The Interaction between Vitamin D and Extracellular Calcium on Osteogenic Differentiation

Dongqing Yang

B.Sc, M.Biotechnology

Thesis submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy

October, 2014

The Discipline of Medicine

School of Medicine

Faculty of Health Sciences

The University of Adelaide

South Australia

Australia

TABLE OF CONTENTS

Thesis abstract ..... **I**

Declaration ..... **III**

Acknowledgements ..... **IV**

Publications and Presentations ..... **VI**

Chapter 1 Literature review: The interaction between vitamin D and extracellular calcium in osteogenic differentiation **1**

1.1 Osteoblast biology **2**

1.1.1 Bone formation by osteoblasts *2*

1.1.2 Differentiation of osteoblasts *3*

a) From mesenchymal stem cells to pre-osteoblasts *4*

b) Pre-osteoblasts to immature osteoblasts *6*

c) Mineralisation by mature osteoblasts and the transition to early osteocytes *6*

d) The final stage of osteoblast differentiation: mature osteocytes *9*

1.1.3 Skeletal site differences *12*

1.2 Nutritional factors interacting with osteoblasts **13**

1.2.1 Vitamin D *13*

a) Vitamin D biology *14*

b) The endocrine role of vitamin D *16*

c) Non-classical actions of vitamin D *18*

d) Vitamin D inhibits osteoblast proliferation *21*

e) Vitamin D activity and osteoblast differentiation *22*

1.2.2 Extracellular calcium concentration *23*

1.3 Conclusions and proposal for study **25**

Aim of study *26*

Hypotheses *26*

Approaches *26*

Chapter 2 Materials and Methods **28**

2.1 Materials **28**

2.1.1 Chemicals and reagents *28*

2.1.2 Molecular biology reagents *28*

2.1.3 Animals *28*

2.2 Nucleotide preparations **29**

2.2.1 DNA extraction *29*

2.2.2 RNA extraction *30*

2.2.3 cDNA synthesis *30*

2.2.4 Conventional PCR (polymerase Chain Reaction) *31*

2.2.5 Real-time PCR *32*

2.3 Isolation of osteoblasts **32**

2.3.1 Osteogenic growth media *32*

2.3.2 Osteogenic differentiation media *37*

2.3.3 Isolation of osteoblast-like cells from neonatal mouse calvarial bones *37*

2.3.4 Cryopreservation and thawing of cells *38*

2.4 Histochemical staining **38**

2.4.1 Alizarin Red staining *38*

2.4.2 Von Kossa staining *39*

2.5 Osteoblast proliferation assay **40**

2.6 Osteoblast differentiation and mineralisation assay **40**

Chapter 3 Differential effects of 1,25-dihydroxyvitamin D on mineralisation and differentiation in two different types of osteoblast-like cultures **42**

Abstract **46**

3.1 Introduction **46**

3.2 Materials and methods **47**

3.2.1 Isolation of osteoblasts from juvenile mouse cortical bones *47*

3.2.2 Isolation of osteoblasts from mouse neonatal calvarial bones *47*

3.2.3 Differentiation assays *47*

3.2.4 Statistics *49*

3.3 Results **49**

3.3.1 Characterisation of cells *49*

3.3.2 In vitro mineralisation and response to 1,25D *50*

3.3.3 1,25D effects on gene expression *50*

3.4 Discussion **50**

References **50**

Erratum **51**

Chapter 4 The regulation of osteogenic differentiation of calvaria-derived osteoblasts in response to 1,25D and calcium, and the influence of the level of VDR expression **52**

4.1 Introduction **52**

4.2 Experimental methods **54**

4.3 Results **60**

4.3.1 Vdr/VDR status and 1,25D metabolism in VDRKO, WT and OSVDR cells ... *60*

4.3.2 Comparison of gene expression profiles of WT and VDRKO cells *63*

4.3.3 Comparison of gene expression profiles of WT and OSVDR cells *72*

4.3.4 Expression levels of osteogenic differentiation-related genes under acute and chronic 1,25D treatments at day 24 of WT and OSVDR cell cultures *72*

4.3.5 Effect of chronic 1,25D treatment on in vitro mineral deposition by VDRKO, WT and OSVDR cells under conditions of total extracellular calcium at either 1.8 mM or 2.8 mM *77*

4.4 Discussion **81**

4.5 Conclusion **90**

Chapter 5 Vitamin D metabolites and extracellular calcium promote mineral deposition by a mature osteoblast cell line MLO-A5 91

Abstract 95

5.1 Introduction 96

5.2 Materials and methods 97

5.2.1 Cell culture 97

5.2.2 Proliferation assay 97

5.2.3 Differentiation/mineralisation assay 97

5.2.4 Statistics analyses 98

5.3 Results 99

5.3.1 Vitamin D receptor activity and vitamin D metabolism in MLO-A5 cells 99

5.3.2 Acute Cyp24a1 induction within 72 hours and 1,25D levels from media supernatants of 25D and 1,25D treated cultures 99

5.3.3 Vitamin D inhibition of MLO-A5 proliferation 100

5.3.4 Vitamin D metabolites enhance mineral deposition by MLO-A5 100

5.3.5 Effects of vitamin D metabolites and extracellular calcium on mRNA levels of genes related to osteogenic differentiation and mineral deposition 101

5.4 Discussion 102

Acknowledgements 105

References 106

Chapter 6 Conclusion remarks 122

References 129

Appendix I 151

Appendix II 156

Appendix III 170

Thesis abstract

While the role of vitamin D in the prevention of rickets in children and osteomalacia in adults has been well demonstrated, its benefit in the treatment of osteoporosis is subject to controversy. Clinical trials of vitamin D supplementation to prevent fractures have been conducted with mixed results and some meta-analyses have indicated limited benefit in reducing fracture risk. The most consistent beneficial effects of vitamin D have been obtained when combined with calcium supplements. 1,25-dihydroxyvitamin D acts on the three major types of bone cells (osteoblasts, osteoclasts and osteocytes) to initiate either catabolic or anabolic actions on bone. To elucidating the potential benefits of vitamin D to bone health, this study examined direct actions of vitamin D metabolites on bone cells focussing on stimulation of *in vitro* osteogenic differentiation. Two cell culture models, representing immature and mature stage of osteoblasts, were employed to investigate the role of vitamin D on osteogenic differentiation. The regulation of a variety of gene expressions and modulation of mineral deposition by these cells, were used as key readouts.

In chapter 3, vitamin D was observed to play an inhibitory role on mineral deposition by the immature calvarial bone-derived osteoblast-like cells (Calvarial cells) but did not exert any suppressive effect on the mature osteoblast/early osteocyte cell line, MLO-A5. Thus the actions of vitamin D appear to be dependent on either the stage of cell maturation or their skeletal origin.

The studies using Calvarial cells were expanded in chapter 4 by utilising cells derived from genetically modified mouse lines, including the global vitamin D receptor (VDR) knockout (VDRKO) and the over-expression of VDR in osteocalcin-expressing cells (OSVDR), in comparison to cells derived from wild-type animals. The active hormone form, 1α,25-dihydroxyvitamin D_3 (1,25D), promoted a mature cell phenotype at

physiological levels (around 30 pM) dependent on the level of *Vdr* mRNA. However, in OSVDR cells with high levels of VDR, a pharmacological concentration of 1,25D (1 nM) appeared to stimulate de-differentiation of the osteoblast phenotype by down-regulating the expression of mature osteoblast/osteocyte genes. *Enpp1* and *Tnap* were identified as key genes to modulate mineral deposition in these models.

In chapter 5, the cell line MLO-A5 was again utilised, here for studying the interaction between vitamin D and extracellular calcium on osteoblasts. Both endogenous and exogenous sources of 1,25D, either alone or interacting with extracellular calcium, increased mineral deposition and the expressions of maturation-related genes. Extracellular calcium altered vitamin D metabolism by MLO-A5 cells. Again, key genes associated with mineral deposition were *Enpp1* and *Tnap*.

Data from this study confirm the stimulatory actions of vitamin D on osteogenic differentiation and identified an interaction with extracellular calcium levels. Mineral deposition was found to be dependent on 1,25D modulation of *Enpp1* and *Tnap* expressions. A highly novel finding was that the extracellular calcium concentration modulates the metabolism of vitamin D and the maturation of these cells. These data help to address the controversy on the actions of vitamin D on osteoblast differentiation and mineralisation and improve our understanding of their biology.

Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint award of this degree.

I give consent to this copy of my thesis when deposited in the University Library, being made available for loan and photocopying, subject to the provisions of the Copyright Act 1968.

The author acknowledges that copyright of published works contained within this thesis resides with the copyright holder (s) of those works.

I also give permission for the digital version of my thesis to be made available on the web, via the University's digital research repository, The Library Search and also through web search engines, unless permission has been granted by the University to restrict access for a period of time.

Signature: Date: 04/02/2015

Dongqing Yang

Acknowledgements

Firstly, I would like to thank my supervisors Professor Howard Morris and Associate Professor Gerald Atkins. Back to the time I was looking for a PhD position, it was very lucky that Howard kindly offered me the chance to study in his laboratory and also Gerald agreed to be my co-supervisor to guide me in the cell biology works. During the study period, both Howard and Gerald made available an incredible amount of intelligent, effort and time to discuss my project, inspire my scientific thinking and improve my academic writing skill. Also, only with their continuous encouragement, could I deal with the difficulty and frustration I encountered during scientific research, enabling me to reach the completion of my PhD study.

Secondly, I would like to forward my acknowledgement to Associate Professor Paul Anderson and Dr Andrew Turner in the Musculoskeletal Biology Research Laboratory, the University of South Australia. Without their patience on teaching me and answering my numerous questions, I would never have been able to accomplish my project. I also would like to thank Ms Rebecca Sawyer and Dr Nga Lam in the Musculoskeletal Biology Research Laboratory, the University of South Australia, as well as Dr Asiri Wijenayaka, Miss Renee Ormsby, Dr Masakazu Kogawa, Dr Nobuaki Ito and Dr Matt Prideaux, from Bone Cell Biology Group, the University of Adelaide, for their kindly guidance on the daily bench work from time to time.

I gratefully acknowledge Professor Lynda Bonewald, University of Missouri, Kansas City, MO, USA, for the provision of the MLO-A5 cell line, made available to Associate Professor Gerald Atkins through a pre-existing collaboration. Especially, I would like to give my acknowledgement to Professor Hong Zhou from the ANZAC Research Institute, Concord, NSW, Australia, for accepting me into her laboratory and generously teaching

me the skill of establishing osteoblast-like culture from neonatal mouse skull bone, which was a very important *in vitro* model in my project.

I am giving my most special thankfulness to my wife Jiangqin Wei for her unconditional understanding, forgiveness and encouragement every single day during this period. Without her love and support both physically and mentally, it would have been absolutely impossible for me to finish my study and make this achievement. Last but not least, I also would like to forward my gratefulness to all of our family members in China for their constant encouragement and support for us both.

Publications and Presentations

Yang D, Atkins GJ, Turner AG, Anderson PH, Morris HA. Differential effects of 1,25-dihydroxyvitamin D on mineralisation and differentiation in two different types of osteoblast-like cultures. *Journal of Steroid Biochemistry and Molecular Biology*. 2013;136:166-70.

[With Erratum to "Differential effects of 1,25-dihydroxyvitamin D on mineralisation and differentiation in two different types of osteoblast-like cultures" J. Steroid Biochem. Mol. Biol. 136 (2013) 166–170]

Yang D, Turner A, Anderson PH, Morris HA, Atkins GJ. Vitamin D metabolites and extracellular calcium promote mineral deposition by the mature osteoblast cell line MLO-A5. (Submitted for publication).

Published Abstracts:

Yang D, Atkins GJ, Anderson PH, Welldon KJ, Morris HA (2011) The role of vitamin D in the proliferation and differentiation of osteoblasts in vitro. *Osteoporosis International* 22, Suppl 4, 268.

Yang D, Atkins GJ, Turner A, Anderson P, Morris HA (2012) Differential effects of 1,25-dihydroxyvitmain D on in vitro mineral deposition: interaction between osteoblast stage and culture medium calcium concentration. Presented at the American Society for Bone and Mineral Research (ASBMR) Annual General Meeting, 2012, Minneapolis, MN, USA. *Journal of Bone and Mineral Research* 27, suppl. 1, S175.

Oral Presentations:

Yang D. The role Vitamin D in the proliferation and differentiation of osteoblasts. The 6th (2010) Clare Valley Bone Meeting, Clare, SA, Australia.

Yang D. The role of calcitrol in the differentiation of osteoblasts *in vitro*. The 7th (2012) Clare Valley Bone Meeting, Clare, SA, Australia.

Yang D. Differential effects of 1,25-dihydroxyvitamin D (1,25D) on *in vitro* mineral deposition: Interaction between osteoblast stage of maturation and culture medium calcium concentration, Australian Society for Medical Research (ASMR) Scientific Meeting, 2012, Adelaide, SA, Australia.

Poster Presentations:

Yang D, Atkins GJ, Anderson PH, Welldon KJ, Morris HA. The role of vitamin D in the proliferation and differentiation of osteoblasts. The 20th Australian and New Zealand Bone and Mineral Society (ANZBMS) Annual Scientific Meeting, 2010, Adelaide, Australia.

Yang D, Atkins GJ, Anderson PH, Welldon KJ, Morris HA. The role of vitamin D in the proliferation and differentiation of osteoblasts *in vitro*. The IOF Regionals 2nd Asia-Pacific Osteoporosis and Bone Meeting, 21st ANZBMS Annual Scientific Meeting combined with the Japanese Society of Bone & Mineral Research, 2011, Gold Coast, QLD Australia.

Yang D, Atkins GJ, Turner AG, Anderson PH, Morris HA. The role of calcitriol in the differentiation of osteoblast *in vitro*. The 15th Workshop on Vitamin D, June 20 – 22, 2012, Houston, TX, USA.