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Osteopontin as a novel substrate for the proprotein convertase 5/6 (*PCSK5*) in bone

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ABSTRACT

Seven proprotein convertases cleave the basic amino acid consensus sequence K/R-X_n-K/R↓ (where $n = 0, 2, 4$ or 6 variable amino acids) to activate precursor proteins. Despite similarities in substrate specificity, basic amino acid-specific proprotein convertases have a distinct tissue distribution allowing for enzymatic actions on tissue-resident substrates. Proprotein convertase 5/6 (PC5/6) has two splice variants – soluble PC5/6A and membrane-bound PC5/6B – and is expressed during mouse development in many tissues including bone and tooth, but little is known about the substrates for PC5/6 therein. Osteopontin (OPN) is an abundant bone extracellular matrix protein with roles in mineralization, cell adhesion and cell migration, and it has putative consensus sequence sites for cleavage by PC5/6, which may modify its function in bone. Since PC5/6-knockout mouse embryos show developmental abnormalities, and reduced overall mineralization, we designed this study to determine whether OPN is a substrate of PC5/6. *In silico* analysis of OPN protein sequences identified four potential PC5/6 consensus cleavage sites in human OPN, and three sites – including a noncanonical sequence – in mouse OPN. *Ex vivo* co-transfections with human OPN revealed complete OPN cleavage reducing full-length OPN (~70 kDa) to an N-terminal fragment migrating at ~50 kDa and two C-terminal fragments at ~18 kDa and ~16 kDa. Direct cleavage of OPN by PC5/6A – the predominant isoform expressed in human osteoblast cells – was confirmed by cell-free enzyme-substrate assays and by mass spectrometry. The latter was also used to investigate potential cleavage sites. Co-transfections of PC5/6 and mouse OPN showed partial cleavage of OPN into a C-terminal OPN fragment migrating at ~30 kDa and an N-terminal fragment migrating at ~29 kDa. Micro-computed tomography of PC5/6-knockout embryos at E18.5 confirmed a reduction in mineralized bone, and *in situ* hybridization performed on cryo-sections of normal mouse bone using *Pcsk5* and *Opn* anti-sense and control-sense cRNA probes indicated the co-localization of the expression of these genes in bone cells. This mRNA expression profile was supported by semi-quantitative RT-PCR using osteoblast primary cultures, and cultured MC3T3-E1 osteoblast and MLO-Y4 osteocyte cell lines. Immunoblotting for OPN from mouse bone extracts showed altered OPN processing in PC5/6-knockout mice compared to wildtype mice. OPN fragments migrated at ~25 kDa and ~16 kDa in wildtype bone and were not present in PC5/6-deficient bone. In conclusion, this study demonstrates that *Pcsk5* is expressed in bone-forming cells, and that OPN is a novel substrate for PC5/6. Cleavage of OPN by PC5/6 may modify the function of OPN in bone and/or modulate other enzymatic cleavages of OPN, leading to alterations in the bone phenotype.

Abbreviations: OPN, osteopontin; FAM20C, family with sequence similarity 20, member C; TNAP, tissue nonspecific alkaline phosphatase; PHEX, phosphate-regulating gene with homologies to endopeptidases on the X chromosome; PC5/6, proprotein convertase 5/6; PC5/6 KO, proprotein convertase 5/6-knockout mouse; Gdf11, growth and differentiation factor 11; CMK, decanoyl-RVKR-chloromethyl ketone; D6R, hexa-D-arginine; Micro-CT, micro computed tomography; RGD, Arg-Gly-Asp; TG2, transglutaminase 2 (tissue transglutaminase).

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1. Introduction

Osteopontin (OPN) is a highly abundant extracellular matrix protein that has been well characterized as a mineralization inhibitor in bone [1–4], in addition to having other functions in cell adhesion, cell migration and cell signaling [5–9]. The abundance of acidic amino acids and the large number of phosphorylations distributed along OPN allows both full-length OPN and its peptides to bind tightly to apatitic mineral crystals, thus inhibiting crystal nucleation and growth [3,4,10,11]. Although OPN is found in a variety of cell types and tissue fluids, its functions have been shown to be highly regulated as a result of post-translational modifications [12,13]. Enzymes such as transglutaminase 2 (TG2; tissue transglutaminase) can introduce conformational change in OPN [14,15] and thrombin, plasmin, cathepsin D and matrix metalloproteases 3, 7 and 9 can expose functional motifs for cell attachment or migration [9,16–18]. Tissue-nonspecific alkaline phosphatase (TNAP) and family with sequence similarity 20, member C (FAM20C) can dephosphorylate and phosphorylate OPN, respectively [3,19,20]. Furthermore, we have recently shown that the enzyme PHEX (phosphate-regulating gene with homologies to endopeptidases on the X chromosome) regulates the mineralization-inhibiting function of OPN by extensively degrading full-length OPN into inactive small peptides, thus allowing normal bone mineralization to proceed [21]. Considering that various enzymes play a significant role in the regulation of OPN function, we sought to discover other potential proteases that might modify OPN in the bone.

Mammalian proprotein convertases are a family of subtilisin-like serine proteinases that activate precursor proteins and peptides throughout the secretory pathway [22,23]. Seven of these proprotein convertases cleave at the basic consensus sequence site K/R-X_n-K/R↓ (where $n = 0$, 2, 4 or 6 variable amino acids), but despite similarities in substrate specificity, basic amino acid-specific proprotein convertases have a distinct tissue distribution allowing for targeted enzymatic actions on specific tissue-resident substrates [22–25]. The proprotein convertase PC5/6 (encoded by the gene *PCSK5*) can exist as secreted soluble PC5/6A or membrane-bound PC5/6B isoform as a result of alternative splicing [26–28]. Proprotein convertase 5/6-knockout (PC5/6 KO) mice die at birth with an altered antero-posterior pattern, including extra vertebrae and lack of tail, kidney agenesis, hemorrhages, collapsed alveoli and retarded ossification [29,30]. Some of these phenotypic features can be explained by the lack of activation of growth and differentiation factor 11 (Gdf11) – a regulator of axial patterning during skeletal development, and a candidate substrate for PC5/6 [29]. *Gdf11*-knockout mice also have additional lumbar and thoracic vertebral segments, and abnormally positioned ribs, similar to the phenotype of PC5/6 KO mice [29,31]; however, not all the skeletal features are identical between these two mutant mice. More specifically, PC5/6 KO mice exhibit delayed mineralization not observed in *Gdf11*-knockout mice.

Pcsk5 (mouse gene) is highly expressed in incisor teeth [32], but little is known about its expression in bone. Four potential PC5/6 consensus sites within the C-terminal half of the human OPN sequence, and three homologous sites are found in mouse OPN (Fig. 1). Since PC5/6 appears to play a role in bone development and mineralization, and that OPN is a well characterized regulator of bone mineralization and other bone formation and remodeling events, the aim of this study was to determine whether OPN was a physiologically relevant substrate for PC5/6.

2. Materials and methods

All reagents were obtained from Thermo Fisher Scientific (Waltham, MA, USA) unless otherwise indicated. Animal procedures were reviewed and approved by the McGill University Institutional Animal Care and Use Committee and all procedures were also approved by the IRCM bioethics committee for animal care, and followed the guidelines of the Canadian Council on Animal Care.

2.1. Osteoblast/osteocyte cell culture

MC3T3-E1 (subclone 14) pre-osteoblast cells – kindly provided by Dr. Renny T. Franceschi (University of Michigan) – were plated at a density of 50,000 cells/cm^2 in complete medium (minimum essential medium with 10% fetal bovine serum [Hyclone, Waltham, MA, USA], 0.225 mM L-aspartic acid, 2 mM L-glutamine and 1 × penicillin/streptomycin). Cultures were allowed to adhere for 24 h, before treatment with complete medium supplemented with 50 μg/mL ascorbic acid and 10 mM β-glycerophosphate for 12 days to induce osteogenic differentiation. MLO-Y4 cells – generously provided by Dr. Lynda Bonewald (University of Missouri-Kansas City) – were cultured on plates coated with rat tail collagen I in α-minimum essential medium with Earle's salts supplemented with 2.5% fetal bovine serum, 2.5% bovine calf serum, and 1 × penicillin/streptomycin until 70% confluency was reached and cells developed connecting dendritic processes as described by Rosser et al. [33].

Primary osteoblasts were isolated by digesting dissected calvariae from 5-day-old wildtype C57BL/6 mouse pups in digestion medium (serum-free minimum essential medium containing 0.1 mg/mL collagenase P [Sigma-Aldrich, Oakville, ON, Canada] and 0.00125% trypsin/EDTA) for 15 min at 37 °C with rapid shaking every 5 min. The digestion medium was changed, and this was repeated once more before incubating in fresh digestion medium for 1 h, with shaking every 15 min. Bone fragments were washed twice in serum-free minimum essential medium, before plating in complete medium to allow bone cells to migrate out of the bone fragments. Cells were trypsinized, passed through a 40 μm cell strainer, and plated at a density of 50,000 cells/cm^2, before treatment with osteogenic medium (same as that described for M3CT3-E1 cell culture) for 21 days. Commercially available, primary human osteoblasts harvested from femoral head cancellous bone were purchased from PromoCell GmbH (Cat. No. C-12720, Heidelberg, Germany), and were cultured and differentiated according to the manufacturer's

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Human  MRIAVICFCL LGITCAIPVK QADSGSSEEK QLYNKYPDAV ATWLNPDPSQ KQNLLAPQNA VSSEETNDFK
Mouse  MRLAVICFCL FGIASSLPVK VTDSGSSEEK -LYSLHPDPI ATWLVPDPSQ KQNLLAPQNA VSSEEKDDFK

Human  QETLPSKSNE SHDHMDDMDD EDDDD--HVD SQDSIDSNDS DDVDDTDDSH QSDESHHSDE SDELVTDFPT
Mouse  QETLPSNSNE SHDHMDDDDD DDDDDGDHAE SEDSVDSDES D--------- ---ESHHSDE SDETVTAS--

Human  DLPATEVFTP VVPTVDTYDG RGDSVVYGLR SKSKKFRRPD IQYPDATDED ITSHMESEEL NGAYKAIPVA
Mouse  --TQADTFTP IVPTVDVPNG RGDSLAYGLR SKSRSFQVSD EQYPDATDED LTSHMKSGES KESLDVIPVA

Human  QDLNAPSDWD SRGKDSYETS QLDDQSAETH SHKQSRLYKR KANDESNEHS DVIDSQELSK VSREFHSHEF
Mouse  QLLSMPSDQD NNGKGSHESS QLDEPSLETH RLEHS----- KESQESADQS DVIDSQASSK ASLEHQSHKF

Human  HSHEDMLVVD PKSKEEDKHL KFRISHELDS ASSEVN
Mouse  HSHKDKLVLD PKSKEDDRYL KFRISHELES SSSEVN
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Fig. 1. Proprotein convertase consensus sites located in OPN sequence. Alignment of human (UniProt protein ID: P10451) and mouse (UniProt protein ID: P10923) OPN sequences with putative proprotein convertase consensus sites highlighted in red/bold. The signal peptide is underlined and the RGD integrin-binding motif is indicated with a box. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)