Epigenetic regulation of histone three lysine twenty seven tri methylation dictates mesenchymal stem cell lineage commitment, lifespan and murine skeletal development

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The Discipline of Medicine

School of Medicine

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Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma 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. I give consent to the copy of my thesis, when deposited at the University Library, being made available for loan and photocopying, subject to the provisions of the Copyright Act 1968. 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 catalogue and also through web search engines, unless permission has been granted by the University to restrict access for a period of time

Signed:

Sarah Hemming

Date: 11-04-2016

Acknowledgments

Firstly I would like to thank my supervisors Stan and Jim for all you help and support over the last four years. You have seen me grow from an honours student to an accomplished PhD student, with many life accomplishments along the way and you both have always encouraging me to succeed. This thesis is not only a reflection of my achievement but yours also. I would like to acknowledge the Australia Postgraduate Award (APA) and Dawes Top-up scholarship, this financial assistant allowed me to focus on my PhD.

I would like to thank the present and past members of the Mesenchymal Stem Cell Laboratory and the Multiple Myeloma Group, your guidance and support has been amazing. I would like to personally recognise the time and help I received from Sharon Paton, Esther Camp-Dotlic, Kim Hynes, Mary Mathews, Kate Vandyke, Steven Fitter and Duncan Hewett. I would like to extend a big thankyou to Ruth and Agatha from Adelaide Microscopy. Thank you, Kencana for helping optimise my antibodies for immunohistochemistry and David Haynes for allowing me to use your laboratory.

I would like to thank my family (Mum, Dad, Matt, Katie and Simon) for your love, support and encouragement throughout my postgraduate studies. Most importantly, I would like to thank my loving and tolerant husband Matt, who throughout this PhD has seen the best and worst of me and still loves me any way. Thank you to Matt's family for all your support and encouragement throughout my PhD.

This has by far been the hardest thing I have accomplished and I couldn't have done it without all of you. Thank you.

Abbreviations

3D	Three dimensional
+/+	Wildtype *Ezh2* alleles
+/-	One wildtype *Ezh2* allele and one floxed *Ezh2* allele
-/-	Two floxed *Ezh2* alleles
Adip	Adipogenic
AIPOQ	Adiponectin
ALK PHOS	Alkaline phosphatase
BFR	Bone formation rate
BFR/BS	Bone formation rate/ Bone surface
BMD	Bone mineral density
BMP	Bone morphogenetic proteins.
BMSC	Bone marrow derived stromal/stem cell
BMSSC	Bone marrow stromal cell
bp	Base pair
BRDU	5-bromo-2deoxyuridine
BS	Bone surface
BSA	Bovine serum albumin
BSP	Bone sialoprotein
BV	Bone volume
BV/TV	Bone volume/ Tissue volume
CamKII	Calcium-calmodulin dependent protein kinase-II
CBFA1	Core binding factor-1 (Gene)
CBFA1	Core binding factor-1 (Protein)
CDK1	cyclin dependent kinase 1
cDNA	Complementary deoxyribonucleic acid

C/EBP-α	CCAAT/Enhancer binding protein alpha (Gene)
C/EBP-α	CCAAT/Enhancer binding protein alpha (protein)
CFU	Colony forming unit
CFU-F	Colony forming unit-fibroblast
ChIP	Chromatin immunoprecipitation
ChIP-Seq	Chromatin immunoprecipitation sequencing
ChIP-on-ChIP	Chromatin immunoprecipitation on Chromatin immunoprecipitation
COL1A1	Collagen type 1A1
Cont	Control
COX2	Cyclooxygenase 2
CTan	Comprehensive TeX Archive Network
Ct.Th	Cortical thickness
DLX5	Distal-less homeobox 5
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DNAse	Deoxyribonuclease
dNTP	Deoxyribonucleotide triphosphate
DNMTs	DNA methyltransferases
DTT	Dithiothreitol
EB	Elution buffer
EDTA	Ethylenediaminetetra-acetic acid
ESC	Embryonic Stem Cells
EZH2	Human enhancer of zeste homolog 2 (Gene)
EZH2	Human & mouse enhancer of zeste homolog 2 (Protein)

Ezh2	Mouse enhancer of zeste homolog 2 (Gene)
$Ezh2^{+/+}$	Tg.Prx-1 Cre +:Ezh2 wt/wt
$Ezh2^{+/-}$	Tg.Prx-1 Cre +:Ezh2 fl/wt
$Ezh2^{-/-}$	Tg.Prx-1 Cre +:Ezh2 fl/fl
FACS	Fluorescence activated cell sorting
FCS	Foetal calf serum
fl	Lox P (flox sites)
g	The number of times the gravitational force
g	Grams
GAPDH	Glutaraldehyde 3-phosphate dehydrogenase
GFP	Green fluorescent protein
GREM1	Gremlin 1
GSK3	Glycogen synthase kinase 3
H&E	Haematoxylin and eosin
HATS	Histone acetyltransferases
HDACs	Histone deacetylases
HBSS	HANKS balanced salt solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HET	Heterozygous
hMSC	Human mesenchymal stem cell
HMTS	Histone methyltransferases
HOM	Homozygous
HSC	Haematopoietic Stem Cell
H3K4	Histone three lysine four
H3K27	Histone three lysine twenty seven

H3K36	Histone three lysine thirty six
H3K79	Histone three lysine seventy nine
H3K9	Histone three lysine nine
H_2O_2	Hydrogen peroxide
IGF	Insulin growth factor
IgG	Immunoglobulin G
IHH	Indian hedge hog
IL-1	Interleukin 1
IL-6	Interleukin 6
IP	Immunoprecipitation
iPSC	Induced Pluripotent Stem Cells
JMJD3	Jumonji domain-containing protein 3
JNK	The c-Jun NH2-terminal kinases
KLF4	Kruppel-like factor 4
LEPR	Leptin receptor
LRP	Lipoprotein related proteins
KMD6A	Lysine demethylase 6A
KDM6b	Lysine demethylase 6B
M	Molar
MAR	Mineral apposition rate
M-CSF	Macrophage colony stimulating factor
Me1	Mono-methylation
Me2	Di-methylation
Me3	Tri-methylation
MEM	Minimum essential medium

Micro-CT	Micro-computed tomography
MITR	Myocyte enhancer factor-2 interacting transcription factor
mm	Miller mitres
MPP	Multipotent progenitor cells
mRNA	Messenger ribonucleic acid
mRNA	Messenger ribonucleic acid
MSC	Mesenchymal Stem Cell
MX1	Myxovirus resistance-1
α-MEM	α-modified Eagle's medium
µM	Micro molar
µm	Microns
NCOR	Nuclear compressor
N	Number
N.Adip/Mar.Ar	Number of adipocytes in marrow area
N.Ob/B.Pm	Number of osteoblasts on bone perimeter
NOD	Normal osteoblast donor
nM	Nano molar
NRecon	high-speed volumetric reconstruction software
Ob	Osteoblast
OC	Osteoclast
OC	Osteocalcin chapter 3
OCN	Osteocalcin
OCR	Osteochondral reticular cells
OCT4	Octamer-binding transcription factor 4
OPG	Osteoprotegerin

OPN	Osteopontin
Oste	Osteogenic
OSX	Osterix
PBND	PCR buffer with non-ionic detergents
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGFRα	Platelet-derived growth factor receptor α
pRUF-EZH2	pRUF-IRES-GFP-EZH2 vector
PK	Proteinase K
Pm	Perimeter
PPARγ2	Peroxisome proliferator-activated receptor gamma
pRUF-GFP	pRUF-IRES-GFP vector
pRUF-KDM6A	pRUF-IRES-GFP-KDM6A vector
PTHRP	Parathyroid hormone-related protein
RANK	Receptor activator of nuclear factor kappa-B
RANKL	Receptor activator of nuclear factor kappa-B ligand
rcf	relative centrifugal force or x g-force
RNA	Ribonucleic acid
ROI	Region of interest
rpm	revolutions per minute
RT	Room temperature
RT-PCR	Real-time polymerase chain reaction
RUNX2	Run related transcription factor
SDEV	Standard deviation
SEM	Standard error of the mean

Sem	Scanning electron microscope
SHH	Sonic hedge hog
siRNA	Small interfering RNA
SOX9	Sex-determining region SRY of the Y chromosome 9
STRO-1	stromal precursor cell surface antigen
Tb.Th	Trabecular thickness
Tb.Sp	Trabecular spacing
TF	Transcription factor
TNF	Tumour necrosis factor
TRAP	Tartrate-resistant acid phosphatase 5
TS	Tissue surface
TSS	Transcription start site
TV	Tissue volume
Tween 20	Polyethylene glycol sorbitan monolaurate
UTX	Ubiquitously transcribed tetra-tricopeptide repeat X
UV	Ultra violet
VSVG	Vesicular stomatitis virus G-protein
WT	Wild type
WNT	wingless-type
w/v	Weight per volume

List of publications

Publications generated during my PhD:

Stem Cells Dev. 2015 Jun 1;24(11):1297-308. doi: 10.1089/scd.2014.0471. Epub 2015 Feb 25. **Cakouros D, Isenmann S, Hemming SE, Menicanin D, Camp E, Zannettino AC, Gronthos S**. Novel basic helix-loop-helix transcription factor hes4 antagonizes the function of twist-1 to regulate lineage commitment of bone marrow stromal/stem cells.

Stem Cells. 2014 Jul; 32(7):1991-2. doi: 10.1002/stem.1710. **Hemming S, Cakouros D, Gronthos S**. Detachment of mesenchymal stem cells with trypsin/EDTA has no effect on apoptosis detection.

Chapter 1

Sarah Elizabeth Hemming, Dimitrios Cakouros and Stan Gronthos. "Epigenetic regulation of mesenchymal stem cell growth and multi-potential differentiation". In: The Biology and Therapeutic Applications of Mesenchymal Cells. Ed K. Atkinson. John Wiley and Sons, Hoboken, New Jersey, USA. 2015, currently in press.

Chapter 2

Hemming S, Cakouros D, Isenmann S, Cooper L, Menicanin D, Zannettino A, Gronthos S. EZH2 and KDM6A act as an epigenetic switch to regulate mesenchymal stem cell lineage specification. Stem Cells. 2014 Mar;32(3):802-15. doi: 10.1002/stem.1573.

Chapter 3

Sarah Hemming, Dimitrios Cakouros, Kate Vandyke, Melissa Davis, Andrew Zannettino, Stan Gronthos. Identification of novel EZH2 targets in osteogenesis. Summited Stem to Cell Reports 2015.

Chapter 4

Sarah Hemming, Dimitrios Cakouros, John Codrington, Kate Vandyke, Andrew Zannettino, Stan Gronthos. EZH2 regulates osteoblast and adipogenic differentiation, bone microarchitecture and bone remodelling in mice. Summited to Journal of Bone and Mineral Research (JBMR).

Conference and awards

Conference Proceedings:

2015

2015 ASSCR

ASSCR, Crown Plaza, Hunter Valley, New South Wales, Australia.

Poster**;** *Methyltransferase Ezh2 regulates newborn skeletal development.*

Sarah Hemming**,** Dimitrios Cakouros, Andrew Zannettino Stan Gronthos.

Winner of the best PhD poster presenter ASSCR 2015.

2015 SAHMRI Research Showcase.

South Australian Health and Medical Research Institute (SAHMRI), Adelaide, Australia**.**

Poster; *Epigenetic modifiers: controlling MSC osteogenic differentiation.*

Sarah Hemming, Dimitrios Cakouros, Melissa Davis, Kate Vandyke, Stan Gronthos.

2015 ASMR Conference

National Wine Centre, Adelaide, Australia.

2015 Keystone and Transcriptional Stem Cell and Epigenetics Meeting

Keystone, Colorado, United States of America.

Poster; *Epigenetic modifiers: controlling MSC osteogenic differentiation.*

Sarah Hemming, Dimitrios Cakouros, Melissa Davis, Kate Vandyke, Stan Gronthos.

2015 Justin Ichiada's ALS Laboratory

Eli and Edythe Broad CIRM Centre for Regenerative Medicine and Stem Cell Research.

University of South California (USC), Los Angeles, USA.

Presented; *Epigenetic Modifiers and Mesenchymal Stem Cells* talk to the Broad CIRM centre.

2014 Florey post-graduate conference

National Wine Centre, Adelaide Australia.

The Faculty of Health Sciences, The University of Adelaide.

Poster; *Epigenetic modifiers: controlling MSC osteogenic differentiation.*

Sarah Hemming, Dimitrios Cakouros, Melissa Davis, Kate Vandyke, Stan Gronthos.
Awarded a Florey poster prize ($300)

2014 ASSCR

ASSCR, Mantra Lorne, Victoria, Australia.
Poster**;** *Epigenetic modifiers: controlling MSC osteogenic differentiation*.
Sarah Hemming**,** Dimitrios Cakouros, Melissa Davis, Kate Vandyke, Stan Gronthos.
Awarded the National Stem Cell Foundation of Australia Conference Education Award. Award covered the travel and registration cost to the conference.

2014 Adelaide Australian and New Zealand Cell and Developmental Biology Meeting

The University of South Australia, Adelaide, Australia.
Poster; *Epigenetic modifiers: controlling MSC osteogenic differentiation*.
Sarah Hemming, Dimitrios Cakouros, Melissa Davis, Kate Vandyke, Stan Gronthos.

2014 ANZSCDB meeting

Australia and New Zealand Society for Cell and Developmental Biology, University of South Australia, Adelaide, Australia.
Poster; *Epigenetic modifiers: controlling MSC osteogenic differentiation*. Sarah Hemming, Dimitrios Cakouros, Melissa Davis, Kate Vandyke, Stan Gronthos.

2013 ASSCR

ASSCR, Brisbane Convention Centre, Brisbane, Queensland, Australia.
Poster; *EZH2 and KDM6A act as an epigenetic switch to regulate Mesenchymal Stem Cell lineage specification***.**
Sarah Hemming, Dimitrios Cakouros, Sandra Isenmann, Lachlan Cooper, Danijela Menicanin, Andrew Zannettino, Stan Gronthos.
I was awarded the National Stem Cell Foundation of Australia Conference Education Award covering travel and registration cost of the conference**.**

2013 Centre for stem cell research (CSCR) postgraduate research day

CSCR, The National Wine Centre, Adelaide, Australia.
Provided by The Robinson Institute, the University of Adelaide.

Poster; *EZH2 and KDM6A act as an epigenetic switch to regulate Mesenchymal Stem Cell lineage specification***.**

Sarah Hemming, Dimitrios Cakouros, Sandra Isenmann, Lachlan Cooper, Danijela Menicanin, Andrew Zannettino, Stan Gronthos. I was awarded Second Place poster prize winner ($500).

2013 ANZOR meeting

ANZOR, Flinder St, Adelaide, Australia.

2013 Three minute thesis competition

Competed in the Faculty of Health Sciences heats.

Title; *Good old Bone*.

Sarah Hemming

2013 ANZSCDB meeting

Australia and New Zealand Society for Cell and Developmental Biology, University of South Australia, Adelaide, Australia

Oral presentation; *EZH2 and KDM6A act as an Epigenetic switch to regulate Mesenchymal Stem Cell Lineage Specification*. Sarah Hemming.

Awarded best PhD student oral presentation

2013 ASMR

Poster Presentation at the 2013 ASMR meeting, for the Australian Society for Medical Research at the Adelaide Convention Centre, Australia.

Poster; *The role of Ezh2 in mesenchymal stem cell growth and multi-differentiation.*

Hemming, S.E**.,** Cakouros, D. and Gronthos, S.

2012 ASSCR

Poster Presentation at the 2012 ASSCR, for the 5th Australasian Society for Stem Cell Research, Adelaide Convention centre, Australia.

Poster; *The role of Ezh2 in mesenchymal stem cell growth and multi-differentiation.*

Hemming, S.E**.,** Cakouros, D. and Gronthos, S.

2012 Health Science Post graduate conference

Poster Presentation at the 2012 Health Science Post graduate conference,
The Adelaide Wine Centre, Australia.
Poster; *The role of Ezh2 in mesenchymal stem cell growth and multi-differentiation.*
Hemming, S.E**.,** Cakouros, D. and Gronthos, S.

2012 Combio

Adelaide Convention Centre, Adelaide, Australia.
Combio containing the Australian society for biochemistry and molecular biology (ASBMB)
Poster; *The role of Ezh2 in mesenchymal stem cell growth and multi-differentiation*.
Hemming, S.E**.,** Cakouros, D. and Gronthos, S.

Accomplishments

2015-Awarded the Medicine travel grant - $5000 for overseas travel.
2014-Awarded best poster prize at Florey Post-Graduate Conference. Awarded $300.
2014-Awarded a National Stem Cell Foundation of Australia Conference Education Award covering travel and registration cost of the ASSCR conference.
2013-Awarded second place for best poster presentation for PhD Student at the Centre for Stem cell research, research day. Awarded $500.
2013-Awarded a National Stem Cell Foundation of Australia Conference Education Award covering travel and registration cost of the ASSCR conference.
2013-Awarded Best PhD oral presentation at the ANZSCDB meeting 2013, at the University of South Australia. Awarded $200.
2015-Awarded the Medicine travel grants 2015. Awarded $5000 for overseas travel.
2015-ASSCR national meeting. Awarded the ASSCR 2015 PhD poster prize $500.
2015-Medical Sciences demonstrator for first yea physiology student's semester 2.
2015-2016 South Australian ASMR committee member.
2016-ASSCR Policy, Ethics and Translation, subcommittee member.

Abstract

Epigenetic modifiers are increasingly being implicated as playing major roles in many cellular and biological processes, such as cell growth, differentiation, lifespan, self-renewal, cancer, and metastasis. Epigenetic modifying proteins such as Enhancer of Zeste homology 2 (EZH2), Lysine demethylase 6A (KDM6A) regulates chromatin structure through the addition or removal histone three lysine twenty seven (H3K27) tri methylation (me3) modification. The presence of H3K27me3 on the promoter of genes leads to the recruitment of chromatin condensation complexes, chromatin compaction and repression of genes transcription. H3K27 demethylases remove the H3K27me3 modification allowing the recruitment of activating transcriptional complexes, opening up of chromatin and gene expression. The Project is based on our initial profiling of histone methylation patterns of genes associated with differentiation and the expression of epigenetic modifying enzymes in MSC clonal populations by cDNA microarray analysis. Our initial studies on the function of EZH2 lineage commitment of human BMSC, suggests that EZH2 is a negative regulator of osteogenesis and a positive regulator of adipogenesis. However, the direct role of the H3K7me3 epigenetic modifiers EZH2 and KDM6A and or KDM6B in BMSC differentiation is unclear, illustrating the importance of determining the epigenetic signatures associated with differentiation and maintenance of MSC. Additionally, EZH2 mutations in one allele of EZH2 methyltransferase SET domain have been identified in patents with Weaver Syndrome. These patients exhibit excess bone growth, aging and mental retardation suggesting the importance of EZH2 in human bone development. Furthermore, with the current use of MSC for Phase II/III clinical trials it's important to understanding of the molecular pathways and epigenetic changes that regulate maintenance and differentiation of MSC aiding in treatment of skeletal tissue disorders/diseases. Therefore this PhD project identifies that EZH2 and KDM6A acts as a switch regulating

MSC lineage commitment. Presence of EZH2 and its H3K27me3 on osteogenic genes such as RUNX2 prevent MSC osteogenic differentiation and intern allows the progression of adipogenic differentiation of MSC. During osteogenesis we believe KDM6A play a role in removing the H3K27me3 off genes critical for osteogenic differentiation. Furthermore during osteogenic differentiation, EZH2 and its H3K27 modifications must be removed from genes such as RUNX2, ZBTB16, MX1 and FHL1 allowing the activation of these genes which are important for osteogenic differentiation. EZH2 conditional deletion in early limb bud mesenchyme reveals EZH2 plays a critical role in skeletal patterning, bone microarchitecture and remodelling.

Student declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution to Sarah Hemming 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. 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.

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***Sarah Elizabeth Hemming, Dimitrios Cakouros and Stan Gronthos. "Epigenetic regulation of mesenchymal stem cell growth and multi-potential differentiation". In: The Biology and Therapeutic Applications of Mesenchymal Cells. Ed K. Atkinson. John Wiley and Sons, Hoboken, New Jersey, USA. 2015, currently in press**

***Hemming, S. *et al.* "EZH2 and KDM6A act as an epigenetic switch to regulate mesenchymal stem cell lineage specification". *Stem cells (Dayton, Ohio)* 32, 802-815, doi:10.1002/stem.1573 (2014).**

***Hemming, S. *et al.* "Identification of novel EZH2 targets in mesenchymal stem/stromal cells which regulate osteogenesis". *Journal of Biological Chemistry. Summited November 2015*.**