EXPRESSION OF RUNT RELATED TRANSCRIPTION FACTOR 2 AND VASCULAR ENDOTHELIAL GROWTH FACTOR IN THE PULP, PERIODONTAL LIGAMENT AND ALVEOLAR BONE: AN IMMUNOHISTOCHEMICAL STUDY USING A RAT ANKYLOTIC MODEL

A thesis submitted in partial fulfilment of the requirements for the degree of

Doctor of Clinical Dentistry (Orthodontics)

Dr Trudy Ann STEWART (BDS Adelaide, BOccThy UQ)

Orthodontic Unit

School of Dentistry

Faculty of Health Science

The University of Adelaide

South Australia

AUSTRALIA

March 2013

1.2 List of Figures:

1.2.1 Literature Review:

1.2.2 Article 1:

1.2.3 Article 2:

1.2.4 Concluding Remarks:

1.3 List of Tables:

1.3.1 Article 1:

1.3.2 Article 2:

1.4 List of Abbreviations:

ABC	Avidin-biotin complex
AEC	3-Amino-9-EthylCarbazole
ALP	Alkaline phophosphatase
ARF	Activation – resorption – formation cycle
bFGF	basic fibroblast growth factor
BGN	Biglycan
BMP	Bone morphogenetic proteins
Cbfα	Core binding factor subunit alpha
Cbfß	Core binding factor subunuit beta
CNC	Cranial neural crest cells
COL1A2	Collagen, Type 1, Alpha 2
CSF-1	Colony stimulating factor 1
CY3	Cyanine 3
DAPI	4',6-diamidino-2-phenylindole
DFC	Dental follicle cells
DFSC	Dental follicle stem cells
DMP1	Dentine matrix acidic phosphoprotein
DPSC	Dental pulp stem cells
DSC	Dental stem cells
DSPP	Dentine sialophosphoprotein
EDTA	Ethylene diamine tetraacetic acid
ECM	Extracellular matrix
EGF	Epithelial growth factor
FGF	Fibroblast growth factor

FGFR	Fibroblast growth factor receptor
FZD	Frizzled receptor
GEE	Generalised estimating equations
GH	Growth Hormone
HSC	Haematopoietic stem cell
HERS	Hertwig's epithelial root sheath
HSC	Haematopoietic stem cells
IGF	Insulin-like growth factor
Ihh	Indian hedgehog
IL	Interleukin
M-CSF	Macrophage colony stimulating factor
MAPK	Mitogen-activated protein kinase cascade
MSC	Mesenchymal stem cells
MSX1/2	Muscle segment homeobox
OB	Osteoblast
Oc	Osteocyte
OC	Osteoclast
OCN	Osteocalcin
OPG	Osteoprotegerin
OSX	Osterix (SP7)
OTM	Orthodontic tooth movement
Pax	Paired box gene
PBS	Phosphate buffered saline
PDGF	Platelet derived growth factor
PDL	Periodontal ligament

PDLSC	Periodontal ligament stem cells
PFE	Primary failure of eruption
PIGF	Placental growth factor
PTH	Parathyroid hormone
PTH1R	Parathyroid hormone receptor gene 1
PTHrP	Parathyroid hormone related peptide
RANK	Receptor activator of nuclear factor kappa-ß
RANKL	RANK ligand
Runx2	Runt related transcription factor 2
SCAP	Stem cells from apical papilla
SHED	Stem cells from human exfoliated deciduous teeth
Shh	Sonic hedgehog
SP7	C2H2 type zinc finger transcription factor (osterix)
TGF-ß	Transforming growth factor-beta
TNF-α	Tumour necrosis factor-alpha
VEGF	Vascular endothelial growth factor
Wnt	Wingless signalling pathway

Measure of Length

mm	millimetre
µm	micrometre

Measure of Volume

ml	millilitre
µl	microlitre

Measure of Weight

µg	microgram
mg	milligram
g	gram
kg	kilogram
kDa	kiloDalton
mw	molecular weight
N	newton

2. ACKNOWLEDGEMENTS

I wish to express my sincere appreciation to the following people for their support in the completion of this thesis:

Professor W. J. Sampson, P.R. Begg Chair in Orthodontics, The University of Adelaide, for his generosity with his time as well as his expert advice and guidance.

Associate Professor C. W. Dreyer, Senior Lecturer in Orthodontics, The University of Adelaide, for his readily available support as well as the use of research books and materials.

Dr Kencana Dharmapatni, School of Medical Sciences, The University of Adelaide, for her expert advice regarding immunohistochemistry, allowing the use of her laboratory for this project, assistance with developing the staining protocols and generous donation of various tissue blocks. Her dedication and enthusiasm for research is truly an inspiration.

Mr Yen Liu, Division of Population Oral Health, The University of Adelaide, for his expert statistical help.

Mr Jim Manavis, Laboratory Manager, Hanson Institute Centre for Neurological Diseases for teaching me how to use the Nanozoomer Digital Microscope

Last but not least, I must thank my wonderful husband David. Your tolerance, support and proofreading is simply amazing and it has made all the hard work worthwhile.

3. THESIS DECLARATION

This thesis contains no material that has been accepted for the award of any other degree or diploma in any other university or 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 for any degree or diploma in any university or other tertiary institution without prior approval of the University of Adelaide.

I give consent for this copy of my thesis, when deposited in the University of Adelaide Library, to be 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 via the University of Adelaide's digital research repository, the University of Adelaide Library catalogue and also through internet search engines, unless permission has been granted by the University of Adelaide to restrict access for a period of time

_____

Dr Trudy Stewart

13th March 2013.

4. ABSTRACT

The current study investigated the expression Runx2 and VEGF in the pulp, periodontal ligament and alveolar bone following hypothermal insult.

Methods and Materials:

Materials from a previous study performed by Tan (2011) were used for this research. The upper right first molars of fifteen eight-week-old male Sprague-Dawley rats were subjected to a single ten minute application of dry ice. The contralateral molar acted as an internal control. The animals were randomly divided into five groups of three and killed 0, 4, 7, 14 and 28 days post hypothermal insult. A further three Sprague-Dawley rats acted as an external control and were humanely killed on day 0 with no hypothermal insult. The maxilla was dissected out, fixed and embedded in paraffin. Coronal sections were cut to include the control and experimental teeth at 5-micron intervals through the furcation region. Sections were then stained with haematoxylin and eosin (H and E) and Runx2 and VEGF immunostains.

Sections were scanned via a Nanozoomer Slide Scanner 2.0 series and viewed on a personal computer (MacBook Pro with 13 inch screen) using the Nanozoomer Digital Pathology (NDP) software. Semiquantitative counting was performed at a magnification of x20 via the ImageJ software. Data was analysed using SAS 9.3 (SAS Institute Inc., Cary, NC, USA). The level of significance was set at $p<0.05$.

Results:

H and E stained sections indicated that ankylosis had developed on the experimental side at days 7 and 14 and one of the three rats at day 28 post hypothermal insult. No ankylosis was present on any control teeth or experimental teeth at days 0 and 4. Disturbance to the pulpal tissues was also noted.

A number of cells stained positively to the Runx2 and VEGF immunostains. Vascular endothelial cells, bone cells (osteoblasts / osteocytes / bone lining cells), osteoclast-like cells, periodontal ligament cells (fibroblasts, epithelial cell rests of Malassez (ERM)) and bone marrow cells in both experimental and control animals were positive for VEGF. Increased staining intensity for VEGF was noted particularly associated with blood vessels and adjacent to regions of ankylosis. Fibroblast-like cells in the pulp and PDL, osteoclasts in resorption lacunae along the PDL, bone lining cells, osteoblasts/osteocyte like cells, cemental cells, odontoblasts, epithelial cell rests of Malassez (ERM), megakaryocytes and vascular endothelial cells were all found to be positive for Runx2 in both the treated and untreated groups.

Statistically significant differences in the percentage of Runx2 positive cells between treated and untreated molar teeth were found in the pulp and alveolar bone but no statistically significant difference was found in the PDL. Although not statistically significant, trends of changing Runx2 expression with time were noted in the pulp and alveolar bone. Runx2 expression increased at days 4, 7 and decreased at days 14 and 28 in the pulp whilst in the alveolar bone expression increased at day 4, decreased at days 7 and 14 and slightly increased at day 28.

In the pulp, a statistically significant difference was found between VEGF positive cells on the experimental side compared to the internal control side, with more VEGF positive cells on the experimental side at day 7 than at days 14 and 28. In the alveolar bone and PDL, although a statistically significant difference was found between the experimental and control sides, there was no significant interaction with time. However, VEGF positive cells appeared to be fewer in the PDL at days 4, 7 and 14 and greater at day 28. In the alveolar bone, more VEGF positive cells were seen at day 4 than at days 7, 14 and 28.

Conclusions:

1) Runx2 and VEGF was expressed by a number of cells within the rat dentoalveolus.

2) When compared to the control groups, changes in Runx2 expression were found in the experimental pulp and alveolar bone, but not in the PDL. Changes in VEGF expression were found in the experimental pulp, PDL and alveolar bone.

3) Changes in Runx2 and VEGF expression also occurred between the internal and external control groups, suggesting that a localised insult may lead to a systemic impact.

4) Post-hypothermal insult, Runx2 and VEGF may play an important role in the development of bony ankylosis.

The null hypothesis, that the expression of Runx2 and VEGF in the rat dentoalveolus does not differ post-hypothermal insult, is rejected.