacent cells [18]. However, this conformation-change induced release is not the only mechanism, as it was reported that LAP2 does not have an RGD-motif and Annes et al. [19] showed that αvβ6-expressing cells do not adhere to LAP2-coated wells.

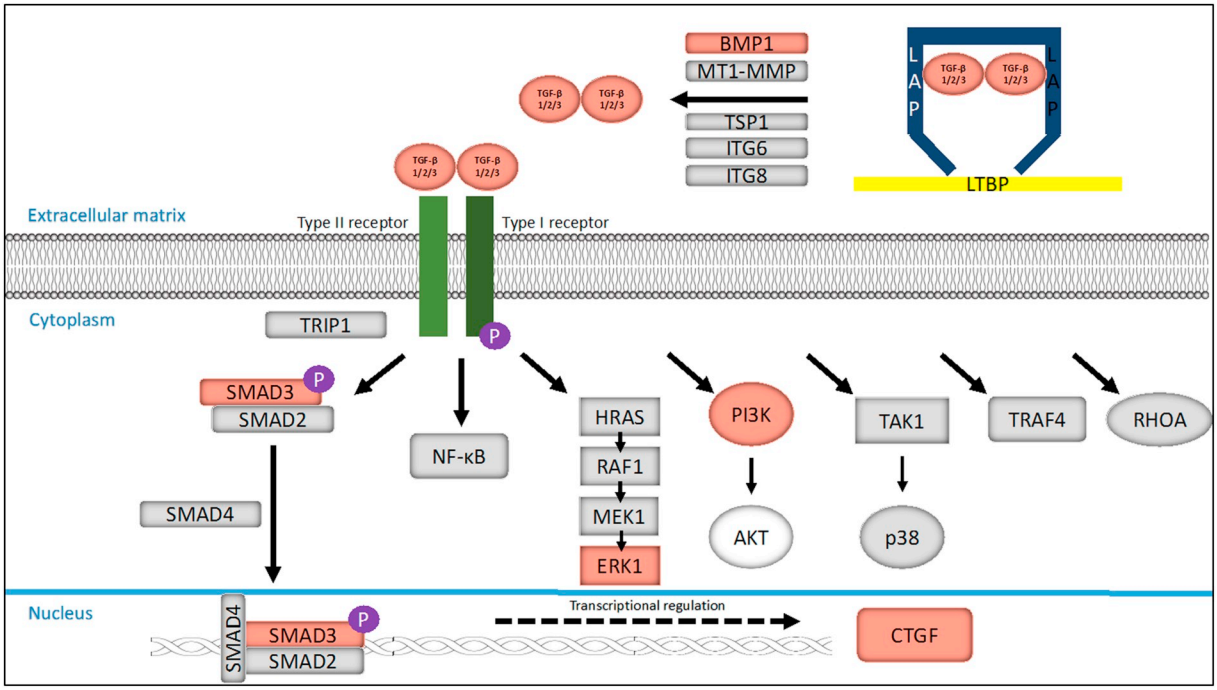

Fig. 1. Schematic representation of TGF-β-associated signaling.
Mediators marked in red are dysregulated in PXE while those marked in grey are unaltered.

A second mechanism for TGF-β activation is mediated by (integrin) protease-dependent activation. Integrin αvβ8 (ITG8) is able to recruit Membrane Type 1-Matrix Metalloproteinase (MT1-MMP) resulting in the degradation of LAP and consecutive release of TGF-β. Other proteinases involved are Matrix Metalloproteinase 2 (MMP2), MMP9, BMP1, plasmin, thrombin, elastase and thrombospondin-1 (TSP-1) [9,15].

Third, reactive oxygen species (ROS) have been suggested to play a role in activation of TGF-β [9,15]. ROS directly induce oxidation of LAP upon which it loses its TGF-β binding capacity [10]. The TGF-β-ROS interaction is however reciprocal, as TGF-β1 has been shown to also increase the production of ROS by inducing NADPH oxidases (NOXs), especially NOX4, and suppress antioxidant systems such as the synthesis of glutathione [20].

Besides these mechanisms, it is well-known that when constituents of the ECM are altered, this will also influence the bioavailability and activity of TGF-β [21]. This is e.g. illustrated by mutations in the *fibrillin-1* (*FBN1*) gene, an elastin-associated protein that causes Marfan syndrome. The altered fibrillin-1 structure will then influence the binding of the LLC to the ECM [22].

After release from its latent complex, TGF-β is able to bind its receptor. Two TGF-β type I and two TGF-β type II receptors have been reported, which are serine/threonine kinases located at the cell surface [23]. They differ in their ligand-binding affinities (e.g. TGF-β receptor 1 has a high affinity for TGF-β1 but low affinity for TGF-β2, while TGF-β receptor 3 has a high affinity for both TGF-β1 and TGF-β2) [23]. Binding of TGF-β dimers to these receptors causes phosphorylation of the inactive type I receptor by the constitutively active type II receptor [23]. Further downstream signaling consists of type I receptor-mediated phosphorylation of SMAD2 and SMAD3 proteins, forming the canonical pathway (Fig. 1). The phosphorylated SMAD2/3 (pSMAD2/3) complex then binds with SMAD4 and translocates to the nucleus, regulating gene expression through binding of SMAD binding elements (SBE) present in the promoter region of target genes [17].

The non-canonical TGF-β signaling pathways include activation of the Rat Sarcoma (RAS) / Rapidly Accelerated Fibrosarcoma (RAF) / Mitogen-activated protein kinase (MEK or MAPK) / Extracellular signal-Regulated Kinase (ERK) pathway, p38, Rho-GTPase (RHOA), TGF-β Activated Kinase 1 (TAK1) / JUN N-terminal Kinase (JNK) pathway, Phosphoinositide 3-Kinase (PI3K) / AK strain Transforming (AKT) pathway, Nuclear Factor-κB (NF-κB) and TNF Receptor Associated Factor 4 (TRAF4) (Fig. 1) [23–25].

Target genes of the TGF-β signaling pathway include proteoglycans, PAI-1 and collagen genes but the most important is CTGF. CTGF plays a major role in ECM production in osteoblasts and has also been linked to angiogenesis, chondrogenesis, wound healing, fibrosis and tumorigenesis [26–29]. Through binding to a yet unknown receptor, CTGF triggers various intracellular pathways such as PI3K/PKB, PKC/p38, MEK/ERK and JNK [26]. Interaction between CTGF and a wide array of integrins (αvβ3, a5β1) has also been demonstrated [26].

Finally, it should be noted that TGF-β signaling itself can also be modulated by these intracellular mechanisms. Lysosomal degradation of the transmembrane TGF-β receptor can terminate signaling, while SMAD proteins can be recruited to TGF-β receptors causing an increase in signaling [18]. TGF-β Receptor Interacting Protein-1 (TRIP-1), an intracellular protein expressed in osteoblasts and fibroblasts, might serve as a positive modulator of TGF-β signaling through its interaction with Type 5b Tartrate Resistant Acid Phosphatase (TRAP) [30,31]. This hypothesis was confirmed by Metz-Estrella et al. [32] who found a 40% decrease in TGF-β signaling activity in a *TRIP-1* knock-out model.

3.1.1. *Activation of TGF-β is increased in PXE*

In human PXE fibroblasts, an upregulation of mostly TGF-β2 was reported [33]. Conversely, no differential TGF-β expression was found in tissues of the *Abcc6*$^{-/-}$ mouse while Dabisch-Ruthe et al. suggested rather a downregulation of TGF-β1 by 50% in PXE fibroblasts supplemented with FCS (Fetal Calf Serum) ID-4 [34]. Though this indicates that TGF-β dysregulation is associated with PXE, the exact nature of this dysregulation remains unclear.

The data on the different mechanisms that can lead to TGF-β dysregulation in PXE is fragmented. While nothing is reported on confirmation-change induced release, the proteases MMP2 and MMP9 are well known to play a role in PXE, as increased serum levels of MMP2 and MMP9 were demonstrated in PXE patients [35,36]. At the cellular

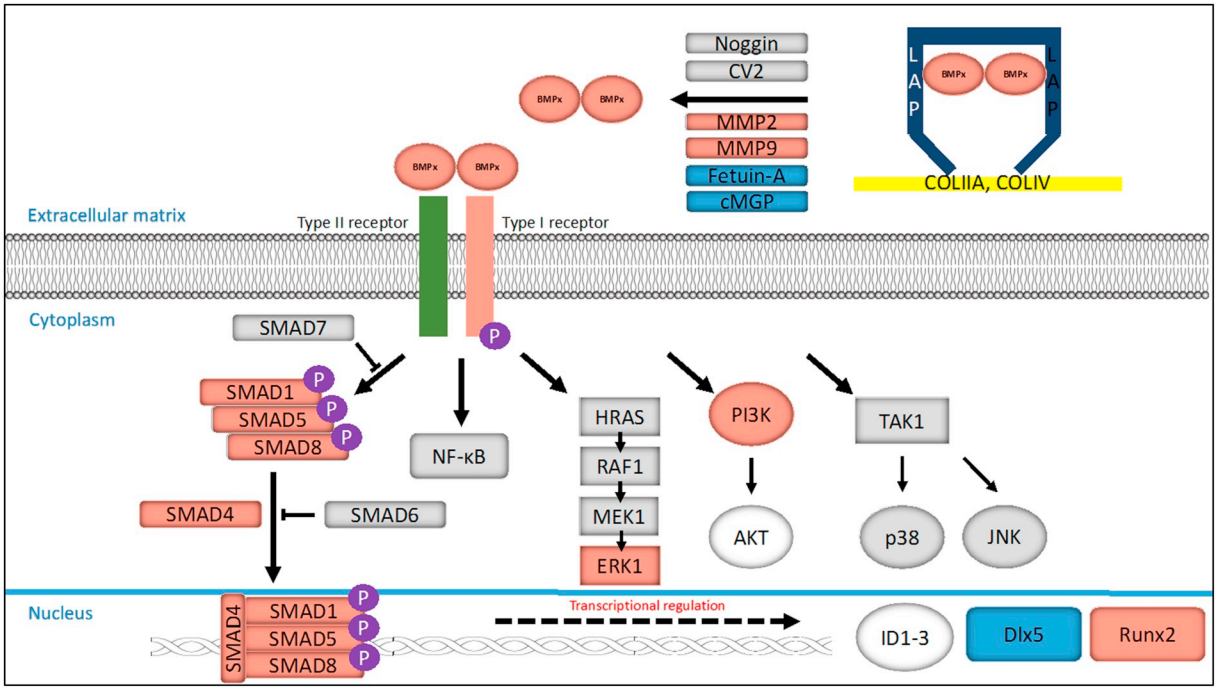

Fig. 2. Schematic representation of BMP-associated signaling.
Mediators marked in red are upregulated in PXE while those in blue are downregulated. Grey markers are unaltered.

level only differential expression of MMP2 and recently also MMP12 could be demonstrated in PXE-fibroblasts [35,37]. The other TGF-β-related proteases have not yet been investigated in PXE.

In contrast, the presence of ROS has been extensively studied in PXE. In PXE patients and mice, the presence of ROS is reflected by elevated serum levels of advanced oxidation protein products, lipid hydroperoxides and enhanced EC-SOD activity [38,39]. It remained unclear however to what extent ROS contributes to or is consequence of elastic fiber mineralization in PXE [40]. A recent study of Miglionico et al. [41] suggests that - in vitro - ROS formation is at least in part a primary mechanism, as they could induce a senescent-like cell phenotype after specific shRNA-induced ABCC6 knockdown in hepG2 cells.

Finally, pathological remodeling of the ECM in PXE is among others reflected by a more infusible XYLT1 mRNA expression in PXE fibroblasts and could thus possibly play a role in TGF-β release [37]. It raises the question whether the TGF-β signaling observed in PXE is a driver mechanism, a consequence of matrix degradation or a combination of both.

3.1.2. Downstream TGF-β signaling in PXE

In PXE, the canonical is TGF-β-pSMAD2/3 axis is upregulated, as demonstrated in skin tissues and cells of patients and the Abcc6$^{-/-}$ mouse [33]. No data have been reported on whether non-canonical TGF-β signaling is of relevance in PXE.

For the target genes of TGF-β, it has long been known that the ECM in PXE is rich in proteoglycans and that PXE fibroblasts display an altered proteoglycan metabolism [42]. The reason for this increased presence of proteoglycans currently remains unclear, and therefore a contribution of TGF-β cannot be excluded. Proteoglycan accumulation is said to play a role in the vascular phenotype of PXE, influencing the thickness and elasticity of arteries [43]. CTGF was found to be significantly upregulated in PXE fibroblasts and skin tissue, potentially being an important link between TGF-β signaling and dysfunctional ECM production in PXE fibroblasts [33]. Interestingly, PXE fibroblasts were reported to be less sensitive to stimulation by exogenous TGF-β1 in vitro which suggests that upregulated TFG-β expression in itself could be a compensatory mechanism [37]. There is no evidence for involvement of collagen genes or PAI-1 in PXE.

3.1.3. Transcriptional regulation of ABCC6 by TGF-β

Besides binding to its receptor, TGF-β has been shown to modulate *ABCC6* promoter activity. By binding to a Sp1 binding site, a significant upregulation of the *ABCC6* promoter could be demonstrated [44,45]. Though it was suggested that this involved cooperation of the transcription factor Sp1 and Smad3, it was demonstrated that Sp1 functions as a distinct TGF-β responsive element for promoter expression and Sp1 by itself can mediate this response. Site-directed mutagenesis of Sp1 abrogates the TGF-β responsiveness [45]. This prominent effect of TGF-β on *ABCC6* expression may again suggest that the increased TGF-β expression and downstream signaling, which is observed in PXE cells, is (partially) the result of ABCC6 deficiency, in an attempt to mitigate this loss. Such compensatory feedback loops have already been demonstrated in fibrosis and carcinogenesis [46,47].

3.2. Bone morphogenic proteins show a tissue- and time-dependent effect in PXE

Bone morphogenetic proteins (BMPs) are a second group of proteins belonging to the TGF-β superfamily, playing a role in a broad variety of developmental and maintenance pathways (gastrulation, organogenesis, angiogenesis, iron homeostasis, inflammation) [48,49]. Though widely distributed, they are predominantly known for their regulatory role in bone homeostasis [50].

BMPs are secreted as hetero- and homodimers, which bind to type I and type II BMP receptors and activate Smad-mediated signaling via phosphorylation of a Smad1/5/8 complex [48]. In bone, this initiates osteogenic differentiation and maturation of chondrocytes, osteoblasts and osteoclasts, which illustrates the important role of BMPs in mineralization. BMP signaling can however be distinct, depending on the formed dimers, the nature of the BMPs, the cell type and the subtypes of receptors involved. This complex regulatory network has been studied most extensively in bone. Receptor preference has been shown for BMPs 2, 4, 5, 6, 7 and 9 - all with significant osteogenic potential in bone - which preferentially bind to different type I receptors: BMP2 and 4 bind

to ALK3 and 6, BMP6 and 7 to ALK2 and BMP9 to ALK1. Similar to TGF-β, this leads to the activation of canonical (Smad-dependent) and non-canonical pathways (Fig. 2) [48,51,52].

Concomitantly expressed with these pro-osteogenic BMPs are BMP3, 13 and 14 that function as antagonists, inhibiting the activity of the other BMPs [48,49]. Besides a balance between propagators and antagonists, also complex feedback loops exist, similar to the TGF-βs [52–54]. BMP2 signaling in osteoblastogenesis commits cells to osteochondral differentiation via expression of ID1–3 and DLX 5, leading to elevated Runx2 and Osterix levels, yet also appears to self-regulate through a negative feedback loop [52–54]. It has been demonstrated that Smad6, an inhibitory Smad protein, is upregulated following Runx2 expression, an end point target of the Smad1/5/8 signaling [55]. Similarly, osteoblasts treated with BMP2 showed increased expression of noggin and gremlin, secreted inhibitory decoys of BMPs [49,56,57]. When osteoclasts are exposed to BMP, Smad1/5/8 signaling upregulates expression of sclerostin and BMP6 which can negatively affect osteoblast mineralization activity [55,58].

Besides bone, BMPs play an essential role in vascular homeostasis [59]. In vascular calcification and soft tissue mineralization in general, involvement of BMPs concentrates on BMP2, that has been found in human calcified atherosclerotic plaques and when cells from such plaques were cultured they continued to express BMP2 in vitro [60]. BMP2 signaling was also shown to regulate the inflammatory response of endothelial cells, which may contribute to vascular mineralization [61]. Furthermore, stimulation of vascular smooth muscle cells resulted in enhanced phosphate uptake via upregulated expression of Pit1 (Phosphate Transporter 1, encoded by SLC20A1; OMIM* 137570) with concomitant Runx2 upregulation and reduced SM22 expression. The latter being an exclusive smooth muscle cell marker, this indicates a putative osteogenic modulation of these cells [60]. Similarly, atherosclerosis and vascular calcifications in the *Ldlr*$^{-/-}$ or *Mgp*$^{-/-}$ mice could be abrogated through treatment of these animals with Bmp signaling inhibitors, indicating that Bmp signaling is pivotal in these processes [62,63].

3.2.1. BMP expression is systemically dysregulated in PXE and underlies pro-osteogenic signaling

In PXE, BMP2 also exerts its crucial role in soft tissue mineralization. BMP2 is abundantly present in mineralization zones in the skin and skin fibroblasts of patients and mice [64]. In *Abcc6*$^{-/-}$ mouse fibroblasts Bmp2 protein expression significantly increased following ROS upregulation after onset of mineralization (at 12 weeks of age) occurred [65]. This was further documented in human fibroblasts and tissues by our group, showing upregulation of the osteogenic BMP2 - SMAD1/5/8 - RUNX2 and ALP pathway, though Osterix expression remained normal [33,65]. Together with increased activity in the MSX2 - Wnt signaling, it was suggested that these cells may undergo partial osteogenic transdifferentiation, similar to the VSMC in vessel mineralization.

However, PXE being a multi systemic disease, it can be anticipated that loss of functional ABCC6 will not be confined to the skin but rather lead to systemic dysregulation of involved signaling cascades. This is nicely illustrated by BMP signaling cascades, which are activated in different tissues (kidneys, heart, aorta, lungs and muscle) of *Abcc6* deficient mice [66,67]. Also the receptors, Alk1 and Alk2 and modulators Hsp70 and CV2 are upregulated [66,67]. That this effect is cell-type or organ specific is illustrated by the fact that in Abcc6 deficient liver, Bmp2 activity is reduced while enhanced Bmp4, 6 and 7, as well as enhanced Bmp-receptor, Alk1 and Alk2, and pSmad1/5/8 protein levels were found [67]. In the heart, dysregulation of Bmp4, Bmp9, Alk2 and endoglin was noted [66]. More recently, we have shown a similar but not identical dysregulation in the *Abcc6*$^{-/-}$ brain with Bmp4 and endoglin upregulation and Alk2 downregulation. This dysregulation is suggested to prime a pro-ischemic state by lowering the threshold for acute ischemic events in the brain [68]. Sera of Abcc6 deficient mice are less capable to inhibit SMAD1/5/8 signaling in response to BMP4 stimulation of Bovine Aortic Endothelial Cells (BAEC) in vitro, again indicating that this pathway is deregulated in PXE and could potentially activate downstream matrix metalloprotease expression [67].

3.2.2. PXE patients suffer from reduced BMP antagonism

The mechanisms underlying BMP dysregulation in PXE have not yet been elucidated; one potential explanation could be that ABCC6-deprived hepatocytes suffer from a lack of BMP antagonist expression. Indeed, fetuin-A and carboxylated MGP (cMGP or Gla-MGP) are known inhibitors of BMP signaling via direct binding to BMPs [69–72], thereby preventing receptor-mediated activation of Smad1/5/8. In PXE, reduced levels of fetuin-A and reduced levels of carboxylated MGP have been reported; for MGP, clustering of ucMGP is co-localized with elastic fiber mineralization [73–77]. Other BMP modulators such as Hsp70 - which antagonizes the binding capacity of MGP to BMPs - and Crossveinless-2 (CV2) - a BMP inhibitor - are respectively up- and down regulated in PXE liver cells [67,78].

Besides this regulatory network, also a possible feedback mechanism should be taken into account. Under normal conditions, cMGP suppresses BMP2 expression [64,67]. Both the loss of cMGP and upregulation of ROS in PXE [40] can enhance BMP2 expression around the time mineralization occurs. In turn, BMP2 induces osteogenic gene expression and ROS formation, thus sustaining its own expression [33,65]. Further, upregulation of ALP activity in PXE affects PPi, OPN and Pi bioavailability, as detailed below. As the latter molecule is a known regulator of BMP2, this may also influence BMP activity [67].

3.3. Disturbed PPi homeostasis: potent but not alone in determining elastic fiber mineralization in PXE

Inorganic pyrophosphate (PPi) is a calcification inhibitor generated by hydrolysis of ATP. Free PPi in the extracellular environment acts as a potent inhibitor of mineralization. In particular, the nucleation and growth of hydroxyapatite (HA) crystals is inhibited through binding of PPi to these sites and antagonizing interaction of Ca^{2+} and Pi [34,79]. However, hydrolysis of PPi results in the formation of inorganic phosphate (Pi) that promotes mineralization. Consequently, not only the levels of PPi and Pi are important, also the ratio PPi/Pi plays a role in both physiological and pathological mineralization [80].

The metabolism of PPi has been explored through crossbreeding of deficient mouse models [80] (Fig. 3). Extracellular PPi is supplied directly through the membrane transporter ANKH [81]. An important quantity of PPi results from hydrolyzation of extracellular ATP to PPi and AMP by the ENPP1 enzyme [82]. PPi can then bind and prohibit crystal growth formations unless it is degraded by (tissue non-specific) alkaline phosphatase (TNAP/ALP). TNAP activity will lead to reduced amounts of free PPi and an increase in Pi. TNAP also dephosphorylates osteopontin (OPN), another potent inhibitor of HA crystal growth, which is downregulated in PXE fibroblasts [65,83]. Degradation of AMP into adenosine and Pi by CD73/NT5E provides an additional source of Pi [84,85]. Free Pi may enter into cells via the NaPi-importers PiT-1 and PiT-2 where it can stimulate ANKH expression, while adenosine may limit cellular TNAP expression [25].

Decreased levels of PPi have been described as a feature in several ectopic mineralization disorders, the most prominent one being Generalized Arterial Calcification of Infancy (GACI, OMIM#208000, 614473), due to mutations of the *ENPP1* gene, in which patients have very low to no detectable PPi in the plasma [86,87]. The idea that PPi may also be of interest for PXE surfaced when biallelic *ENPP1* mutations were found to be a rare cause of PXE and, vice versa, in some GACI patients biallelic *ABCC6* mutations were found [4]. This hypothesis proved to be correct as reduced levels of circulating PPi were demonstrated in patients as well as in *Abcc6*$^{-/-}$ murine and rat models [4,82,88]. Serum TNAP/ALP activity in PXE patients is within normal,

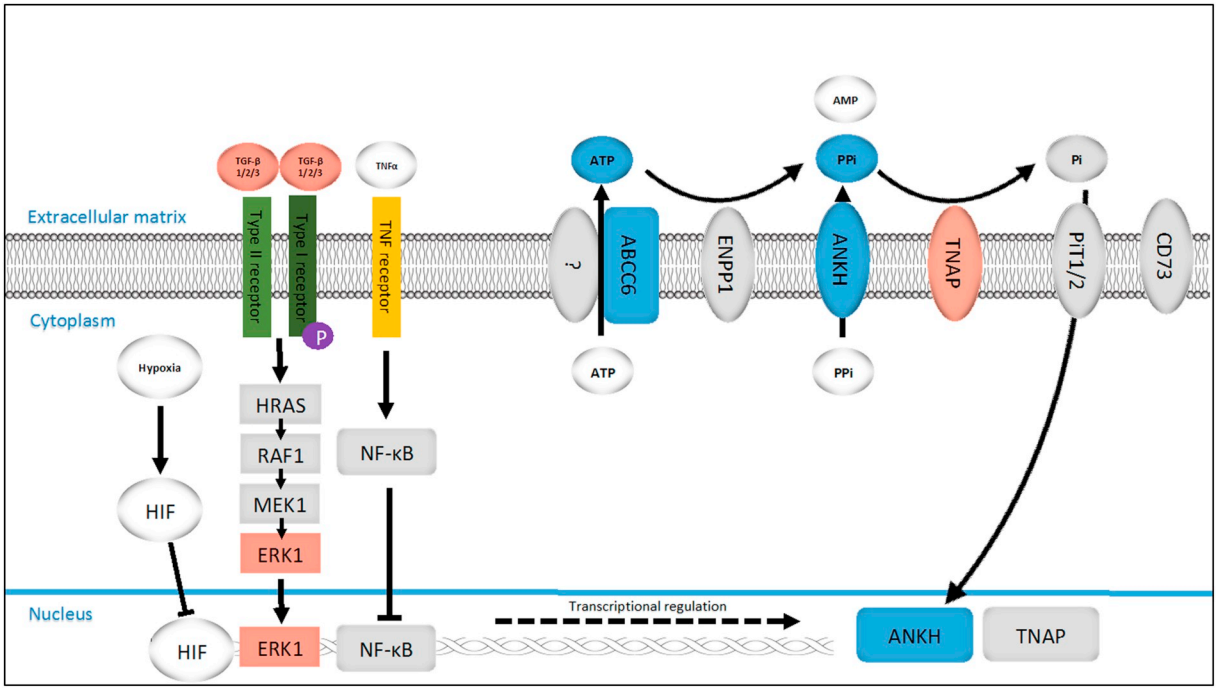

Fig. 3. Schematic representation of PPi homeostasis.
Mediators in red are upregulated in PXE, in blue are downregulated in PXE while those marked in grey are unaltered.

physiological ranges, indicating that PPi is not excessively catabolized in circulation [77]. Though a higher TNAP activity was seen in fibroblasts, it was said to be insufficient to induce significant mineralization [77,89]. Besides tissue-dependency, the alterations in expression of PPi-related proteins are also different according to the time-point of mineralization: in a later calcification phase upregulation of e.g. ENPP1 occurs in an effort to diminish the mineralization process [77,90].

3.3.1. *Extracellular delivery of PPi might be affected in PXE*

In vivo liver perfusion experiments confirmed that the ABCC6 transporter is involved in ATP transport from the liver cells to the circulation and thus regulates that largest quantity of circulating ATP [82]. While it has been suggested that ABCC6 may transport ATP itself, so far there has not been convincing evidence of this hypothesis. Nonetheless, it seems clear that the majority of extracellular ATP is depending on a normal functioning ABCC6 transporter to be in circulation. Besides the ABCC6-dependent ATP transport, extracellular PPi is also depending on transport capacity of the ANKH protein, an efflux transmembrane transporter of ATP which is involved in the trans-golgi network trafficking and endocytosis [91,92]. No data are available on ANKH expression in the PXE liver, but in human PXE fibroblasts ANKH expression was reported to be unaltered [34,90]. In contrast, in *Abcc6*$^{-/-}$ mouse fibroblast Ank expression was downregulated, even before the onset of mineralization, potentially suggesting species-specific differential expression [65].

Though all these observations clearly demonstrate that the PXE ECM is a pro-calcifying environment and that PPi dysregulation is an important propagator of mineralization, recent studies have suggested that the lower PPi levels probably do not completely explain the complex pathophysiology of PXE [41,93–95]. Among others, treating Abcc6$^{-/-}$ mice with bisphosphonates - synthetic PPi analogues - was not able to prevent mineralization completely. This again suggests the synergistic effect of multiple mechanisms in PXE.

4. Addressing discrepancies and gaps in current knowledge: TGF-β signaling and PPi transport

When evaluating the data on TGF-β, BMP and PPi signaling in PXE, it became clear that for TGF-β conflicting results have been reported - mainly as to which isoforms are up- or downregulated - as well as unclarities regarding the mechanisms underlying the differential expression. On the contrary for BMPs and PPi, the reported data seem very consistent, except for the expression of the transmembrane transporter ANKH.

4.1. *TGF-β is overexpressed in PXE, as a consequence of pre-existing deregulation of or crosstalk with the BMP signaling cascade*

We re-evaluated the expression of TGF-β1, TGF-β2 and TGF-β3 in native PXE fibroblasts. We repeatedly found all three isoforms to be upregulated in PXE fibroblasts compared to controls which overlaps with data for cardiovascular calcification in literature (Table 1) [12,13].

Despite upregulated expression, TGF-β remains biologically inactive if it is sequestered to the extracellular matrix. As limited data on the release of TGF-β are available in PXE, we subsequently evaluated the known release mechanisms. First, we evaluated confirmation-change induced release of TGF-β and found expression levels for ITG6 were normal in PXE cells, suggesting that this mechanism might not play a role in PXE (Table 1). Second, we investigated mediators for protease-dependent activation of TGF-β. We noted significant differential expression for a single metalloprotease, BMP1 (Table 1). BMP1 activates TGF-β1 by cleaving LTBP1 leading to consequent LAP cleavage by MMP2. Together with increased MMP2 activity, BMP1 upregulation indicates a potentially important role of the BMP1-MMP2 cascade in TGF-β activation in PXE [96]. Of interest, by cleaving the BMP2 antagonist chordin, BMP1 also enables activation of BMP2 and induction of the pSMAD1/5/8 signaling cascade, illustrating a potential crosstalk between TGF-β and BMP signaling in PXE [96].

Among the canonical pathway mediators, we confirmed SMAD2 over expression as previously reported. For the non-canonical

Table 1
Relative expression of the investigated mediators of TGF-β signaling and ANKH in PXE fibroblast versus controls.

	Relative expression in PXE fibroblasts Experiment 1 [SEM] Experiment 2 [SEM]	**Relative expression in control fibroblasts** Experiment 1 [SEM] Experiment 2 [SEM]	***P*-value** Experiment 1 Experiment 2	**PXE (n) / control (n)**
TGF-β isoforms				
TGF-β1	1.427 [1.10–1.95]	0.608 [1.10–1.95]	**0.0029**	n = 7 / n = 5
	1.426 [1.05–1.91]	0.608 [1.05–1.91]	**0.0032**	
TGF-β2	1.246 [1.00–1.59]	0.864 [0.67–1.12]	**0.031**	n = 7 / n = 5
	1.208 [0.92–1.60]	0.868 [0.65–1.18]	0.080	
TGF-β3	1.350 [0.91–2.00]	0.657 [0.40–1.09]	**0.025**	n = 7 / n = 5
	1.387 [1.00–1.95]	0.632 [0.41–0.92]	**0.0035**	
TGF-β activators				
BMP1	1.643 [1.20–2.10]	0.475 [0.39–0.85]	< **0.001**	n = 7 / n = 5
	1.613 [1.38–2.05]	0.546 [0.30–0.77]	< **0.001**	
ITG6	1.129 [0.80–1.85]	0.850 [0.60–1.18]	0.32	n = 7 / n = 5
	0.924 [0.80–1.51]	1.089 [0.72–1.60]	0.58	
ITG8	0.882 [0.55–1.40]	1.134 [0.70–1.80]	0.42	n = 7 / n = 5
	0.911 [0.35–1.80]	1.098 [0.35–2.80]	0.75	
TSP1	1.150 [0.73–1.87]	0.846 [0.50–1.39]	0.36	n = 7 / n = 5
	1.067 [0.75–1.52]	0.937 [1.61–1.48]	0.64	
MT1-MMP	1.089 [0.80–1.50]	0.888 [0.60–1.31]	0.39	n = 7 / n = 5
	1.176 [0.92–1.52]	0.850 [0.55–1.30]	0.20	
TGF-β signaling - Canonical pathway				
SMAD3	1.643 [1.00–3.00]	0.742 [0.50–1.00]	**0.024**	n = 4 / n = 5
	1.575 [0.90–2.75]	0.739 [0.51–1.00]	**0.020**	
SMAD2	0.880 [0.60–1.30]	1.136 [0.90–1.40]	0.24	n = 4 / n = 5
	1.199 [0.80–1.90]	0.876 [0.70–1.10]	0.19	
SMAD4	1.156 [1.00–1.33]	0.856 [0.60–1.21]	0.092	n = 4 / n = 5
	1.102 [0.95–1.30]	0.856 [0.60–1.21]	0.29	
TGF-β signaling - Non-canonical pathways				
ERK1	1.470 [1.00–2.38]	0.735 [0.60–1.00]	**0.015**	n = 5 / n = 5
	1.390 [0.90–2.20]	0.821 [0.65–1.11]	**0.050**	
PI3K	1.551 [1.15–2.10]	0.769 [0.60–0.99]	**0.0012**	n = 5 / n = 5
	1.448 [1.05–2.00]	0.801 [0.65–1.05]	**0.005**	
HRAS	0.933 [0.80–1.18]	1.072 [0.80–1.40]	0.40	n = 5 / n = 5
	0.924 [0.75–1.18]	1.083 [0.80–1.40]	0.35	
RAF1	1.094 [0.90–1.43]	0.919 [0.65–1.30]	0.39	n = 5 / n = 5
	1.039 [0.83–1.30]	0.962 [0.65–1.40]	0.72	
MEK1	0.974 [0.80–1.18]	1.029 [0.65–1.61]	0.81	n = 5 / n = 5
	0.867 [0.72–1.305]	1.195 [1.00–1.43]	0.015	
TAK1	1.214 [0.70–2.20]	0.856 [0.63–1.20]	0.28	n = 5 / n = 5
	0.918 [0.55–1.50]	1.097 [0.90–1.40]	0.49	
TRAF4	0.993 [0.72–1.30]	1.007 [0.80–1.31]	0.93	n = 5 / n = 5
	0.955 [0.75–1.20]	1.051 [0.82–1.30]	0.53	
RHOA	1.024 [0.85–1.25]	0.976 [0.95–1.25]	0.75	n = 5 / n = 5
	0.955 [0.78–1.20]	1.047 [0.80–1.35]	0.58	
NF-κB	1.025 [0.80–1.42]	0.967 [0.75–1.23]	0.77	n = 5 / n = 5
	1.031 [0.78–1.43]	0.966 [0.77–1.20]	0.74	
p38	0.984 [0.80–1.20]	1.016 [0.75–1.40]	0.63	n = 5 / n = 5
	0.961 [0.82–1.10]	1.041 [0.75–1.45]	0.65	
TGF-β modulators				
TRIP1	1.056 [0.83–1.23]	0.947 [0.75–1.27]	0.48	n = 5 / n = 5
	1.038 [0.90–1.23]	0.963 [0.71–1.30]	0.64	
TGF-β gene targets				
CTGF	1.665 [1.50–2.49]	0.621 [0.60–1.53]	**0.0072**	n = 5 / n = 5
	2.026 [1.35–3.10]	0.568 [0.30–1.00]	< **0.001**	
Markers of chondrocyte differentiation				
SOX9	0.410 [0.13–1.26]	2.793 [1.92–4.05]	< **0.001**	n = 5 / n = 5
DKK1	1.582 [1.25–2.00]	0.317 [0.24–0.47]	< **0.001**	n = 5 / n = 5
ATP transporters				
ANKH	0.640 [0.50–0.77]	1.43 [1.19–1.76]	< **0.001**	n = 5 / n = 5
	0.825 [0.58–1.15]	1.212 [0.95–1.53]	< **0.001**	

For each target, the results of two independent experiments are shown in respectively PXE fibroblasts and controls. Standard error of the mean (SEM) is mentioned between brackets. For each set of experiments, the number of patients is mentioned. Significance was set at $p < .05$. Significant *p*-values are marked in bold. TGF-β = transforming growth factor beta, BMP = bone morphogenetic protein, ITG = integrin, TSP1 = thrombospondin-1, MT1-MMP = membrane type 1-matrix metalloproteinase, ERK = extracellular signal-regulated kinase, PI3K = phosphoinositide 3-kinase, HRAS = harvey rat sarcoma viral oncogene homolog, RAF1 = rapidly accelerated fibrosarcoma, MEK1 = mitogen-activated protein kinase, TAK1 = TGF-β activated kinase 1, TRAF4 = TNF receptor associated factor 4, RHOA = Rho-GTPase, NF-κB = nuclear factor-κB, TRIP1 = TGF-β receptor interacting protein-1, CTGF = connective tissue growth factor, SOX9 = SRY-BOX 9, DKK1 = dickkopf 1, ANKH = homolog of ANK.

downstream targets of TGF-β, we demonstrated that ERK1 and PI3K were activated. However, mediators in between TGF-β and ERK1 were not overexpressed, suggesting that in PXE TGF-β is not directly responsible for ERK1 activity. Rather, increased activity of Connective Tissue Growth Factor (CTGF) signaling and/or BMP2 overexpression may predominantly contribute to ERK1 activity [26,27]. Similarly, it currently remains unclear whether PI3K activation is due to TGF-β activation or CTGF [26]. Regardless of the origin, both ERK1 and PI3K activity may contribute to the expression of chondrocyte differentiation markers. It has been shown that BMP2 and TGF-β3 can synergistically affect chondrogenic differentiation [97,98] and Kita et al. identified PI3K/Akt as a driving mechanism for chondrogenesis, resulting in up-regulation of *SOX9* and other markers [99]. In line with these arguments we found *SOX9* and inhibitory factor Dickkopf1 (*DKK1*) to be dysregulated in PXE (Table 1).

Finally, we did not detect differential expression of *TRIP-1* suggesting that TGF-β signaling in PXE is not modulated by the mechanism described by Metz-Estrella et al. [32] (Table 1).

4.2. ANKH down regulation in PXE suggests that PPi transport to the extracellular environment is diminished

When evaluating the expression of ANKH in PXE fibroblasts, we found ANKH RNA expression to be significantly downregulated compared to control fibroblasts and this reduction was confirmed on skin biopsies through immunofluorescence staining (Fig. 4). This suggests that both PPi transport as well as PPi metabolism are dysfunctional in PXE.

The expression of ANKH, which is ubiquitously available in mesenchymal cells, is regulated by cytokines as TGF-β, TNF-α and IL-1β and environmental factors such as mechanical stress and hypoxia [24,81,92,100]. Oca et al. demonstrated that TGF-β stimulates both ANKH expression and PPi release through activation of ERK1/2 and PKCα pathways, independently from Smad activity [25]. Although ERK1/2 overexpression is demonstrated in PXE, it is unlikely to be the result of TGF-β overexpression (see above). ANKH expression is down regulated by TNF-α, IL-1β and hypoxia [101]. To date, little is known about the role of inflammatory cytokines in PXE, though their relevance for soft tissue mineralization in general and vascular mineralization specifically is well recognized [102]. It has been shown that TNF-α and IFN-γ are able to suppress the *ABCC6* promotor activity [45]. There is limited evidence suggesting that inflammation may play a role in development or evolution of PXE lesions, as PET-CT scan of the skin showed uptake of 18F-FDG - a marker for inflammation - in the neck and axillary folds of a PXE patient [103]. Further, PXE-like skin lesions have been encountered in a number of metabolic, both acquired and heritable, disorders, some of which involve immunologic and inflammatory aberrations [104–106]. Altogether, it cannot be excluded that pro-inflammatory cytokines participate in the complex pathomechanisms underlying PXE.

5. Discussion and conclusion

Based on current knowledge, the cellular signaling associated with PXE mainly revolves around TGF-β, BMPs and PPi homeostasis, which may synergistically contribute to ectopic calcification. Interpreting the many results that have been obtained in the past years and integrating them into a single model is however challenging because of differences in experimental set-up, because of the intrinsic variability of the PXE phenotype - particularly when working with human tissues - and because of the complexity of the signaling network itself. Evident experimental issues that should be taken into account when translating in vitro data to the clinic, or when evaluating apparent inconsistencies between in vitro data and in vivo results, is that often in vitro observations in PXE are done in 'pro-calcifying' circumstances: experiments are done on cells that are grown in matrix calcification-inducing conditions, e.g. by inducing changes in extracellular calcium-phosphate product. This does not reflect the physiological conditions of the PXE ECM and any of these results should be verified in under 'normal' pathological conditions. The same is true for animal experiments where a mineralization-inducing diet is used or non-physiological methods such as cryo-injury are applied. Though the data mentioned above show that often results under these circumstances are reproducible in PXE patients, this is not always the case and these differences may be (partly) due to the experimental set-up.

However, the complexity of the PXE phenotype and the absence of

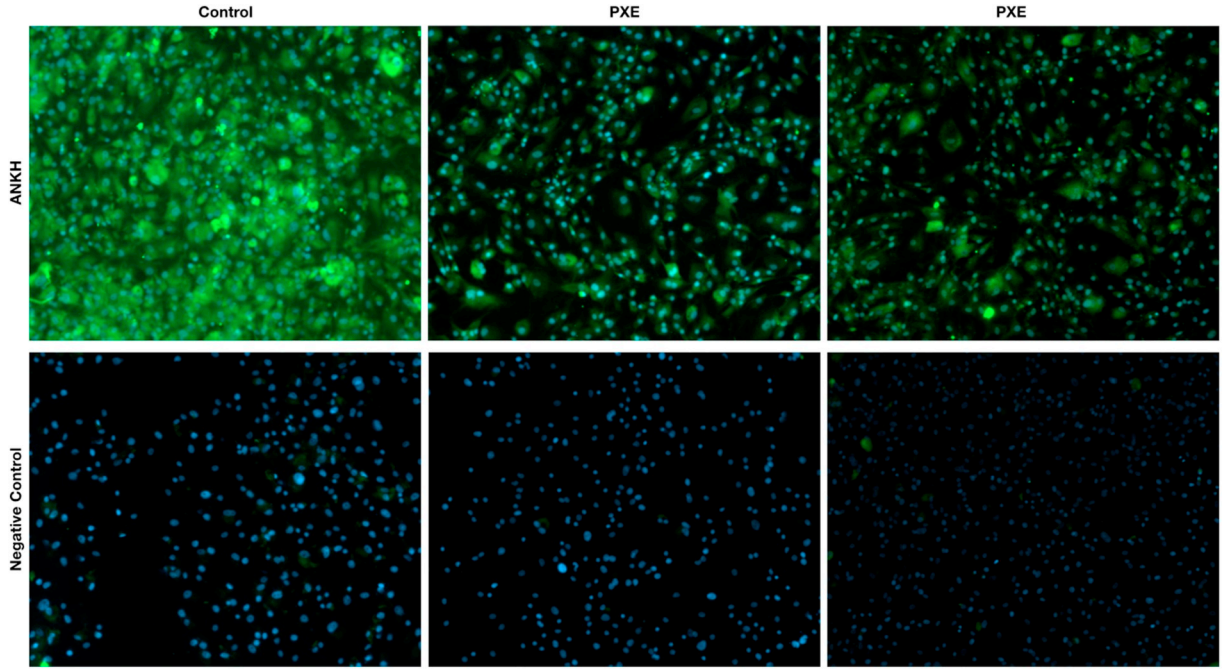

Fig. 4. Downregulation of ANKH in PXE fibroblasts.
Immunofluorescent labeling of control fibroblasts and PXE fibroblasts shows that staining is less strong in PXE (magnification x400).

genotype-phenotype correlations itself is probably a much more decisive challenge than experimental issues. Human PXE cells, mostly fibroblasts, that are used for signaling experiments are usually matched for age and sex compared to their controls. Very often however these cells have a different *ABCC6* genotype - certainly when comparing results of different studies. Though one can argue that the absence of a solid genotype-phenotype correlation may render the stratification of fibroblasts according to their *ABCC6* genotype less utile, the high phenotypic variability in PXE suggests that care should be given on comparing fibroblasts of patients with at least a similar phenotype, which can be evaluated using tools such as the PXE Phenodex. Indeed, the molecular background of PXE is most probably much more complex than just the causal gene, and may encompass large and small effect modifier genes and/or epigenetic modifications. Variations in these will not only have an effect on the clinical phenotype of the patient but most probably also on biological processes in the cells. Similar considerations should be made when pooling of samples - e.g. serum samples of different PXE patients - is done for some experiments. As in experimental genomic analysis, careful and deep phenotypic of patients included in experiments remains of the utmost importance.

The observation that cellular dysregulation due to ABCC6 deficiency is similar but not identical in different tissues is both interesting and challenging. Interesting, because it implies that the deficient ABCC6 transporter, mainly confined to liver and kidney, influences the signaling in different tissues through a number of potentially circulating mediators. This suggests that ABCC6 can be expected to turn up as a relevant player in other (disease) processes, as recently demonstrated for cholesterol homeostasis and brain ischemia [68,107]. At the same time, tissue-specific effects will need to be taken into account when thinking about therapies for PXE which may target or influence these signaling cascades. As multiple mechanisms seem to play a role simultaneously in an individual patient, efficient treatment is likely to target several of these, if not all. An alternative approach could be to target one or more signaling points of convergence. Different elements of the disease signaling cascades can already be pharmacologically targeted. Though points of convergence are still mostly speculative, it can already be envisaged that influencing these may result in different outcomes in various tissues.

Recent studies on PPi-Related proteins have indicated that there is not just tissue dependency but that some mechanisms and mediators are also time-dependent, with crucial timeframes before mineralization, at the start of mineralization and during advanced mineralization. Future studies should ideally also study these different moments in time separately, to find points of convergence that would allow to prevent and restore mineralization at the same time and/or to identify compensatory mechanisms which could be put to advantage in controlling the mineralization.

Despite the significant amount of signaling data summarized in this paper, an interesting gap in PXE research is the elastic fiber homeostasis. Indeed, it can be argued that resolving the mineralization in PXE will not solve the elastic fiber fragmentation. Regardless of which comes first - which has not been completely resolved to date - even in a pathophysiological scenario where the elastic fiber fragmentation is the result of mineralization, the fiber degradation that is present will remain and continue to lead to disease symptoms. It will therefore be of great interest to study the abundance and function of factors involved in elastic fiber biogenesis, maintenance and degradation in PXE and which of these could be potentially used to stabilize or even improve ECM quality when the mineralization has been stopped.

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