RESEARCH REPORT

Vitamin K reduces hypermineralisation in zebrafish models of PXE and GACI

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ABSTRACT

The mineralisation disorder pseudoxanthoma elasticum (PXE) is associated with mutations in the transporter protein ABCC6. Patients with PXE suffer from calcified lesions in the skin, eyes and vasculature, and PXE is related to a more severe vascular calcification syndrome called generalised arterial calcification of infancy (GACI). Mutations in *ABCC6* are linked to reduced levels of circulating vitamin K. Here, we describe a mutation in the zebrafish (*Danio rerio*) orthologue *abcc6a*, which results in extensive hypermineralisation of the axial skeleton. Administration of vitamin K to embryos was sufficient to restore normal levels of mineralisation. Vitamin K also reduced ectopic mineralisation in a zebrafish model of GACI, and warfarin exacerbated the mineralisation phenotype in both mutant lines. These data suggest that vitamin K could be a beneficial treatment for human patients with PXE or GACI. Additionally, we found that *abcc6a* is strongly expressed at the site of mineralisation rather than the liver, as it is in mammals, which has significant implications for our understanding of the function of ABCC6.

KEY WORDS: ABCC6, Mineralisation, PXE, Vitamin K, Zebrafish

INTRODUCTION

Pseudoxanthoma elasticum [PXE; Online Mendelian Inheritance in Man (OMIM) #264800] is a congenital disorder which causes ectopic mineralisation of the eyes, skin and arterial walls, and, in most cases, is associated with mutations in the *ABCC6* gene (Bergen et al., 2000; Chassaing et al., 2005; Uitto et al., 2014). The closely related disease generalised arterial calcification of infancy (GACI; OMIM #208000) is characterised by severe vascular mineralisation that is usually lethal in the neonatal period (Cheng et al., 2005). Mutations in either *ABCC6* or *ENPP1* can cause GACI, and it has been suggested that PXE and GACI represent a spectrum of symptoms from the same underlying causes (Nitschke et al., 2012; Nitschke and Rutsch, 2012). For a recent review of these and similar mineralisation disorders, see Li et al. (2014).

Much of the research into PXE has focused on the role of the protein encoded by *ABCC6*, a putative efflux transporter of an unknown factor. In mice, *Abcc6* is expressed in arterial endothelial cells, cornea, retina, neurons and kidney proximal tubules, but the highest expression is found in the liver (Beck et al., 2003; Matsuzaki et al., 2005; Scheffer et al., 2002). Homozygous *Abcc6*^{-/-} mice feature spontaneous calcification of the eye, vasculature and skin

(Gorgels et al., 2005; Klement et al., 2005). Several experiments using these models have given weight to the ‘metabolic hypothesis’ that PXE is a disorder caused by insufficient levels of a circulating agent, excreted from the liver by ABCC6. Transplanting skin from wild-type mice onto *Abcc6*^{-/-} animals resulted in mineralisation of the grafted tissue, whereas the reverse operation did not (Jiang et al., 2008). Surgical joining (parabiosis) of *Abcc6*^{-/-} and wild-type mice halted ectopic mineralisation in the knockout animal (Jiang et al., 2010b), suggesting that the blood of wild-type mice carries an as-yet-unknown anti-mineralisation factor. One candidate for this factor is fetuin-A (alpha-2-HS-glycoprotein/Ahsg), a small liver-derived protein which can inhibit hydroxyapatite precipitation (Schäfer et al., 2003). Serum levels of fetuin-A are reduced in both human PXE patients and *Abcc6*^{-/-} mice, and recombinant overexpression of fetuin-A successfully restored normal mineralisation in knockout mice (Jiang et al., 2010a, 2007).

Another candidate for this unknown factor is vitamin K (Borst et al., 2008). Patients with PXE have reduced levels of serum vitamin K (Vanakker et al., 2010), a cofactor required by the enzyme gamma-glutamyl carboxylase (GGCX) to convert glutamic acid into gamma-carboxyglutamate (Gla) in certain proteins, conferring a high affinity for calcium. Three Gla proteins are directly implicated in bone or soft tissue mineralisation: osteocalcin (OC; bone gamma carboxyglutamate protein/Bglap – Mouse Genome Informatics), matrix Gla protein (MGP) and Gla-rich protein (GRP) (Theuvsen et al., 2012; Viegas et al., 2008). Functional loss of *GGCX* results in symptoms very similar to PXE (Vanakker et al., 2006), and in mice, the pathological mineralisation phenotype of the *Abcc6*^{-/-} genotype is accelerated by the concomitant knockout of *Ggex* (Li and Uitto, 2010). Arterial calcification has been reported in patients using vitamin K antagonists such as warfarin, probably due to under-carboxylation of OC, GRP and especially MGP (Chatrou et al., 2012; Theuvsen et al., 2012). Similar results were obtained in *Abcc6*^{-/-} mice (Li et al., 2013). However, ectopic mineralisation in *Abcc6*^{-/-} mice was not reduced by dietary administration of vitamin K₁ or K₂ (Brampton et al., 2011; Gorgels et al., 2011; Jiang et al., 2011) despite successfully raising the vitamin K concentration in tissues and serum; interestingly, this increase was significantly subdued in knockout mice and was accompanied by hepatic lesions, suggesting that *Abcc6*^{-/-} mice have an impaired ability to absorb, metabolise or distribute vitamin K (Brampton et al., 2011).

Here, we describe a zebrafish mutant, *gräte* (*grt*; *abcc6ahu4958*), identified in a forward genetic screen, with a mutation in the *abcc6a* gene. *gräte* fish show signs of excessive mineralisation in the craniofacial and axial skeleton but appear otherwise normal. A transgenic reporter revealed unexpected *abcc6a* expression at craniofacial bone elements and in the notochord, but not in the liver. Significantly, administration of vitamin K counteracted the hypermineralisation phenotype of *abcc6a*^{-/-} and *enpp1*^{-/-} embryos, whereas administration of warfarin exacerbated the phenotype in both lines.

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RESULTS AND DISCUSSION

Characterisation of the *gräte* phenotype as a model for PXE

Zebrafish homozygous for the *gräte* allele featured hypermineralisation of the axial skeleton, resulting in mineralised structures appearing in the intervertebral space (Fig. 1A). Adult mutants survived for at least one year, but were shorter than siblings (Fig. 1B). Craniofacial bone elements within embryos appeared to be more mineralised (Fig. 1C). Elsewhere, ectopic mineralisation is infrequently present in skin on the ventral side; interestingly, this is equally common in *grt*^{+/−} and *grt*^{−/−} embryos (Fig. 1D). Quantifying Alizarin Red staining (Fig. 1E) revealed that mineralisation was significantly more advanced in *grt*^{−/−} embryos as early as 6 dpf, and that heterozygous embryos had an intermediate phenotype (Fig. 1F,G). Compared with siblings, juveniles at 6 weeks featured an undulating spine and vertebrae were shorter (mutant, 162±2.1 µm; sibling, 174±1.8 µm; s.e.m., *P*<0.001) and thicker (mutant, 106±6.0 µm; sibling, 85±3.8 µm; s.e.m., *P*=0.01) than

those in siblings, with large mineralised nodules developing on the margins of the intervertebral space (Fig. 1H–K).

Using whole-genome sequencing (Mackay and Schulte-Merker, 2014), a T→G mutation was detected at chr6:10974761 in the *abcc6a* gene. The *gräte* phenotype co-segregated with markers flanking the *abcc6a* locus (Fig. 2A). This gene encodes a putative efflux transporter of the ATP-binding cassette (ABC) superfamily, with two transmembrane domains and two catalytic nucleotide-binding domains (NBD) (Fig. 2B). The identified mutation results in the substitution L1429R in a highly conserved region of NBD-2 containing the Walker B motif (Fig. 2C). This motif of four hydrophobic residues is essential for binding to ATP (Geourjon et al., 2001; Walker et al., 1982). Most of the known human PXE-causing mutations are in the second ABC or NBD domains (Le Saux et al., 2001) (Fig. 2B), including an I1424T substitution immediately preceding the human equivalent of zebrafish L1429

Fig. 1. The *gräte* mutant phenotype is characterised by hypermineralisation in the skin and axial skeleton. (A) Alizarin Red staining of embryos at 8 days post-fertilisation (dpf), demonstrating hypermineralisation along the vertebral column (arrowheads). Scale bar: 1 mm. (B) Adult fish are viable, but feature a curved spine and reduced length. Scale bar: 1 cm. (C) *grt*^{−/−} embryos (ventral view) with enhanced mineralisation in craniofacial elements (arrowheads). (D) Skin mineralisation is infrequently seen in *grt*^{−/−} and *grt*^{+/−} embryos (D', ventral view). (E) Representative images showing the area quantified by the mineralisation assay used in this and in subsequent figures. (F) Vertebral mineralisation in *grt*^{−/−} embryos proceeds faster than in wild type, leading to (G) vertebral fusion from 6 dpf onwards. (H,J) Alizarin Red staining at 6 weeks post fertilisation (wpf) reveals a thickened, curved spine in *grt*^{−/−} fish; confocal images (I,K) of the boxed regions reveal mineralised nodules on the margins of the intervertebral space (arrowheads). Scale bars: 0.1 mm.

Fig. 2. *gräte* encodes an *abcc6a* allele. (A) Diagram of the genomic region linked to the *grt*^{-/-} phenotype. The number of recombinants (out of total embryos tested) is shown above three markers used in meiotic mapping. The candidate gene *abcc6a* is shown in blue. (B) Structure of the *Abcc6a* protein. Transmembrane helices (dark green) are organised into two transmembrane domains (TM; green). Two nucleotide-binding domains (NBD; light blue) each contain a highly conserved ABC signature motif (blue) and two Walker motifs (yellow). Known PXE-causing mutations in *ABCC6* are shown in their relative locations on the zebrafish sequence (white triangles) along with the common mutation R1141* (black triangle), the common deletion of exons 23–29 (horizontal line) and the *gräte* L1429R substitution (red triangle). Shaded boxes represent alternating exons. The conservation score for each residue is shown on an area plot (grey). (C) Multiple alignment of *ABCC6* genes across different vertebrates; colours represent amino acid classes. The Walker B motif (underlined) contains four hydrophobic residues. Leucine-1429 (red triangle) is substituted with a hydrophilic arginine in the *gräte* allele.

(L1425). The detectable phenotype in heterozygous embryos (not reported in human patients or the mouse model) suggests L1429R to be highly deleterious. Importantly, the phenotype was considerably variable from clutch to clutch, indicating that external factors can influence the extent of ectopic mineralisation. This is congruent with the variable human symptoms of PXE even among families with the same genetic lesion in *ABCC6* (Uitto et al., 2014).

In contrast to the phenotype described above, morpholino knockdown of *abcc6a* has previously been reported to cause oedemas and high mortality in embryonic zebrafish (Li et al., 2010), even though expression was reduced by only 54–81%. The discrepancy between morpholino and *grt*^{-/-} phenotypes might be attributed to off-target effects of the morpholinos, even though co-injection of morpholino with wild-type mouse *Abcc6* mRNA caused a complete rescue of the phenotype (Li et al., 2010).

Superficially, the *gräte* phenotype does not match that of human patients with PXE, who experience mineralisation of the skin and angioid streaks in the retina (Finger et al., 2009), with no reported vertebral abnormalities. Knockout mice mirror the human symptoms (Klement et al., 2005). A possible cause of this discrepancy is the greater propensity for mineralisation to occur in the fibrillar collagen of the notochord sheath. Similarly, the *enpp1*^{-/-} zebrafish (*dragonfish*) features extensive hypermineralisation of the axial skeleton, unlike human patients with GACI (Apschner et al., 2014).

***abcc6a* is expressed in osteoblasts and not the liver**

In situ hybridisation (ISH) at 5 dpf showed *abcc6a* expression in regions of developing bone such as the lateral-ventral edge of the operculum (Fig. 3A), but not in the liver (unlike mammalian *ABCC6*). Two orthologues of *ABCC6* exist in the zebrafish genome, but whole-mount ISH revealed *abcc6b* expression in the operculum and parasphenoid as well as the cartilage of the ear, with no hepatic expression (Fig. 3B). Fetuin-A (*ahsg*) was highly expressed in the liver (Fig. 3C). Expression of either *abcc6a* or *ahsg* was not altered by the *gräte* allele (data not shown) and expression patterns of the vitamin K-dependent genes *ggcx* and *vkor* were not associated with bone elements (supplementary material Fig. S1).

To facilitate further analysis of *abcc6a* expression, a reporter construct was prepared by introducing the GAL4 element into the

start codon of the *abcc6a* gene via BAC recombineering (Bussmann and Schulte-Merker, 2011). The mosaic expression of *Tg(abcc6a:gal4;uas:gfp)* at 7 dpf revealed GFP in the notochord and in cells near the operculum and cleithrum (Fig. 3D; supplementary material Fig. S3). Crossing the stable GAL4 line with a line expressing UAS:RFP and the early osteoblast marker *osterix:GFP* (Spoorendonk et al., 2008) confirmed that *abcc6a* was co-expressed with *osterix* (*osx*; *Sp7 transcription factor/sp7* – Zebrafish Model Organism Database) in some cells in the operculum and cleithrum (Fig. 3E,F); expression in these bone elements first appeared at 4 dpf, about one day after that of *osx*. By contrast, expression of *abcc6a* appeared very early (24 hpf) in the notochord and neural tube (Fig. 3E). In older fish (20 dpf), *osx*⁺ osteoblasts were present in the developing neural and hemal arches of the vertebrae, whereas *abcc6a* was strikingly expressed in the intervertebral disc regions (Fig. 3G,I), structures that are affected most by the *gräte* allele (Fig. 1K). The late osteoblast marker *osteocalcin:GFP* is co-expressed with *abcc6a* in some, but not all, cells around the operculum (Fig. 3H). Based on these observations, we believe that *abcc6a* labels a population of mature osteoblasts. *ABCC6* expression in mammalian osteoblasts or in other cells at the site of mineralisation has not been reported in the literature.

It has been hypothesised that *Abcc6* has an endocrine role, exporting a ligand from the liver into the circulation. Our results show that zebrafish *Abcc6a* functions locally at the site of mineralisation, suggesting that the transported ligand in fish is not liver derived. Jansen et al. recently reported that *ABCC6* overexpression induces nucleotide release *in vitro* (Jansen et al., 2013). These nucleotides are rapidly converted by ENPP1 into pyrophosphate (PPi), a potent inhibitor of mineralisation (Jansen et al., 2013; Nitschke et al., 2012). In a follow-up study, Jansen et al. reported that PPi secretion from the livers of *Abcc6*^{-/-} mice was dramatically lower than that of wild-type mice, and they suggested that *ABCC6* is an ATP efflux transporter (Jansen et al., 2014). In line with this compelling hypothesis, we postulate that zebrafish *Abcc6a* secretes ATP from cells at the site of mineralisation, increasing PPi locally, in contrast with the hepatically derived PPi in mammals.

We have recently described a zebrafish allele, *dragonfish* (*dgf*), with a nonsense mutation in *enpp1*, resulting in ectopic

Fig. 3. *abcc6a* is expressed at sites of mineralisation, but not the liver. (A) *abcc6a* transcripts are detected near the opercula (op) of 5 dpf embryos. (B) *abcc6b* expression appears in the opercula (op), parasphenoid (ps), cleithrum (cl) and cartilage of the ear. Neither gene is detected in the liver, in contrast to (C) fetuin-A (*ahsg*). A,B,C, lateral views; A',B',C', ventral views. (D) A transgenic reporter for *abcc6a* in a 7 dpf embryo stained with Alizarin Red. GFP is seen in the notochord (arrowhead), operculum (D') and cleithrum (D''). (E,F) The *abcc6a* transgenic reporter in an embryo also expressing the osteoblast marker *osterix:GFP*, demonstrating *abcc6a* expression in some osteoblasts of the operculum. Expression can also be seen in the neural tube (arrowhead in E). (G) In juvenile (20 dpf) vertebrae, *abcc6a* is expressed in the centra, whereas *osx* is expressed in the arches. (H) Some *abcc6a*⁺ osteoblasts also co-express the mature osteoblast marker *osteocalcin:GFP*. Dotted outline approximates the extent of the operculum at this stage. (I) In juvenile zebrafish, *abcc6a* is expressed in the intervertebral disc region, craniofacial bone elements and fins. Scale bars: 10 μ m in F,H.

mineralisation of the skin and axial skeleton in embryos, and the eyes and bulbus arteriosus of the heart in adults similar to GACI symptoms (Apschner et al., 2014). The axial phenotype of *dgf* mutants is considerably more severe than *grt* mutants, but *dgf*^{+/-} embryos are phenotypically normal. We did not observe an additive effect from these two alleles: *grt*^{+/-}; *dgf*^{+/-} embryos were indistinguishable from *grt*^{+/-}; *dgf*^{+/+}, and the axial hypermineralisation phenotype of *dgf* mutants was not exacerbated by the *grt* genotype (data not shown). Furthermore, transgenic overexpression of ENPP1 reduced mineralisation in all embryos, regardless of *grt* genotype (not shown). Both of these results suggest that zebrafish *Abcc6a* is one of several sources of nucleotides for Enpp1.

Vitamin K reduces hypermineralisation

Patients with PXE are reported to have low serum concentrations of vitamin K (Vanakker et al., 2010), but vitamin K was not effective in treating PXE in mouse models. We tested the effect of vitamin K on *gräte* embryos by supplementing the media from 4–8 dpf with 80 μ M

phylloquinone (vitamin K₁). Vitamin K₁ reduced hypermineralisation in *grt*^{-/-} and *grt*^{+/-} embryos, resulting in significant rescue of the *gräte* phenotype (Fig. 4A,B).

Warfarin is a potent antagonist of vitamin K, reducing serum levels by inhibiting its recycling. As warfarin has been reported to accelerate the mineralisation phenotype of *Abcc6*^{-/-} mice (Li et al., 2013), we raised *grt*^{-/-} zebrafish embryos in the presence of sodium warfarin from 4–8 dpf. Mortality was observed at concentrations exceeding 120 μ M. At 60 μ M, warfarin stimulated an approximately twofold increase in mineralisation in embryos of all genotypes, resulting in dramatic hypermineralisation of *grt*^{-/-} embryos (Fig. 4A,C). Warfarin also stimulated ectopic mineralisation in the ventral skin in ~20% of treated embryos regardless of genotype (supplementary material Fig. S2).

To test whether the observed activity of vitamin K is specific to that of *abcc6a*, we administered vitamin K₁ (80 μ M) to *dragonfish* (*enpp1*^{-/-}) embryos, which feature extensive axial hypermineralisation due to an inability to produce PPI (Apschner et al., 2014; Huitema et al., 2012). In these embryos, vitamin K₁

Fig. 4. Vitamin K reduces hypermineralisation in *gräte* and *dragonfish* mutants. (A) Representative images of *grt*^{-/-} embryos after treatment with vitamin K or warfarin from 4–8 dpf, showing that vitamin K reduces hypermineralisation, whereas warfarin exacerbates it. (B,C) Quantification of Alizarin Red staining in A reveals significant rescue of the phenotype by vitamin K ($n = 20$ embryos per group). (D) *Dgf*^{-/-} embryos treated with vitamin K or warfarin from 4–8 dpf. (E,F) Quantification of Alizarin Red staining in D reveals a significant beneficial effect of vitamin K; results are similar to those seen in *grt*^{-/-} embryos ($n = 24$ per group). (G) Alizarin Red staining of *dgf*^{-/-} embryos exhibiting ectopic mineralisation in the ventral skin. Administration of vitamin K did not affect the incidence (H) or the extent (I) of this mineralisation.

administration from 4–8 dpf provided the same protective effect as seen in *gräte*, and warfarin similarly exacerbated the phenotype (Fig. 4D–F). *Dragonfish* embryos exhibit ectopic mineralisation in the skin much more frequently than *gräte* embryos, enabling the effect of vitamin K to be tested on this phenotype. Curiously, vitamin K did not reduce the incidence or extent of skin mineralisation, which affected about half of the *dgf*^{-/-} embryos regardless of treatment (Fig. 4G–I).

These results show for the first time that vitamin K supplementation reduces hypermineralisation caused by mutations in either of the genes implicated in PXE and GACI. We propose that the reduced serum level of vitamin K seen in PXE patients is a consequence of its utilisation by GGCX in an attempt to restrict mineralisation, and not a consequence of the loss of ABCC6; subsequent administration of vitamin K could increase MGP carboxylation by GGCX, thus preventing hypermineralisation. This could also explain why *Abcc6*^{-/-} mice were more resistant to increases in serum levels caused by a vitamin K-rich diet (Brampton et al., 2011). One prediction of this model is that serum vitamin K would also be depleted in GACI patients (ENPP1^{-/-}).

The potential of vitamin K as a treatment for PXE or GACI is of enormous clinical significance, but the positive results shown here

contrast with negative results seen in mice from three separate research groups. We believe this contrast unlikely to be simply a matter of bioavailability. It is possibly related to the difference in affected tissues; vitamin K-dependent anti-mineralisation factors such as MGP might have a bigger impact in the axial skeleton than in the skin. In any case, the near-identical response of *abcc6a* and *enpp1* mutants to vitamin K and warfarin treatment renders strong support for the notion that the human PXE and GACI syndromes are closely related clinical entities. Finally, the expression of *abcc6a* in non-hepatic organs, which we report here, sheds new light on the cellular source of the ABCC6 ligand.

MATERIALS AND METHODS

Zebrafish husbandry

Zebrafish were maintained in standard husbandry conditions (Nüsslein-Volhard and Dahm, 2002) in accordance with Dutch regulations for ethical treatment of experimental animals. Embryos were raised in E3 media (5 mM NaCl, 17 μ M KCl, 330 μ M CaCl₂, 330 μ M MgSO₄) at 28°C.

Identification of the *gräte* mutation

A forward-genetic screen was performed using Alizarin Red staining as described previously (Spoorendonk et al., 2010). The mutation in *abcc6a* was detected using whole-genome sequencing, as reported elsewhere

(Mackay and Schulte-Merker, 2014). Subsequent genotyping was performed using the KASP SNP detection assay mix (LGC Genomics) with the following primers:

wild type: 5'-GAAGGTGACCAAGTTCATGCTAGACAAAAGTT-CTGGTGCT-3'; mutant: 5'-GAAGGTGCGAGTCAACGGATTGAC-AAAAGTTCTGGTGCG-3'; common reverse: 5'-GTCCAGTGCAGC-TGTTGCCCTCAT-3'.

Whole-mount ISH and BAC transgenesis

ISH was performed as described (Schulte-Merker, 2002; Thisse and Thisse, 2008), and details are provided in the supplementary material methods.

To generate the *abcc6a:gal4* transgene, the *GAL4FF* transcriptional activator was recombined in place of the ATG site of *abcc6a* in bacterial artificial chromosome (BAC) #DKEY-252I22, as described (Bussmann and Schulte-Merker, 2011). The following primers were used: *Abcc6a_gal4_f*: 5'-GAAGCAGGATACAGCAGGGATAGAGACAGCCTCAGGACACG-ACGAGTGACCATGAAGCTACTGTCTTCTATCGAAC-3'; *Abcc6_neo_r*: 5'-GAAATGTTTGCTCACCCATAGAGGGTCAAGTCCACTTAGACT-GCAAAGGTCAGAAGAACTCGTCAAGAAGGCG-3'.

Vitamin K/warfarin treatment and mineralisation assay

Vitamin K₁ or sodium warfarin (Sigma-Aldrich) were dissolved in 1:1 DMSO:ethanol or water to a working stock concentration of 40 or 60 mM, respectively. Embryos (4 dpf) were incubated with compounds in E3 media in the dark until 8 dpf. Embryos were fixed, bleached with H₂O₂, stained with Alizarin Red (0.005%) in 1% KOH and 0.2% Triton X-100 (Sigma-Aldrich), and imaged using an Olympus SZX16 stereomicroscope. To quantify Alizarin Red staining, images were processed using ImageJ (NIH). Mineralisation in each genotype-treatment group was compared by a two-tailed *t*-test.

Acknowledgements

S.S.-M. acknowledges support from the Smart Mix Programme of the Netherlands Ministry of Economic Affairs. L. Lleras provided valuable advice.

Competing interests

The authors declare no competing or financial interests.

Author contributions

E.W.M. performed experiments and analysed data. A.A. constructed the *abcc6a* BAC transgene. E.W.M. and S.S.-M. conceived experiments and wrote the manuscript.

Funding

This work was supported by the Deutsche Forschungsgemeinschaft (DFG), Cells-in-Motion Cluster of Excellence [EXC 1003 – CiM]. A.A. received a DOC Fellowship (Austrian Academy of Sciences).

Supplementary material

Supplementary material available online at <http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.113811/-DC1>

References

- Apschner, A., Huitema, L. F. A., Ponsioen, B., Peterson-Maduro, J. and Schulte-Merker, S. (2014). Pathological mineralization in a zebrafish *enpp1* mutant exhibits features of Generalized Arterial Calcification of Infancy (GACI) and Pseudoxanthoma Elasticum (PXE). *Dis. Models Mech.* **7**, 811–822.
- Beck, K., Hayashi, K., Nishiguchi, B., Saux, O. L., Hayashi, M. and Boyd, C. D. (2003). The distribution of *Abcc6* in normal mouse tissues suggests multiple functions for this ABC transporter. *J. Histochem. Cytochem.* **51**, 887–902.
- Bergen, A. A. B., Plomp, A. S., Schuurman, E. J., Terry, S., Breuning, M., Dauwerse, H., Swart, J., Kool, M., van Soest, S., Baas, F. et al. (2000). Mutations in *ABCC6* cause pseudoxanthoma elasticum. *Nat. Genet.* **25**, 228–231.
- Borst, P., van de Wetering, K. and Schlingemann, R. (2008). Does the absence of *ABCC6* (Multidrug Resistance Protein 6) in patients with Pseudoxanthoma elasticum prevent the liver from providing sufficient vitamin K to the periphery? *Cell Cycle* **7**, 1575–1579.
- Brampton, C., Yamaguchi, Y., Vanakker, O., Van Laer, L., Chen, L.-H., Thakore, M., De Paepe, A., Pomozi, V., Szabó, P. T., Martin, L. et al. (2011). Vitamin K does not prevent soft tissue mineralization in a mouse model of pseudoxanthoma elasticum. *Cell Cycle* **10**, 1810–1820.
- Bussmann, J. and Schulte-Merker, S. (2011). Rapid BAC selection for *tol2*-mediated transgenesis in zebrafish. *Development* **138**, 4327–4332.
- Chassaing, N., Martin, L., Calvas, P., Le Bert, M. and Hovnanian, A. (2005). Pseudoxanthoma elasticum: a clinical, pathophysiological and genetic update including 11 novel *ABCC6* mutations. *J. Med. Genet.* **42**, 881–892.
- Chatrou, M. L., Winckers, K., Hackeng, T. M., Reutelingsperger, C. P. and Schurgers, L. J. (2012). Vascular calcification: The price to pay for anticoagulation therapy with vitamin K-antagonists. *Blood Rev.* **26**, 155–166.
- Cheng, K.-S., Chen, M.-R., Ruf, N., Lin, S.-P. and Rutsch, F. (2005). Generalized arterial calcification of infancy: different clinical courses in two affected siblings. *Am. J. Med. Genet. A* **136A**, 210–213.
- Finger, R. P., Issa, P. C., Ladewig, M. S., Götting, C., Szliska, C., Scholl, H. P. N. and Holz, F. G. (2009). Pseudoxanthoma Elasticum: genetics, clinical manifestations and therapeutic approaches. *Surv. Ophthalmol.* **54**, 272–285.
- Geourjon, C., Orelle, C., Steinfelds, E., Blanchet, C., Deléage, G., Di Pietro, A. and Jault, J.-M. (2001). A common mechanism for ATP hydrolysis in ABC transporter and helicase superfamilies. *Trends Biochem. Sci.* **26**, 539–544.
- Gorgels, T. G. M. F., Hu, X., Scheffer, G. L., van der Wal, A. C., Toonstra, J., de Jong, P. T. V. M., van Kuppevelt, T. H., Levelt, C. N., de Wolf, A., Loves, W. J. P. et al. (2005). Disruption of *Abcc6* in the mouse: novel insight in the pathogenesis of pseudoxanthoma elasticum. *Hum. Mol. Genet.* **14**, 1763–1773.
- Gorgels, T. G. M. F., Waarsing, J. H., Herfs, M., Versteeg, D., Schoensiegel, F., Sato, T., Schlingemann, R. O., Ivandic, B., Vermeer, C., Schurgers, L. J. et al. (2011). Vitamin K supplementation increases vitamin K tissue levels but fails to counteract ectopic calcification in a mouse model for pseudoxanthoma elasticum. *J. Mol. Med.* **89**, 1125–1135.
- Huitema, L. F. A., Apschner, A., Logister, I., Spoorendonk, K. M., Bussmann, J., Hammond, C. L. and Schulte-Merker, S. (2012). *Entpd5* is essential for skeletal mineralization and regulates phosphate homeostasis in zebrafish. *Proc. Natl. Acad. Sci. USA* **109**, 21372–21377.
- Jansen, R. S., Küçüksomanoğlu, A., de Haas, M., Sapth, S., Otero, J. A., Hegman, I. E. M., Bergen, A. A. B., Gorgels, T. G. M. F., Borst, P. and van de Wetering, K. (2013). *ABCC6* prevents ectopic mineralization seen in pseudoxanthoma elasticum by inducing cellular nucleotide release. *Proc. Natl. Acad. Sci. USA* **110**, 20206–20211.
- Jansen, R. S., Duijst, S., Mahakena, S., Sommer, D., Szeri, F., Váradi, A., Plomp, A., Bergen, A. A. B., Elferink, R. P. J. O., Borst, P. et al. (2014). ATP-binding cassette subfamily C member 6-mediated ATP secretion by the liver is the main source of the mineralization inhibitor inorganic pyrophosphate in the systemic circulation. *Atertio. Thromb. Vasc. Biol.* **34**, 1985–1989.
- Jiang, Q., Li, Q. and Uitto, J. (2007). Aberrant mineralization of connective tissues in a mouse model of pseudoxanthoma elasticum: systemic and local regulatory factors. *J. Invest. Dermatol.* **127**, 1392–1402.
- Jiang, Q., Endo, M., Dibra, F., Wang, K. and Uitto, J. (2008). Pseudoxanthoma elasticum is a metabolic disease. *J. Invest. Dermatol.* **129**, 348–354.
- Jiang, Q., Dibra, F., Lee, M. D., Oldenburg, R. and Uitto, J. (2010a). Overexpression of fetuin-A counteracts ectopic mineralization in a mouse model of pseudoxanthoma elasticum (*Abcc6*–/–). *J. Invest. Dermatol.* **130**, 1288–1296.
- Jiang, Q., Oldenburg, R., Otsuru, S., Grand-Pierre, A. E., Horwitz, E. M. and Uitto, J. (2010b). Parabiotic heterogenetic pairing of *Abcc6*–/–/*Rag1*–/– mice and their wild-type counterparts halts ectopic mineralization in a murine model of pseudoxanthoma elasticum. *Am. J. Pathol.* **176**, 1855–1862.
- Jiang, Q., Li, Q., Grand-Pierre, A. E., Schurgers, L. J. and Uitto, J. (2011). Administration of vitamin K does not counteract the ectopic mineralization of connective tissues in *Abcc6* (–/–) mice, a model for pseudoxanthoma elasticum. *Cell Cycle* **10**, 701–707.
- Klement, J. F., Matsuzaki, Y., Jiang, Q.-J., Terlizzi, J., Choi, H. Y., Fujimoto, N., Li, K., Pulkkinen, L., Birk, D. E., Sundberg, J. P. et al. (2005). Targeted ablation of the *Abcc6* gene results in ectopic mineralization of connective tissues. *Mol. Cell. Biol.* **25**, 8299–8310.
- Le Saux, O., Beck, K., Sachsinger, C., Silvestri, C., Treiber, C., Göring, H. H. H., Johnson, E. W., De Paepe, A., Pope, F. M., Pasquali-Ronchetti, I. et al. (2001). A spectrum of *ABCC6* mutations is responsible for pseudoxanthoma elasticum. *Am. J. Hum. Genet.* **69**, 749–764.
- Li, Q. and Uitto, J. (2010). The mineralization phenotype in *Abcc6*–/– mice is affected by *Gcgc* gene deficiency and genetic background – a model for pseudoxanthoma elasticum. *J. Mol. Med.* **88**, 173–181.
- Li, Q., Sadowski, S., Frank, M., Chai, C., Váradi, A., Ho, S.-Y., Lou, H., Dean, M., Thisse, C., Thisse, B. et al. (2010). The *abcc6a* gene expression is required for normal zebrafish development. *J. Invest. Dermatol.* **130**, 2561–2568.
- Li, Q., Guo, H., Chou, D. W., Harrington, D. J., Schurgers, L. J., Terry, S. F. and Uitto, J. (2013). Warfarin accelerates ectopic mineralization in *Abcc6*–/– mice: clinical relevance to pseudoxanthoma elasticum. *Am. J. Pathol.* **182**, 1139–1150.
- Li, Q., Jiang, Q. and Uitto, J. (2014). Ectopic mineralization disorders of the extracellular matrix of connective tissue: molecular genetics and pathomechanisms of aberrant calcification. *Matrix Biol.* **33**, 23–28.
- Mackay, E. W. and Schulte-Merker, S. (2014). A statistical approach to mutation detection in zebrafish with next-generation sequencing. *J. Appl. Ichthyol.* **30**, 696–700.

- Matsuzaki, Y., Nakano, A., Jiang, Q.-J., Pulkkinen, L. and Uitto, J. (2005). Tissue-Specific Expression of the ABCC6 Gene. *J. Invest. Dermatol.* **125**, 900-905.
- Nitschke, Y. and Rutsch, F. (2012). Generalized arterial calcification of infancy and pseudoxanthoma elasticum: two sides of the same coin. *Front. Genet.* **3**, 302.
- Nitschke, Y., Baujat, G., Botschen, U., Wittkamp, T., du Moulin, M., Stella, J., Le Merrer, M., Guest, G., Lambot, K., Tazarourte-Pinturier, M.-F. et al. (2012). Generalized arterial calcification of infancy and pseudoxanthoma elasticum can be caused by mutations in either ENPP1 or ABCC6. *Am. J. Hum. Genet.* **90**, 25-39.
- Nüsslein-Volhard, C. and Dahm, R. (2002). *Zebrafish*. Oxford University Press, Oxford.
- Schäfer, C., Heiss, A., Schwarz, A., Westenfeld, R., Ketteler, M., Floege, J., Müller-Esterl, W., Schinke, T. and Jähnen-Dechent, W. (2003). The serum protein α 2-Heremans-Schmid glycoprotein/fetuin-A is a systemically acting inhibitor of ectopic calcification. *J. Clin. Invest.* **112**, 357-366.
- Scheffer, G. L., Hu, X., Pijnenborg, A. C. L. M., Wijnholds, J., Bergen, A. A. B. and Scheper, R. J. (2002). MRP6 (ABCC6) detection in normal human tissues and tumors. *Lab Invest.* **82**, 515-518.
- Schulte-Merker, S. (2002). Looking at embryos. In *Zebrafish: A Practical Approach* (ed. C. Nüsslein-Volhard and R. Dahm). Oxford University Press, Oxford.
- Spoorendonk, K. M., Peterson-Maduro, J., Renn, J., Trowe, T., Kranenbarg, S., Winkler, C. and Schulte-Merker, S. (2008). Retinoic acid and Cyp26b1 are critical regulators of osteogenesis in the axial skeleton. *Development* **135**, 3765-3774.
- Spoorendonk, K. M., Hammond, C. L., Huitema, L. F. A., Vanoevelen, J. and Schulte-Merker, S. (2010). Zebrafish as a unique model system in bone research: the power of genetics and in vivo imaging. *J. Appl. Ichthyol.* **26**, 219-224.
- Theuvsen, E., Smit, E. and Vermeer, C. (2012). The role of vitamin K in soft-tissue calcification. *Adv. Nutr. Res.* **3**, 166-173.
- Thisse, C. and Thisse, B. (2008). High-resolution in situ hybridization to whole-mount zebrafish embryos. *Nat. Protocols* **3**, 59-69.
- Uitto, J., Jiang, Q., Váradi, A., Bercovitch, L. G. and Terry, S. F. (2014). Pseudoxanthoma elasticum: diagnostic features, classification and treatment options. *Expert Opin. Orphan Drugs* **2**, 567-577.
- Vanakker, O. M., Martin, L., Gheduzzi, D., Leroy, B. P., Loeys, B. L., Guerci, V. I., Matthys, D., Terry, S. F., Coucke, P. J., Pasquali-Ronchetti, I. et al. (2006). Pseudoxanthoma elasticum-like phenotype with cutis laxa and multiple coagulation factor deficiency represents a separate genetic entity. *J. Invest. Dermatol.* **127**, 581-587.
- Vanakker, O. M., Martin, L., Schurgers, L. J., Quaglino, D., Costrop, L., Vermeer, C., Pasquali-Ronchetti, I., Coucke, P. J. and De Paepe, A. (2010). Low serum vitamin K in PXE results in defective carboxylation of mineralization inhibitors similar to the GGCX mutations in the PXE-like syndrome. *Lab. Invest.* **90**, 895-905.
- Viegas, C. S. B., Simes, D. C., Laizé, V., Williamson, M. K., Price, P. A. and Cancela, M. L. (2008). Gla-rich Protein (GRP), a new vitamin K-dependent protein identified from sturgeon cartilage and highly conserved in vertebrates. *J. Biol. Chem.* **283**, 36655-36664.
- Walker, J. E., Saraste, M., Runswick, M. J. and Gay, N. J. (1982). Distantly related sequences in the alpha- and beta-subunits of ATP synthase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold. *EMBO J.* **1**, 945-951.