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ABCC6 deficiency is associated with activation of BMP signaling in liver and kidney

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ABSTRACT

Mutations in ABCC6 (ATP-binding cassette, subfamily C, member 6), an orphan transporter expressed in the liver, are the cause of pseudoxanthoma elasticum. Since ABCC6 was reported to affect matrix Gla protein (MGP), an inhibitor of bone morphogenetic proteins (BMPs), we studied BMP signaling and expression in various tissues of mice with and without functional ABCC. Enhanced BMP signaling was found in all examined tissues in the absence of ABCC6. Despite this, the expression of particular BMP proteins varied widely between tissues. Interestingly, the expression of most BMP proteins in the liver moved in the opposite direction to the same BMP proteins in kidneys in response to ABCC6 alterations. Thus, ABCC6 deficiency stimulates BMP signaling by acting on the expression of multiple BMPs.

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1. Introduction

ABCC6 (ATP-binding cassette, subfamily C, member 6) is an orphan transporter mainly expressed in the liver [1]. Mutations in the human *ABCC6/MRP6* gene is a known cause of pseudoxanthoma elasticum (PXE) [2], a heritable recessive disorder characterized by calcification of elastin fibers in connective tissue, including the heart, vasculature, skin and eyes [3]. In addition to vascular calcification [4], the mice with *Abcc6* mutations also exhibit other vascular abnormalities including lower elasticity and increased myogenic tone [5]. It is believed that the ABCC6 substrate mediates the ectopic calcification via the circulation since ABCC6 is absent or minimally expressed in the calcified tissue resulting from the deficiency. This is consistent with the finding that parabiotic combination of blood circulation between *Abcc6* knockout (*Abcc6*$^{-/-}$) and wild type mice rescued the vascular calcification [6]. Previous work on ABCC6 deficiency suggests that the tissue effects are mediated by multiple signaling pathways such as the transforming growth factor beta (TGFβ) family and bone morphogenetic proteins (BMPs) [7].

BMP signaling is a pro-mineralizing pathway that has been associated with ABCC6 deficiency and could promote osteochondrogenic transitions in susceptible cells. Indeed, Hosen et al. reported an up-regulation of the BMP2-SMADs-RUNX2 as well as the TGFβ2-SMAD2/3 pathways at the mineralization sites in ABCC6-deficent mice [7], and Meng et al. reported that ABCC6 correlated with the BMP2-Wnt signaling pathway [8]. Matrix Gla protein (MGP) acts as an inhibitor of BMP2, BMP4, BMP6 and BMP7 [9–11] (unpublished data for BMP6), and depends on correctly gamma-carboxylated glutamate residues for optimal function [10]. Low levels of gamma-carboxylation in MGP and low serum levels of MGP have been reported in ABCC6 deficiency [12,13], which might lessen the BMP inhibitory function of MGP. It has also been reported that the gamma-carboxylation system in the vasculature is less efficient than in the liver [14], potentially putting the vasculature at higher risk for calcification if gamma-carboxylation is impaired. Our previous studies suggested that BMP signaling is increased in the ABCC6-deficient heart [15] further supporting that BMP signaling is a downstream target of ABCC6.

Abbreviations: ABCC6, ATP-binding cassette, subfamily C, member 6; BAEC, bovine aortic endothelial cells; BMPs, bone morphogenetic proteins; MGP, matrix Gla protein; PXE, pseudoxanthoma elasticum; TGFβ, transforming growth factor beta

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Because of the widespread tissue effects of ABCC6 deficiency, we examined if ABCC6, mainly expressed in the liver, affected BMP signaling in other organs, focusing on liver, kidneys, and aorta, and if the same BMP-related genes were activated. We found a general activation of BMP signaling in these organs in the absence of ABCC6. However, the induction of BMP and their receptors varied between tissues, making it difficult to attribute the BMP activation to a particular factor. We conclude that although the presence (or absence) of genetic deficiency of ABCC6 has a systemic effect on BMP signaling, but is caused by tissue-specific induction of BMP-related genes. Targeting BMP signaling for treatment purposes may therefore have to be tailored to the respective organs.

2. Methods

2.1. Ethics statement

Use of animals and all experimental procedures were review and approved by the University of California, Los Angeles (UCLA) Chancellor's Animal Research Committee (ARC). The investigation conformed to the National Research Council, Guide for the Care and Use of Laboratory Animals, Eighth Edition (Washington, DC: The National Academies Press, 2011).

2.2. Animals and cells

C3H/HeJ (C3H) and C57BL/6J (BL6) mice were purchased from the Jackson Laboratory. *Abcc6*-Tg mice on C3H background were generated as described [8] and contain an *Abcc6* BAC transgene derived from C57BL/6J. *Abcc6* knockout (*Abcc6*$^{-/-}$) mice were obtained from Dr. A.A.B. Bergen [4] and backcrossed for 10 generations on a C57BL/6J background. Littermates were used as wild type controls. All mice were fed a standard chow diet (Diet 8604, HarlanTeklad, Laboratory). All mice were used for experiments at 3–4 months of age.

Bovine aortic endothelial cells (BAEC) were cultured as previously described [16], and BMP4 (0–40 ng/ml, R&D Systems) in culture medium with 10% fetal bovine serum (FBS), or serum from C3H or C3H-*Abcc6*^{Tg} mice was added at the time of plating.

2.3. RNA analysis

Quantitative (q)PCR was performed as previously described [16,17]. The primers and probes used for qPCR for mouse activin receptor-like kinase (ALK) 1, ALK2, BMP2, BMP4, BMP6, BMP7, heat shock protein (HSP)70, MGP, Noggin, and crossveinless-2 (CV2) were pre-designed and obtained from Applied Biosystems (Foster City, CA) as part of Taqman® Gene Expression Assays.

2.4. Immunofluorescence

Tissue sections were fixed in 4% paraformaldehyde and processed as previously described [18]. Immunofluorescence was performed in detail as previously described [19]. We used specific antibodies for pSMAD1/5/8 (Santa Cruz Biotechnology), total SMAD and ALK2 (both from Santa Cruz Biotechnology), ABCC6 (MRP6 antibody S-20, Santa Cruz Biotechnology), albumin (Bethyl Laboratories). The nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) (Sigma–Aldrich) [19].

2.5. Immunoblotting

Immunoblotting was performed as previously described [20]. Equal amounts of cellular protein or culture medium were used. Results were then analyzed by immunoblotting using specific antibodies to VEGF (200 ng/ml; R&D Systems). Blots were incubated with specific antibodies to pSMAD1/5/8, pSMAD2/3 (both 400 ng/ml; Cell Signaling Technology), total SMAD, BMP-4, ALK1, ALK2, (all 400 ng/ml; Santa Cruz Biotechnology), BMP-2, ALK3 and ALK6 (all 200 ng/ml; Santa Cruz Biotechnology), BMP-6, BMP-7, BMP receptor type II (BMPRII), CV2, and Noggin (all 200 ng/ml; R&D Systems), HSP70 (100 ng/ml; Stressgen). β-Actin (1:5000 dilution; Sigma–Aldrich) was used as loading control.

2.6. Statistical analysis

Data was analyzed for statistical significance by ANOVA with post hoc Tukey's analysis. The analyses were performed using GraphPad Instat®, version 3.0 (GraphPad Software). Data represent mean ± SD. *P*-values less than 0.05 were considered significant, and experiments were repeated a minimum of three times.

3. Results

3.1. ABCC6 deficiency is associated with BMP activation in multiple organs

Previous observations suggested that BMP signaling increased in ABCC-deficient hearts [15]. Therefore, we hypothesized that deficiency of ABCC, which is expressed mainly in the liver, had the ability to affect BMP signaling also in other organs.

To study BMP signaling, we used C3H wild type mice, which lack functional ABCC6 due to a natural mutation [21], and *Abcc6* transgenic mice on the same background (C3H-*Abcc6*^{Tg} mice). The expression of ABCC6 in liver was about 0.03-fold and 3-fold in C3H and C3H-*Abcc6*^{Tg} mice, respectively, compared to BL6 mice, which express functional ABCC6 as previously reported [8]. We were able to detect ABCC6 by immunofluorescence in the liver of the C3H-*Abcc6*^{Tg} and BL6 mice, but not in the C3H mice (Fig. 1A). Albumin staining is shown for comparison. We were unable to detect ABCC6 expression in other organs by immunofluorescence including kidneys (Fig. 1B), aorta, heart, muscle and lungs (data not shown). We then compared BMP activity in these organs, which revealed that liver, kidneys, heart, aorta, lungs, and muscle all had increased levels of activated phosphorylated (p)SMAD1/5/8 as determined by immunoblotting (Fig. 2A). No changes were detected in the levels of pSMAD2/3, which mediates TGFβ-signaling, or the levels of total SMAD proteins. To confirm these results, we used BL6 wild type mice and BL6 mice with Abcc6 gene deletion (Abcc6$^{-/-}$ mice). As expected, the BL6 mice had less pSMAD1/5/8 in the tested organs when compared to BL6-*Abcc6*$^{-/-}$ mice (Fig. 2B). In all subsequent experiments, we included the BL6 to ensure consistency with the C3H-*Abcc6*^{Tg} mice.

We also compared the ability of serum from C3H and C3H-*Abcc6*^{Tg} mice to enhance BMP4 signaling in BAECs. The BAECs were treated for 20–24 h with BMP4 (0–40 ng/ml) in culture medium containing 10% FBS, or serum from C3H or C3H-*Abcc6*^{Tg} mice. The levels of pSMAD1/5/8 were compared by immunoblotting. The results showed that the serum from the C3H-*Abcc6*^{Tg} mice caused less activation of SMAD1/5/8 in response to BMP4 than did C3H serum or FBS (Fig. 2C). Together, the results suggest that functional ABCC6 regulates BMP signaling in the organs that were tested, even though ABCC6 expression was only detected in the liver.

3.2. Altered expression of ALK2 in presence of the Abcc6 transgene

To further examine the BMP activation, we first assessed the expression of ALK2, a type I BMP receptor, in the heart, aorta, muscle, kidneys, lungs and liver of C3H, C3H-*Abcc6*^{Tg}, and wild type BL6 mice. ALK2 is widely expressed and can regulate expression of MGP through the ALK1 receptor [22]. The results revealed that

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