Bone 50 (2012) 670–680

Contents lists available at SciVerse ScienceDirect

Bone

journal homepage: www.elsevier.com/locate/bone

Original Full Length Article

Ucma is not necessary for normal development of the mouse skeleton

Nicole Eitzinger [a,c], Cordula Surmann-Schmitt [a], Michael Bösl [b], Georg Schett [c], Klaus Engelke [d], Andreas Hess [e], Klaus von der Mark [a], Michael Stock [a,c,*]

[a] Department of Experimental Medicine I, Nikolaus-Fiebiger Centre of Molecular Medicine, University of Erlangen—Nuremberg, 91054 Erlangen, Germany
[b] Max Planck Institute of Neurobiology, 82152 Martinsried, Germany
[c] Department of Internal Medicine 3, Erlangen Medical School, University of Erlangen—Nuremberg, 91054 Erlangen, Germany
[d] Institute of Medical Physics, University of Erlangen—Nuremberg, 91054 Erlangen, Germany
[e] Institute of Pharmacology and Toxicology, University of Erlangen—Nuremberg, 91054 Erlangen, Germany

ARTICLE INFO

Article history:
Received 24 August 2011
Revised 4 November 2011
Accepted 9 November 2011
Available online 2 December 2011

Edited by: Bjorn Olsen

Keywords:
Ucma
Knockout
Gla
Skeletal development
Gene expression

ABSTRACT

Ucma (Upper zone of growth plate and Cartilage Matrix Associated protein) is a highly conserved tyrosine-sulphated secreted protein of Mw 17 kDa, which is expressed by juvenile chondrocytes.
To evaluate the physiological function of this novel cartilage protein, we generated a Ucma-deficient mouse strain by introducing a *lacZ*/*neoR*-cassette into the first exon of the *Ucma* gene. This mutation results in the complete loss of Ucma mRNA and protein expression. Surprisingly, however, although previous *in vitro* studies implied a role for Ucma in calcification and ossification, these processes were not affected in Ucma-deficient mice during normal development. Likewise, cartilage development was normal. While in previous works Ucma was mainly detected in the cartilage of embryonic and young mice, we detected Ucma expression also in the adult cartilage of the ribs using the *lacZ* cassette under the control of the *Ucma* promoter. Moreover, Ucma protein was specifically detected in adult growth plate cartilage by immunohistochemistry. Considering that skeletal development in Ucma-deficient mice is not significantly impaired, protein expression in adult cartilage indicates that Ucma might be involved in skeletal homeostasis and in the mechanical properties of the skeleton during challenging conditions such as ageing or disease.

Introduction

Ucma (Upper zone of growth plate and Cartilage Matrix Associated protein) was recently identified as a gene downregulated in chondrocytes after retinoid acid-dependent dedifferentiation [27]. The gene codes for a highly conserved secreted protein of app. 17 kDa, which exhibits no structural similarities to any other known proteins according to bioinformatic analyses. The secreted part of Ucma is further proteolytically processed by a furin-like protease resulting in a C-terminal Ucma fragment of 9.5 kDa (Ucma-C). This C-terminal domain exhibits the highest grade of sequence conservation, and a high number of charged amino acids and sulphation of 1–2 tyrosine residues render Ucma-C highly hydrophilic [27,29].

The murine *Ucma* gene is composed of five exons with the translation start located in exon 1. To date there is no evidence for other transcription initiation sites using an alternative first exon [27]. Yet, alternative splicing has been reported resulting in transcripts missing exons 2 and/or 4 [15]. In previous studies significant Ucma expression in mice was confined to cartilage. Highest Ucma expression levels were detected in juvenile chondrocytes. *In situ*, mRNA expression of *Ucma* is most profoundly detected in young vertebral, rib, and epiphyseal cartilage, where expression is observed from embryonic day E13.5 onwards, peaks perinatally and decreases after birth. Expression is strongest in distal chondrocytes while it declines with chondrocyte maturation towards hypertrophy. The uncleaved precursor protein can be detected in similar regions, whereas the cleaved isoform, Ucma-C, appears to migrate through the matrix and exhibits a broader distribution pattern. Interestingly, Ucma protein was found to be localised in close association with collagen type II fibres within the cartilage matrix, suggesting a role in the architecture of the cartilage matrix [27,29].

Functional *in vitro* studies have shown that Ucma-C impairs the osteogenic differentiation of calvarial cells and mesenchymal stem cells, indicating that Ucma might be involved in bone development [27]. In this respect it is intriguing that Ucma harbours a consensus recognition site for γ-carboxylase and that Ucma-C from sturgeon (*Acipenser nacarii*) appears to contain γ-carboxyglutamic acid (Gla) residues [31]. However, whether murine Ucma contains Gla is yet to be demonstrated. Mouse Ucma prepared as recombinant protein in HEK 293 cells in the presence of γ-glutamyl-carbvoxylase, VKORC1 (vitamin K epoxide reductase complex, subunit 1) and vitamin K does not contain Gla

Abbreviations: Ucma, Upper zone of growth plate and Cartilage Matrix Associated protein; dpc, days *post coitum*; E15.5, E17.5 etc., embryonic day 15.5, 17.5 (15.5 dpc, 17.5), respectively; P1, P10 etc., postnatal days 1, 10, respectively; ISH, in situ hybridisation; IHC, immunohistochemistry; ko, knockout; wt, wild-type.

* Corresponding author at: University of Erlangen—Nuremberg, Medical School, Nikolaus-Fiebiger-Zentrum für Molekulare Medizin, Department of Internal Medicine 3, Glückstr. 6, 91054 Erlangen, Germany. Fax: +49 9131 8526341.
E-mail address: mstock@molmed.uni-erlangen.de (M. Stock).

8756-3282/$ – see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.bone.2011.11.017

(M. Stock, unpubl. observation). Gla has been shown to be highly effective in calcium binding. Consequently, the Gla-containing skeletal proteins osteocalcin and matrix Gla protein (MGP) have been shown to control mineralisation of skeletal tissues [5,9,17,23].

Based on these previous studies we hypothesised that Ucma may have a physiological role in cartilage development and calcification processes during vertebrate skeleton formation. However, the actual role of Ucma in mammalian development is still unknown, and therefore we generated a Ucma-deficient mouse strain to address this question.

In this study we present the analysis of the first loss-of-function *in vivo* model for Ucma in mammals. Surprisingly, Ucma-deficient mice develop normally and do not exhibit overt defects in skeletal development or in calcification of cartilage and bone. They do not differ from wild-type littermates in viability, nor in size or weight. Histological analyses did not reveal any significant changes in cartilage and bone development as assessed by histochemical and gene expression analyses. Interestingly, we detected Ucma protein, in particular the processed isoform Ucma-C, in mouse cartilage of all ages. While mRNA expression levels of *Ucma* peak around birth, as reported earlier [27], we demonstrate here the presence of Ucma protein in cartilage of adult mice using a new antibody raised against a C-terminal Ucma peptide. This finding is consistent with specific β-galactosidase staining in cartilage of adult Ucma-deficient mice, which carry the *lacZ* gene under the control of the *Ucma* promoter. These data implicate that the physiological role of Ucma may not be focused on development, but rather on postnatal events, and that the importance of Ucma may become apparent under challenging or pathologic conditions.

Materials and methods

Construct design

Genomic fragments of the murine *Ucma* gene were amplified by long-range PCR using genomic DNA from R1 ES cells as a template. To obtain a direct fusion of the *lacZ* gene to the start codon of *Ucma*, a chimeric *Ucma*/*lacZ* fragment was generated by fusion PCR and introduced into the targeting vector pSK + TAG3-IRES-LacZpA-MC1NeopA, thereby replacing the IRES sequence of the targeting vector [22]. A left arm of homology of app. 4.5 kb (corresponding to nt. 1892662–1897300 in NT_039202.7) and a right arm of homology of app. 3.4 kb (corresponding to nt. 1897303–1900681 in NT_039202.7) were cloned into the targeting vector by standard techniques. The resulting targeting construct pUcma-KO thus contained a *lacZ* gene with an SV40 polyadenylation signal inserted in frame directly after the start codon of *Ucma* in the first exon, followed by a neomycin phosphotransferase gene (MC1Neo polyA). This *lacZ*/*neoR* cassette disrupts the *Ucma* mRNA sequence, inactivates the gene and provides a positive selection marker.

Transfection and screening of murine embryonic stem cells and Generation of a Ucma-deficient mouse strain

R1 embryonic stem cells (1×10^7/0.8 ml) were transfected with 35 μg *Kpn*I-digested targeting vector pUcma-KO (Biorad GenePulser; 240 V, 480 μF). After recovering for 15 min at RT transfected ES cells were plated onto feeder cell (γ-irradiated primary murine embryonic fibroblasts; PMEF) layers at a density of 2×10^6/10 cm dish in ES cell medium (DMEM with 10% FCS, nonessential amino acids, L-glutamine, penicillin–streptomycin, 0.1 mM 2-mercaptoethanol, 1000 U/ml leukaemia inhibitory factor [ESGRO, Chemicon]). Positive selection of recombinant clones was started after 24 h using 250 μg/ml G418, and G418-containing ES cell medium was replaced daily. A total of 240 clones were picked and grown. Isolated DNA was screened by Southern blotting and hybridization for correct homologous recombination using a DNA probe located 3′ of the knockout construct (corresponding to nt. 1900711–1901204 in NT_039202.7; Fig. 1). Two ES cell clones containing the correctly targeted *Ucma* allele (clones 1B and 5D) were expanded, injected into C57BL/6 blastocysts, and used for the generation of chimera. Chimeric offspring were screened for germ line transmission by coat colour and PCR as well as by Southern blot analysis. For both clones correct transmission of the mutated

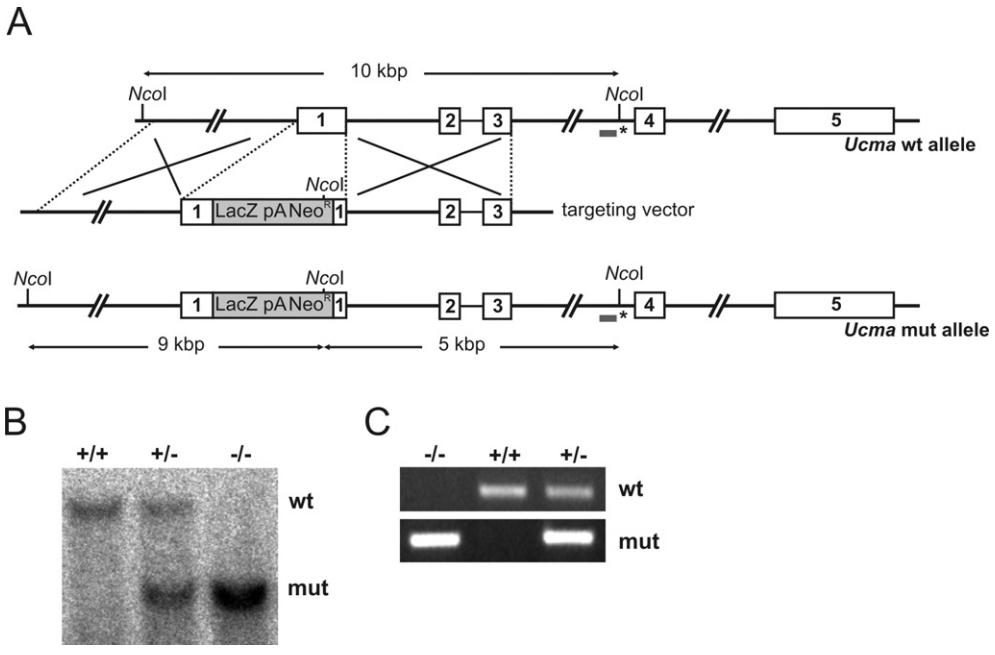

Fig. 1. Generation of Ucma-null mutant mice. A: Targeting strategy for the generation of a *Ucma* ko-allele in embryonic mouse stem cells. A *lacZ*/*neoR* cassette disrupts the *Ucma* gene in the first exon directly after the translation start site. The probe for Southern blot genotyping (*) detects a 10 kb fragment in case of a wt and a 5 kb fragment in case of a mutant (mut) allele. B: Southern blot of *Nco*I-digested genomic DNA from wild type, heterozygous and knockout specimen hybridised with the probe indicated in A (*). C: PCR genotyping of Ucma-deficient, wt, and heterozygous mice using genomic DNA as a template and specific primers for mutant (mut) and wt alleles.