

The Regulation of Osteoprotegerin and Dickkopf-1 Production in Osteoblastic Cells

Thesis submitted in accordance with the requirements of the
University of Chester for the degree of Doctor of Philosophy by

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June 2011

University of Chester

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ACKNOWLEDGEMENTS

I would like to thank both Dr Michael J Marshall, my supervisor at the Charles Salt Research Centre and Professor John Williams, my supervisor at Chester University for their knowledge, guidance, support and patience during my time here at the Robert Jones and Agnes Hunt Orthopaedic Hospital, Oswestry. Thanks to Dr Michael Davie, Director of Research at the Charles Salt Centre both for allowing me to undertake my PhD in his research laboratory and for providing my funding. I would also like to thank Dr DC Mangham for the fracture callus and Paget bone tissue sections and all the laboratory staff in both the Pathology and Histopathology departments for performing the immunohistochemistry on such sections.

The biggest thanks of all must go to both my parents and my partner Luke for their constant encouragement, understanding, patience and emotional support (or in Luke's words, "putting up with me"!) both throughout my PhD and whilst writing this thesis.

DECLARATION

The work in this thesis is original and has not been submitted previously in support of any qualification or programme.

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ABSTRACT

Bone is a highly specialised living tissue and has both mechanical and metabolic functions. Remodelling of the bone ensures a healthy bone mass and is regulated by a trio of secreted proteins, namely receptor-activator of NF κ B (RANK), receptor-activator of NF κ B ligand (RANKL) and osteoprotegerin (OPG). OPG, a major regulator of osteoclastogenesis, bone resorption and vascular calcification, is produced by various cell types including mesenchymally derived cells, particularly osteoblastic cells.

Wnt signalling also plays a role in maintaining healthy bone mass. Dickkopf-1 (DKK-1) is a soluble inhibitor of Wnt signalling and its excessive expression contributes to bone loss in rheumatoid arthritis and multiple myeloma. Recently, DKK-1 has been demonstrated to be over-produced in osteoblasts of patients with Paget's disease of bone (PDB).

The osteoblastic cell lines MG63 and Saos-2 were subjected to a series of different growth factors, hormones and cytokines to investigate the production of OPG, DKK-1 and the expression of various *Wnt* proteins. These results demonstrate that during standard culture conditions, both OPG and DKK-1 production in osteoblastic cells depend on a factor present in serum. Serum deprivation resulted in the up-regulation of *Wnt4* and *Wnt11*, while down-regulating the expression of *Wnt7b*. Serum-induced OPG and DKK-1 production and Wnt expression was found to be regulated via a number of different signalling pathways.

OPG production and expression was stimulated by platelet-derived growth factor-AB (PDGF-AB) not only in MG63 and Saos-2 osteosarcoma cells, but also a mouse pre-osteoblastic cell line (MC3T3-E1) and human bone marrow stromal cells (BMSC). PDGF-AB was shown to act through the PDGF receptor, PKC, PI3K, ERK and P38 and not via NF κ B or JNK. PDGF isoforms AA, BB and AB demonstrated a similar stimulation of OPG production. The importance of PDGF in fracture healing suggests a role for OPG production in countering bone resorption during the early phase of this process.

BIO, an inhibitor of canonical Wnt signalling resulted in the down-regulation of *DKK-1* and the up-regulation of *Wnt5a*. Phorbol ester (PE), a known stimulator of PKC resulted in the up-regulation of *DKK-1*, *Wnt4*, *Wnt7a* and *Wnt16*. The effects of PE were inhibited by bisindolymaleamide but not staurosporine. DKK-1 production, but not expression, was observed to be stimulated by calcium along with an up-regulation of *Wnt7b* and a down-regulation of *Wnt10a* and *Wnt11*. Incubation of pre-stimulated cells with Triton-X demonstrated the ability of calcium to increase DKK-1 secretion.

DKK-1 was shown to be significantly elevated in the serum of PDB patients compared to healthy controls and did not correlate with ALP levels. Immunohistochemistry demonstrated that DKK-1 production is increased in both osteoblasts and fibrotic cells within the marrow cavity in PDB patients compared to fracture callus. β -catenin was found to be localised to intercellular membranes of plump osteoblasts, demonstrating its alternate role as a cell adhesion protein. DKK-1 therefore may be a useful biomarker of PDB and that *Dkk-1* may play a central role in the aetiology of PDB.

In summary, the results presented in this thesis have investigated the ways in which OPG and DKK-1 production in osteoblastic cells can be modulated with various effectors and the effect of Wnt signalling. These results may therefore be beneficial to increase the understanding of bone biology, improve fracture repair and generate further research into the role DKK-1 and the osteoblast in the aetiology of PDB to enable improved treatments to be developed.

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ABBREVIATIONS

1,25(OH) ₂ D ₃	1,25 dihydroxyvitamin D ₃
αMEM	Minimal essential medium, alpha
ALP	Alkaline phosphatase
ANOVA	Analysis of variance
APC	Adenomatous polyposis coli
ATP	Adenosine triphosphate
BGP	Sodium beta-glycerophosphate
BIM	Bisindolylmaleamide
BIO	6-Bromoindirubin-3'-monoxime
BMD	Bone mineral density
BMP-2	Bone morphogenetic protein-2
BMP-7	Bone morphogenetic protein-7
BMSC	Bone marrow stromal cell
BMU	Bone multicellular unit
BP	Bisphosphonate
BSA	Bovine serum albumin
BSP	Bone sialoprotein
Ca ²⁺	Calcium ions
CaCl ₂	Calcium Chloride
cAMP	Cyclic adenosine monophosphate
CaSR	Calcium sensing receptor
cDNA	Copy deoxyribonucleic acid
CRD	Cysteine-rich domain
CREB	Cyclic adenosine monophosphate response element-binding
CT	Cycle threshold
CTX	Carboxy-terminal crosslinked telopeptide of type I collagen
DAB	3,3' diaminobenzidine
DEXA	Dual energy X-ray absorptiometry
DKK-1	Dickkopf-1
DKK-2	Dickkopf-2
DKK-3	Dickkopf-3
DKK-4	Dickkopf-4
DMEM	Dulbecco's modified essential medium
DNA	Deoxyribonucleic acid

Dvl	Dishevelled protein
ECM	Extracellular Matrix
EDTA	Diaminoethanetetra-acetic acid diammonium salt
ELISA	Enzyme-linked immunosorbant assay
ERK	Extracellular signal-related kinase
ESH	Expansile skeletal hyperphosphatasia
FCS	Foetal calf serum
FEO	Familial expansile osteolysis
FFPE	Formalin fixed paraffin embedded
FGF-1	Fibroblast growth factor-1
FGF-2	Fibroblast growth factor-2
Fzd	Frizzled receptor
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GH	Growth hormone
GHSH	Growth hormone stimulating hormone
GIOP	Glucocorticoid induced osteoporosis
GS3K β	Glycogen synthase kinase-3 beta
HCl	Hydrochloric acid
hESC	Human embryonic stem cell
HGF	Hepatocyte growth factor
hMSC	Human mesenchymal stems cell
HSPG	Heparin sulphate proteoglycan
IGFII	Insulin-like growth factor-II
IL-1	Interleukin-1
IL-6	Interleukin-6
IL-11	Interleukin-11
JNK	c-Jun N-terminal kinase
JPD	Juvenile Paget's disease of bone
LEF	Lymphoid enhancing factor
LRP5/6	Low density lipoprotein related protein 5/6
M-CSF	Macrophage-colony stimulating factor
MM	Multiple myeloma
MMP-13	Matrix metalloproteinase-13
mRNA	Messenger ribonucleic acid
MSC	Mesenchymal stem cell
n	Number of replicates
ND	Not detected

NF-AT	Nuclear factor of activated T-cells
NFκB	Nuclear-factor kappa B
NHS	National health service
NTX	Amino-terminal crosslinked telopeptide of type I collagen
OA	Osteoarthritis
OC	Osteocalcin
OCIF	Osteoclastogenesis inhibitory factor
ODAR	Osteoclast differentiation and activation receptor
ODF	Osteoclast differentiation factor
OPG	Osteoprotegerin
OPGL	Osteoprotegerin ligand
OPPG	Osteoporosis pseudoglioma
P	Probability factor
p38 MAPK	p38 mitogen-activated protein kinase
PAR-2	Protease-activated receptor-2
PBS	Phosphate buffered saline
PCP	Planar cell polarity
PCR	Polymerase chain reaction
PDB	Paget's disease of bone
PDGF-AA	Platelet-derived growth factor-AA
PDGF-AB	Platelet-derived growth factor-AB
PDGF-BB	Platelet-derived growth factor-BB
PDGFRα	Platelet-derived growth factor receptor alpha
PDGFRβ	Platelet-derived growth factor receptor beta
PE	Phorbol ester
PGE ₂	Prostaglandin E ₂
PI	Propidium iodide
Pi3K	Phosphoinositide-3OH kinase
PICP	Carboxy-terminal propeptide of type I procollagen
PINP	Amino-terminal propeptide of type I procollagen
PKA	Protein kinase A
PKC	Protein kinase C
PTH	Parathyroid hormone
RA	Rheumatoid arthritis
RANK	Receptor activator of nuclear-factor kappa B
RANKL	Receptor activator of nuclear-factor kappa B ligand
RNA	Ribonucleic acid

RT-PCR	Reverse transcription polymerase chain reaction
SCF	Stem cell factor
SD	Standard deviation
sFRP-1	Secreted Frizzled-related protein-1
sFRP-2	Secreted Frizzled-related protein-2
sFRP-3	Secreted Frizzled-related protein-3
sFRP-4	Secreted Frizzled-related protein-4
siRNA	Short interfering ribonucleic acid
SOST	Sclerostin
SQSTM1	Sequestosome 1
sRANKL	Soluble RANKL
STS	Staurosporine
TACE	TNF- α convertase enzyme
TBS	Tris buffered saline
TCF	T-cell factor
TGF β	Transforming growth factor-beta
TMB	3,3',5,5' tetramethylbenzidine
TNFR	Tumour necrosis factor receptor
TNF α	Tumour necrosis factor-alpha
Tph1	Tryptophan hydroxylase I
TRAIL	TNF-related apoptosis-inducing ligand
TRANCE	TNF-related activation-induced cytokine
TRAP	Tartrate resistant acid phosphatase
tsALP	Total serum alkaline phosphatase
UBA	Ubiquitin-associated
VDR	Vitamin D receptor
VEGF	Vascular endothelial growth factor
VSCC	Voltage-sensitive calcium channel
WIF-1	Wnt inhibitory factor-1
WISP	Wnt-induced secreted protein

UNITS OF MEASUREMENT

<i>g</i>	Gravitational force
g	Gram
mg	Milligram
μg	Microgram
ng	Nanogram
L	Litre
mL	Millilitre
μL	Microlitre
M	Molar
mM	Millimolar
μM	Micromolar
nM	Nanomolar
cm	Centimetre
mm	Millimetre
μm	Micrometre
nm	Nanometre
U/ml	Units per millilitre