CHANGING THE FACE OF CRANIOSYNOSTOSIS

The role of RBP4 in osteogenesis and suture fusion

Victoria Dawn Leitch
Bachelor of Science (Biomedical Science), Bachelor of Science (Honours)

A thesis submitted for the degree of Doctor of Philosophy in Medicine
School of Paediatrics and Reproductive Health
Faculty of Health Sciences
University of Adelaide
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ABSTRACT

Craniosynostosis is the premature fusion of cranial sutures and results in the compensatory malformation of the skull to accommodate the rapid growth of the brain during early childhood. This PhD thesis aims to look at the molecular mechanisms at play during this premature fusion; in particular it follows on from a recent microarray study of craniosynostosis tissue conducted in this laboratory. This study showed a 37x down regulation of RBP4 in fused sutures in humans. RBP4 is a retinol binding protein whose function is to transport retinol in the blood to target tissue, where it is metabolised to retinoic acid. This is of interest as retinoic acid is known to have an influence on bone growth. In this PhD project we have used animal and cell culture models to assess the levels of RBP4 during suture fusion and osteogenesis and its possible role in this process.

Expression of Rbp4, Stra6 and other markers of osteogenesis were assessed using quantitative PCR in a mouse model of Saethre-Chotzen craniosynostosis syndrome (Twist1+/−). This demonstrated an initial correlation between suture fusion and Rbp4 down regulation as well as an inverse relationship between Rbp4 and Stra6 expression. However, sutures that did not fuse and parietal bone also displayed downregulation of Rbp4 at later timepoints. Histology showed that this might be related to parietal bone thickening. Multiple cell culture models were trialed, but proved unsuitable for RBP4 studies in osteogenesis. The commonly used mouse pre-osteoblastic cell line, MC3T3-E1, mineralised but did not express Rbp4. Primary coronal suture cells were isolated from mice, which expressed Rbp4, but failed to mineralise. Subsequently, primary cell cultures from human sutures were tested in osteogenesis assays and showed a decrease in RBP4 levels during mineralisation.
Immunocytochemistry was used to determine the localisation of RBP4 in suture cells compared to Huh7 cells, a liver carcinoma cell line with known secretion of RBP4. Results showed that RBP4 is localised to the endoplasmic reticulum in suture cells, differing to the localization seen in Huh7 cells. Western blot analysis also demonstrated that unlike liver cells, human suture cells do not secrete detectable levels of RBP4. Finally, functional studies to analyse the role of RBP4 in osteogenesis using a lentiviral delivery system for over expression of RBP4 showed no effect on the ability of human suture cells to mineralise. A high level of overexpression was achieved however there were issues with infection efficiency which may have affected the outcome of these experiments.

These studies demonstrate some unique characteristics of RBP4 in suture cells and extend its role beyond a simple serum transporter of retinol. In addition to a role in suture fusion, these results could be a reflection of a broader function of RBP4 in normal bone growth and osteogenesis.

**KEYWORDS:** RBP4, retinoic acid, Saethre-Chotzen, suture fusion, craniosynostosis, osteogenesis.
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 Victoria Dawn Leitch 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.

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Signed: __________________________

Victoria D. Leitch

Date: __________________________
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**Australian Rotary Health**

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# ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>α-MEM</td>
<td>minimal essential media alpha modification</td>
</tr>
<tr>
<td>μl</td>
<td>microlitre</td>
</tr>
<tr>
<td>μm</td>
<td>micrometre</td>
</tr>
<tr>
<td>μM</td>
<td>micromolar</td>
</tr>
<tr>
<td>+/-</td>
<td>heterozygous knockout</td>
</tr>
<tr>
<td>-/-</td>
<td>homozygous knockout</td>
</tr>
<tr>
<td>3D</td>
<td>three dimensional</td>
</tr>
<tr>
<td>°C</td>
<td>degrees celsius</td>
</tr>
<tr>
<td>A (nucleic)</td>
<td>adenine</td>
</tr>
<tr>
<td>A (amino acid)</td>
<td>alanine</td>
</tr>
<tr>
<td>ALP</td>
<td>alkaline phosphatase</td>
</tr>
<tr>
<td>Arg</td>
<td>arginine</td>
</tr>
<tr>
<td>ATCC</td>
<td>American type culture collection</td>
</tr>
<tr>
<td>BCO</td>
<td>bone chip outgrowth</td>
</tr>
<tr>
<td>BMP</td>
<td>bone morphogenetic protein</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>C (nucleic)</td>
<td>cytosine</td>
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<tr>
<td>C (amino acid)</td>
<td>cysteine</td>
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<tr>
<td>CANX</td>
<td>calnexin</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>cm</td>
<td>centimetre</td>
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<tr>
<td>CO2</td>
<td>carbon dioxide</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>CRABP</td>
<td>cellular retinoic acid binding protein</td>
</tr>
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<td>CRBP</td>
<td>cellular retinoid binding protein</td>
</tr>
<tr>
<td>Ct</td>
<td>cycle threshold</td>
</tr>
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<td>CT</td>
<td>computed tomography</td>
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<tr>
<td>CYPA</td>
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<td>DAPI</td>
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<td>DEPC</td>
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<td>DMEM</td>
<td>Dulbecco's modified eagle medium</td>
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<tr>
<td>FACS</td>
<td>fluorescence-activated cell sorting</td>
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<td>fetal bovine serum</td>
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<tr>
<td>FD</td>
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<td>fibroblast growth factor</td>
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<td>fibroblast growth factor receptor</td>
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g grams
G (nucleic) guanine
G (amino acid) glycine
G (lentiviral vector) phCMV-G
gagpol phCMV-gagpol
GFP green fluorescent protein
H histidine
H&E haematoxylin and eosin
HCl hydrochloric acid
HPLC high pressure/performance liquid chromatography
HPRT hypoxanthine-guanine phosphoribosyltransferase
hRBP4 human RBP4
HRP horse radish peroxidase
I isoleucine
H₂O water
IgG immunoglobulin
IHH Indian hedgehog
iLBP intracellular lipid binding protein
IRES internal ribosome entry site
kb kilobase
LB broth Luria Bertani broth
LN2 liquid nitrogen
LV lentivirus
M molar (concentration)
M (amino acid)  methionine
MgCl₂  magnesium chloride
microCT  Microcomputerised tomography
ml  millilitre
mM  millimolar (concentration)
MSX2  muscle segment homeobox 2
mRNA  messenger RNA
NaCl  sodium chloride
ng  nanograms
nM  nanomolar (concentration)
OC  osteocalcin
OG  osteogenic
O/N  overnight
P  proline
PBS  phosphate buffered saline
PCR  polymerase chain reaction
PD  partial digestion
pen/strep  penicillin and streptomycin
PF  posterior frontal
PFA  paraformaldehyde
Pro  proline
Q  glutamine
R  arginine
RA  retinoic acid
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<td>RARE</td>
<td>retinoic acid response element</td>
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<tr>
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<td>T (amino acid)</td>
<td>threonine</td>
</tr>
<tr>
<td>Tat</td>
<td>pcDNA3.1Tat</td>
</tr>
<tr>
<td>TBS</td>
<td>tris buffered saline</td>
</tr>
<tr>
<td>TGF-β</td>
<td>transforming growth factor β</td>
</tr>
<tr>
<td>Tris</td>
<td>tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>TTBS</td>
<td>tween20 tris buffered saline</td>
</tr>
<tr>
<td>TTR</td>
<td>transthyretin</td>
</tr>
<tr>
<td>V (amino acid)</td>
<td>valine</td>
</tr>
</tbody>
</table>
V  volts
v/v  volume per volume
W  tryptophan
WCHRI  Women’s and Children’s Health Research Institute
WT  wildtype
w/v  weight per volume
Y  tyrosine
Chapter 1

General Literature Review
1.1 INTRODUCTION

Craniosynostosis is a pathological condition resulting from premature fusion of cranial sutures. It is a relatively common medical condition with approximately 1 in 2500 live births being affected. Patients have cranial suture fusion which may be accompanied by a number of other developmental abnormalities or as part of a syndrome. The Women’s and Children’s Hospital in Adelaide admitted 40 patients for craniosynostosis surgeries in the 2005/06 financial year incurring significant cost to the Australian health care system. More important than financial concerns however, is the effect on the development of the child. The current treatment is life threatening transcranial surgery and some children with craniosynostosis will require multiple hospital visits, repeated operations, and ongoing observation to correct the resultant malformations arising from premature fusion of sutures. No cure for craniosynostosis is available. In order to improve quality of life for patients with less intrusive treatments, an improved understanding of the process of normal suture fusion and the disturbances to this process that result in premature fusion must be developed.

This introduction will discuss the current state of knowledge of the process of craniosynostosis and highlight deficiencies in this knowledge. In particular it will provide background information on syndromic and non-syndromic craniosynostosis and the possible genetic mechanisms at play. The role of retinoic acid (RA) in osteogenesis and the potential role of retinol binding protein 4 (RBP4) in craniosynostosis will also be discussed.
1.2 BONE

The skeleton, made up of bone, provides both structure for the body and protection for internal organs. Bone is a living tissue with the ability to remodel and regenerate in response to injury and for everyday maintenance (Manolagas, 1999). In humans it is estimated that the skeleton is completely replaced every 10 years by this process of remodelling (Parfitt, 1994, Alman et al., 2011). The ability of bone to behave in this manner is dependent on the balance between the different cell types located in bone.

Bone is comprised of numerous different cells including osteoprogenitor cells, preosteoblasts, osteoblasts, osteocytes and osteoclasts, as well as extracellular bone matrix (Sherwood et al., 2001). Osteoprogenitor cells are multipotent stem cells that have been directed to a bone cell fate. These osteoprogenitor cells differentiate into osteoblasts, which are responsible for the synthesis of bone matrix components, such as collagen and proteoglycans. After becoming trapped in newly formed bone, osteoblasts become osteocytes, mature bone cells which are the most commonly found cell type in cortical bone (Plotkin et al., 2005). Although unable to divide mitotically like osteoblasts and progenitor cells, osteocytes are still capable of some bone maintenance (Barragan-Adjemian et al., 2006) often in response to mechanical strain (Lanyon, 1993, Aarden et al., 1994) and damage (Verborgt et al., 2000). Osteocytes have also been implicated in the control of osteoclasts (Tanaka et al., 1995, Kogianni et al., 2008).

A distinct transition between these stages of differentiation in bone cells is difficult to define. Many different potential morphological and biochemical markers of the different stages of differentiation have been identified. Morphologically, osteoblasts appear to be a relatively
larger and cuboidal cell whereas osteocytes are generally smaller, have larger sized nuclei and multiple cytoplasmic extensions (Sommerfeldt and Rubin, 2001, Gupta et al., 2010) (Figure 1.1). Biochemically, the acquisition of osteocalcin (OC) is a universally accepted marker of a fully differentiated osteoblast (Desbois and Karsenty, 1995, Ducy, 2000, Yamaguchi et al., 2000), making it a late marker of differentiation (Morike et al., 1995, Garcia et al., 2002). Induction of alkaline phosphatase (ALP) activity is also a commonly used marker (Butler, 1989, Napoli, 1996, Yamaguchi et al., 2000). In contrast to OC, ALP is an early marker of osteoblast differentiation, with higher expression seen in younger cells (Morike et al., 1995, Garcia et al., 2002) (Figure 1.1). Other markers of the transition from osteoprogenitor to osteoblast cells are bone morphogenetic proteins (BMPs) (Yamamoto et al., 2002), collagen 1 (Gupta et al., 2010), fibroblast growth factor receptor 1 (FGFR1) (Liu et al., 2003), FGFR2 (Iseki et al., 1999), Msx2 (Alappat et al., 2003) and Runx2 (Nacamuli et al., 2002).

Unlike osteocytes and osteoblasts, osteoclasts are derived from a monocyte/macrophage lineage and their main function is to resorb the surface of bone into its constituent minerals using acid filled vesicles. An in-depth review on the role of osteoclasts has been published by Li (Li et al., 2006). Overproduction of osteoclasts leads to excess bone degradation resulting in the imbalance of bone homeostasis as seen in conditions such as osteoporosis (Krane, 2005). Hence a balance between all cell types is required to maintain healthy bone and skeleton.

The signalling events that initiate and maintain these stages of osteogenic differentiation are also of great interest to bone biologists. Growth factors such as fibroblast growth factors (Marie, 2003, Gupta et al., 2010), BMPs (Yamaguchi et al., 2000)
Figure 1.1: The osteoblast lineage and the commonly used markers of their differentiation.

Adapted from Alberts (Alberts et al., 2002).
and parathyroid hormone (Shih, 2004), and minerals and vitamins such as calcium (Zayzafoon, 2006), vitamin D (van Driel M et al., 2004), Runx2 (Lian et al., 2004) and inorganic phosphate (Beck, 2003) all play a role in osteoblast differentiation.

Additionally, differentiation has also been attributed to mechanical factors (Smartt et al., 2007). Recently, reports have also suggested that the origin of osteoprogenitor cells may have an influence on the efficiency of this differentiation (Aghaloo et al., 2010). Mature bone is comprised of approximately 70% minerals and 30% organic matrix (Boivin and Meunier, 2003). The skeleton is the body’s main repository for mineral storage and is referred to as the bone matrix. Mineralisation involves the deposition of mineral crystals onto the matrix secreted by osteoblasts. The main constituent of bone matrix is calcium hydroxyapatite (up to 80%) (Li et al., 2006), but it also contains phosphate, sodium and magnesium (Kartsogiannis and Ng, 2004). A primary deposition of minerals occurs first, followed by a slower secondary increase in mineral levels (Follet et al., 2004), a process controlled in part by ALP (Orimo, 2010).

1.2.1 CRANIOGENESIS

The genesis of the human skull is a complex process beginning at approximately 8 weeks gestation. A full account can be found in “Craniofacial Development” (Sperber and Sperber, 2001) but key events in skull development are discussed here.

The skull is thought to develop in two distinct areas, the cranial base and the calvaria (Gruber
and Brockmeyer, 2003). The cranial base, or viscerocranium, arises from both the ectodermal neural crest and mesoderm (McBratney-Owen et al., 2008). In this process, multiple distinct foci of cartilage form and ultimately create the viscerocranium by endochondral ossification. This process, also involved in long bone formation, requires a cartilage template which is subsequently invaded by osteoclasts and osteoblasts, resulting in bone formation. This cartilage template is produced by chondrocytes, a cell type not commonly recognised in the calvaria.

The neurocranium, or calvaria, consists of the occipital, parietal and frontal bones. These arise from a mesodermal lineage and here the bone develops by intramembranous ossification (Rana, 1984). Formation of these bones occurs as osteoblasts secrete matrix directly onto the desired area, independently of a cartilaginous template (Rice et al., 2003). Extracellular matrix, particularly collagen type 1 and proteoglycans (Cole and Hanley, 1991) is mineralised and rapid ossification at the edges of the new cranial bones occurs (Alberius and Johnell, 1990), allowing outgrowth of these bones. The presence of the brain is required for this growth, with examples of deficient calvarial growth when the brain is absent (Bianca et al., 2005, Koukoura et al., 2006). Growth of these bones occurs with ossification beginning from a ossification centre allowing bone to grow radially. These ossification centres form at approximately 8 weeks post conception in the case of parietal and frontal bone and form the basis of growth of these bones (Sperber and Sperber, 2001). The fibrous regions separating these bones, and containing the growth fronts, arise from 2 specific lineages of cells. In particular the sagittal, lambdoid and coronal sutures derive from the interface between the neural crest and mesoderm cells; and the metopic suture is derived from neural crest alone (Morriss-Kay and Wilkie, 2005).
The study of the embryogenesis of the skull is highly reliant on animal models, due to the inherent ethical issues of using human tissue for this analysis. Although these models provide useful insights into the development of the skull, it is important to remember that there are also some species specific differences during craniofacial growth and that this may be reflected during early craniogenesis. For example, varying origins of the cells that develop into the cranial vault in mice (mesoderm and neural crest) and avian (neural crest alone) models can cause confusion (Couly et al., 1993, Jiang et al., 2002).

Despite widespread acceptance of calvarial development by intramembranous ossification, there have been reports that challenge this dogma. The presence of cartilage at the growth sites of cranial bones, suggests a possible role for endochondral bone formation at these sites. Cartilage has been identified in human lambdoid sutures (Coussens et al., 2007), human metopic suture (Manzanares et al., 1988), mouse coronal sutures in a model of Apert syndrome (Wang et al., 2005), mouse posterior frontal sutures (Sahar et al., 2005) and in the sagittal suture of $Axin2$ knockout mice (Maruyama et al., 2010). Cartilage specific markers have also been reported in human sagittal and lambdoid sutures (Coussens et al., 2007) and rat coronal sutures (Alberius and Johnell, 1990). It has also been well characterised that calvarial cells from mice can undergo differentiation to either osteoblasts or cartilage secreting chondrocytes under induction in vitro (Aberg et al., 2005). It remains to be seen whether this cartilage stage of growth is a less-recognised later stage of normal cranial development, or whether this may be a key factor in disorders of excess bone growth such as craniosynostosis.

Between the growing calvaria are areas of connective tissue, which allow for the rapid brain growth experienced in childhood. Four main fontanelles are present at birth, the anterior,
posterior and two lateral, but these areas are generally no longer evident by 2 years of age (Tunnessen, 1990). Fontanelles are present at the intersection of the other fibrous regions of tissue in the calvaria, sutures. The location of these fontanelles and sutures can be seen in Figure 1.2.

1.2.2 SUTURES

Sutures are the fibrous connections between the bones of the skull. There are normally 6 cranial sutures in the newborn human skull (two coronals, a sagittal, a metopic and two lambdoids). Many animal skulls also contain these, or similar sutures, allowing them to be used as an in vivo model of suture development (Figure 1.3).

Cranial sutures have multiple functions. These include allowing for the rapid bone growth required to accommodate the increase in size experienced by the brain during childhood (Baer, 1954). Sutures also allow the movement of the calvarial bones to permit passage through the birth canal (Warren and Longaker, 2001b) and may help to cushion common “playground” impacts which most children encounter in early life. To function, sutures must remain patent; i.e. maintain active bone growth without fusion. Excluding the metopic suture, which closes shortly after birth, the remaining sutures stay patent until early adulthood (Cohen and Maclean, 2001). The metopic suture is analogous to the rodent posterior frontal suture, which fuses within the first 2 months in the life of a rodent (Song et al., 2004b).
Figure 1.2: Diagrammatical representation of the locations of sutures, fontanelles and bone in the human neonate. Adapted from (Verklan and Walden, 2004).
Figure 1.3: Diagrammatical comparison of the location of sutures in human (left) and murine (right) skull. Adapted from Jackson Laboratories (www.jax.org).
The different sutures arise from different developmental origins. In mouse, the posterior frontal and sagittal sutures are resultant of cells from the neural crest (Behr et al., 2010). The coronal suture has been shown to arise from the mesoderm and due to its location is an important barrier between the mesoderm derived parietal bone and the neural crest derived frontal bone (Jiang et al., 2002). The different developmental origins of the sutures may be a reason for differing rates of abnormal suture fusion, however this does raise the question of the posterior frontal and the differences in fusion between it and the sagittal suture, of the same origin. This may indicate that the origin of the suture alone can not dictate its fate.

1.3 CRANIOSYNOSTOSIS

Scientists and clinicians have debated the link between fusion of cranial sutures and malformation of the skull for well over two centuries. In 1791, an association was made between premature suture fusion and skull malformation (Sommering, 1791) and by 1852 the term craniostenosis was widely used and accepted as the condition resulting from premature fusion of sutures (Virchow, 1851).

The suture is heterogeneous, consisting of many different cell types. The majority of the cells in the suture are derived from the multipotent osteoprogenitor cell lineage, and these will be at different stages of differentiation. The main structures present at the suture are dura mater, bone, osteogenic fronts, mesenchyme and the pericranium (Warren and Longaker, 2001b) (Figure 1.4). The osteogenic fronts of bones in sutures are the proliferatively active areas where bone growth occurs (Decker and Hall, 1985).
Figure 1.4: Diagram demonstrating different cell types present in a suture. (a) MicroCT image demonstrating a human fusing and unfused suture. The scale bar represents 1mm. (Coussens, 2007). (b) Stylised diagram demonstrating type and location of cells in an unfused suture. Adapted from Liu (Liu et al., 1995).
This bone growth is balanced by apoptosis of osteoblasts at these fronts (Furtwangler et al., 1985). The dura mater, a thick layer of collagenous membrane containing nerves and blood vessels, was originally thought to only be a protective layer for the brain. However, studies have demonstrated the dura’s ability to express osteogenic factors (Bradley et al., 1997, Greenwald et al., 2000a) (see Section 1.5.1). Premature fusion of the osteogenic fronts across a suture interferes with growth and causes abnormal compensatory mechanisms to begin in the skull (Virchow, 1851, Enlow, 1986). This premature fusion of sutures is identified as Craniosynostosis.

The premature fusion of sutures in the skull prevents the normal development of the growth fronts of the calvarial bones. The inability of the skull to accommodate the growing brain can result in mental retardation, increased intracranial pressure, blindness and deafness (Warren and Longaker, 2001b). The continued growth of the brain causes craniofacial deformities. The extent of cranial malformation and the secondary effects of fusion depend on the number and type of the sutures affected, and the timing of the fusion. Craniosynostosis can be classified as either syndromic or non-syndromic, in addition to other terms describing the nature of the fusion, as listed in Table 1.1. Research is constantly being undertaken to elucidate the possible causes of craniosynostosis. Despite this, the causes remain largely unknown and are poorly understood.

1.3.1 SUTURE-SPECIFIC OBSERVATIONS

The sagittal suture is the most commonly affected suture, accounting for about 40% of cases,
Table 1.1: Table of common terms classifying craniosynostosis

<table>
<thead>
<tr>
<th>TERM</th>
<th>DEFINITION</th>
</tr>
</thead>
<tbody>
<tr>
<td>Simple</td>
<td>Involving fusion of only one suture</td>
</tr>
<tr>
<td>Compound</td>
<td>Involving fusion of two or more sutures</td>
</tr>
<tr>
<td>Syndromic</td>
<td>Suture fusion occurring as a result of a underlying cause (syndrome), generally accompanied by other abnormalities</td>
</tr>
<tr>
<td>Non-syndromic/Isolated</td>
<td>Suture fusion occurring independently from abnormalities and from an unknown cause</td>
</tr>
<tr>
<td>Primary</td>
<td>A simple or compound suture fusion</td>
</tr>
<tr>
<td>Secondary</td>
<td>A suture fusion occurring secondary to a known disorder</td>
</tr>
</tbody>
</table>
or approximately 1 in 4000 live births (Lajeunie et al., 1996). The sagittal suture is an end-to-end suture and as fusion prevents the lateral growth of the head, children present with elongated, narrow skulls. As the sagittal is a relatively long suture, fusion may not always affect the whole length, with anterior or posterior fusion along partial lengths also having an effect on craniofacial morphology (Jane et al., 2000). Current opinion in relation to surgical intervention is divided (Fearon et al., 2006) with a study suggesting a reduced need for surgery as a result of normal social, behavioural and academic development in untreated children (Boltshauser et al., 2003). However more recent reports have suggested that sagittal synostosis may affect intracranial volume and therefore retard brain development (Terner et al., 2011) supporting previous findings which also suggested a need for surgery to aid neurological development in these patients (Magge et al., 2002).

Coronal synostosis may involve one (unilateral) or both (bilateral) coronal sutures, although bilateral cases are more commonly found in cases of syndromic craniosynostosis. It is one of the more commonly affected sutures, seen in up to 30% of cases (Cohen, 1980). The morphology of the resultant malformation is dependent on whether the synostosis is unilateral or bilateral. Fusion of the coronal suture can have devastating effects on the appearance of a patient, in particular ipsilateral frontal plagiocephaly and nasal deviation (Barone and Jimenez, 2004). The timing and method of surgical treatment is controversial in cases of coronal craniosynostosis (Marchac, 1987, Bartlett et al., 1990). However, unlike sagittal synostosis, there is not a well defined link between intracranial volume and coronal synostosis (Hill et al., 2011).

The metopic suture is interesting as it naturally fuses considerably earlier than other sutures in
the skull (Manzanares et al., 1988). Fusion has usually occurred by 3 years of age in humans (Weinzweig et al., 2003) although there are cases of metopic sutures remaining patent into adulthood (Bademci et al., 2007). There appears to be an ethnic correlation with these cases of long term metopic patency (Ajmani et al., 1983, Bilodi et al., 2004). Premature fusion of the metopic suture accounts for approximately 10% of all cases of craniosynostosis. The result is ridging of the nasal bridge, narrow forehead, hypotelorism (Eppley and Sadove, 1994) and a characteristic triangle shaped head, referred to as trigonocephaly. Surgery to correct metopic craniosynostosis is almost always successful (Collmann et al., 1996). Links have been identified between metopic synostosis and cognitive and behavioural problems in affected children and although the studies looking at this phenomenon have shown a significant link (Sidoti et al., 1996) the relationship requires clarification. A well documented side effect of craniosynostosis (particularly syndromic) is sleep apnea (Mitsukawa et al., 2004, Bannink et al., 2010). The link between obstructive sleep disorders and behavioural and cognitive problems in children has been well established and more recently this has been linked with craniosynostosis (Bannink et al., 2010). This may indicate that learning difficulties are a secondary side effect of the breathing difficulty rather than a direct result of the suture fusion.

The lambdoid suture is the suture most rarely affected by the process of craniosynostosis, with reported incidence of 2-5% of cases (Losee and Mason, 2005), however the incidence is significantly less in patients treated at the Australian Craniofacial Unit (personal communication, A/Prof Peter Anderson). Like the coronal, craniosynostosis can also involve one or both of the lambdoid sutures (unilateral or bilateral). It results in a trapezoid shaped head, ipsilateral ear movement (Helms et al., 1997) craniofacial asymmetry and occipital
flattening (Smartt et al., 2007). Lambdoid synostosis may often be confused with deformities related to positional deformational plagioccephaly (Wu et al., 1996). The best way to truly distinguish between lambdoid synostosis and plagioccephaly is computed tomography (CT) scanning. Although rare, malformations from this type of synostosis can be very severe (Leboucq et al., 1993). Lambdoid synostosis is generally non-syndromic but, although no genetic factors have been identified, there have been reports of familial lambdoid synostosis (Kadlub et al., 2008).

In craniosynostosis, any or all of the cranial sutures may be affected with varying frequency, and all result in distinct malformations. Examples of some resultant skull shapes can be seen in Figure 1.5. Estimates of incidence vary greatly in the literature (sagittal synostosis up to 60% and coronal as low as 20%) (Kabbani, 2004). When comparing incidences, the sample size and possibility of one family with an inherited genetic craniosynostosis-causing mutation biasing statistics in an area must be considered. The differing incidences of the types of suture fusion may also indicate that fusion may occur in a suture-specific fashion. Further research to elucidate the possibilities of differences in the mechanisms of fusion are warranted, particularly to compare the metopic with the other sutures, due to it’s natural pattern of fusion during seemingly early human life.

1.3.2 NON-SYNDROMIC CRANIOSYNOSTOSIS

The most common form of craniosynostosis is non-syndromic (Warren and Longaker, 2001b) accounting for approximately 80% of reports (Ferreira et al., 2006). Despite the higher
Figure 1.5: Examples of synostoses of the different sutures. a and b) demonstrates sagittal synostosis, c and d) demonstrates metopic synostosis, e and f) demonstrates coronal synostosis and g and h) demonstrates lambdoid synostosis. Arrows in pictures indicate the area of fused suture. Images are adapted from (Aldridge et al., 1994, Benson et al., 1996, Boyle and Rosenblum, 1997, Sze et al., 2005).
incidence the aetiology of these cases is the least understood. Non-syndromic craniosynostosis involves the fusion of a suture, independent of other abnormalities or syndromes. The causes remain unknown, but genetics, teratogens and mechanical factors have all been implicated (Speltz et al., 2004).

Although genetic mutations are often well characterised in syndromic craniosynostosis (see Section 1.7), the genetic mutations that have been found in non-syndromic craniosynostosis are less defined.

Mutations in *TWIST1* have been found in non-syndromic sagittal, metopic, (Boyadjiev, 2007) and coronal synostosis (Seto et al., 2007, Boyadjiev, 2007). An *FGFR1* mutation has been identified in two cases of non-syndromic craniosynostosis (Kress et al., 2000) and mutations in *FGFR2* have been found in non-syndromic sagittal craniosynostosis (Boyadjiev, 2007). An *FGFR3* mutation has also been reported in cases of non-syndromic craniosynostosis (Golla et al., 1997, Tsai et al., 2005). A mutation in the *EFNA4* gene, important in ephrin signalling, has been reported in 3 patients with non-syndromic craniosynostosis (Merrill et al., 2006). Recently, 5 different mutations have also been identified in insulin-like growth factor 1 receptor in cases of non-syndromic craniosynostosis- a gene not yet linked to syndromic craniosynostosis (Cunningham et al., 2011). It is highly likely that other mutations exist that are yet to be identified and these could be contributing to the cases that cannot be attributed to a known mutation.

Despite these reports, a large number of patients that present with non-syndromic craniosynostosis do not test positive for these genetic abnormalities (Boyadjiev, 2007,
Anderson et al., 2007). Diagnosis is often difficult because of the large overlap and variance of phenotypic abnormalities in syndromes. This can make diagnosis of non-syndromic versus syndromic craniosynostosis complicated. There are recorded cases of craniosynostosis which were initially reported as non-syndromic but later re-evaluated as syndromic synostosis (Bellus et al., 1996, Muenke et al., 1997).

In Adelaide, Australia, genetic testing is performed on patients with suspected non-syndromic craniosynostosis for TWIST1, FGFR1-exon7, FGFR2-exons 8,10,11, FGFR3-exons 7,10 (Anderson et al., 2007).

1.3.3 SYNDROMIC CRANIOSYNOSTOSIS

Syndromic craniosynostosis is rarer than non-syndromic craniosynostosis. It accounts for only approximately 20% of reported cases. Syndromic craniosynostosis is fusion that occurs as a symptom of a condition or disorder and can often be attributed to an underlying genetic anomaly. Currently at least 150 genetic syndromes with craniosynostosis have been classified (Schell et al., 1995). The varied genotypes and ranging phenotypes mirror the generally poorly understood nature of syndromic craniosynostosis. Genes affected in the known underlying mutations and associated phenotypes of the major syndromic craniosynostosis are listed in Table 1.2 and discussed in more depth in Section 1.7.

Cases of syndromic craniosynostosis are often accompanied by skeletal and limb abnormalities. All Apert’s patients present with syndactyly of the limbs
Table 1.2: Table of craniosynostotic syndromes

<table>
<thead>
<tr>
<th>Syndrome</th>
<th>Incidence</th>
<th>Recognised</th>
<th>Gene mutated</th>
<th>Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apert Syndrome</td>
<td>1 in 16-20,000</td>
<td>1906 (Apert, 1907)</td>
<td>FGFR2</td>
<td>Suture fusion, high prominent forehead, syndactyly of digits, depressed nasal bridge, midface hypoplasia, ocular hypertelorism, narrow palate, teeth crowding.</td>
</tr>
<tr>
<td>Saethre-Chotzen syndrome</td>
<td>1 in 25-50,000</td>
<td>1931-2 (Chotzen, 1932)</td>
<td>TWIST1</td>
<td>Suture fusion, craniofacial and limb abnormalities, facial asymmetry, brachydactyly, low frontal hairline, ptosis, small ears, broad nasal bridge</td>
</tr>
<tr>
<td>Boston type craniosynostosis</td>
<td>1 in 1,000,000</td>
<td>1993 (Warman et al., 1993)</td>
<td>MSX2</td>
<td>Suture fusion, suprobital repression, short first metatarsals.</td>
</tr>
<tr>
<td>Pfeiffer syndrome</td>
<td>1 in 100,000</td>
<td>1964 (Pfeiffer, 1964)</td>
<td>FGFR2</td>
<td>Suture fusion, broad thumbs and great toes, partial syndactyly, midface hypoplasia, ocular proptosis, hearing loss.</td>
</tr>
<tr>
<td>Crouzon syndrome</td>
<td>1 in 25,000</td>
<td>1912 (Crouzon, 1912)</td>
<td>FGFR2 and 3</td>
<td>Suture fusion, ocular hypertelorism, short upper lip, hypoplastic maxilla, midface deficiency</td>
</tr>
<tr>
<td>Beare-Stevenson syndrome</td>
<td>1 in 3,000,000,000</td>
<td>1969 (Beare et al., 1969)</td>
<td>FGFR2</td>
<td>Suture fusion, ear defects, cutis gyrata, skin tags, prominent umbilical stump, acanthosis nigricans</td>
</tr>
<tr>
<td>Muenke Syndrome</td>
<td>1 in 30,000</td>
<td>1996 (Muenke et al., 1997)</td>
<td>FGFR3</td>
<td>Suture fusion, often confused with Saethre-Chotzen (can sometimes be distinguished by deafness, but generally requires genetic testing), carpal and tarsal fusions, coned epiphyses of digits.</td>
</tr>
<tr>
<td>Shprintzen-Goldberg syndrome</td>
<td>Not reported</td>
<td>1982 (Shprintzen et al., 1978)</td>
<td>FBN1</td>
<td>Suture fusion, connective tissue defects, arachnodactyly, camptodactyly, mental retardation, low set ears, exophthalmos, marfanoid body type</td>
</tr>
<tr>
<td>Antley-Bixler Syndrome</td>
<td>Not reported</td>
<td>1975 (Antley and Bixler, 1975)</td>
<td>FGFR2</td>
<td>Like severe Pfeiffer, easily fractured, midface hypoplasia, trapeziodicephaly, synostosis of humerus and radius</td>
</tr>
</tbody>
</table>
Saethre-Chotzen syndrome may also cause syndactyly in hands, and broad toes and thumbs can be seen with Pfeiffer syndrome (Wilkie, 1997). Arachnodactyly and camptodactyly are features of Shprintzen-Goldberg syndrome. Syndromic craniosynostoses are more likely to be accompanied by mental retardation, heart defects and ear malformation (Cohen, 1980). Syndromic craniosynostosis is also more likely to result in raised intracranial pressure than non-syndromic craniosynostosis, therefore more often requiring further transcranial surgical intervention with all the attendant risks.

1.4 TREATMENT OF CRANIOSYNOSTOSIS

If left untreated, craniosynostosis may lead to impaired physical health and psychological problems for the child. Hence, surgery to allow normal brain growth and restoration of aesthetics to the cranium and face is necessary. Surgical intervention is currently the only option for treatment of craniosynostosis. The timing of treatment is controversial (Warren and Longaker, 2001b) and depends greatly on the age and development of the child. It is, however, likely in the case of syndromic craniosynostosis that children will need repeated surgery as they continue to grow. Treatment while the child is still young is beneficial, as normal growth of the child’s brain can assist in the reformation of a normal head shape (Speltz et al., 2004).

Linear craniectomy was the first recorded example of surgical intervention, performed in the 1890’s, particularly by Lane (Lane, 1892). The removal of the suture in this manner was often
ineffective as it failed to address the related problems that occur with the compensatory
growth of other areas of the skull. This surgery is still commonly used today but is
accompanied by re-alignment of other bones in the skull to correct the secondary effects of
craniosynostosis. Recently there has been a push towards less invasive treatments.
Endoscopic procedures have proven successful in treating sagittal craniosynostosis when use
in conjunction with a postoperative helmet (Brown and Proctor, 2011, Berry-Candelario et al.,
2011). However, there has also been evidence to suggest that the helmet alone (irrespective of
surgery) can assist in the correction of the malformations associated with sagittal synostosis
(Sood et al., 2011).

1.5 SUTURE MAINTENANCE AND FUSION

As early as 1851, scientists had begun suggesting possible causes for suture fusion, initially
believing that the osteogenic fronts in sutures behaved autonomously, not receiving influence
from surrounding tissues (Virchow, 1851). However, since then, focus has been directed to
the likelihood of paracrine instruction from the surrounding mesenchyme, dura mater and
pericranium inducing suture fusion.

1.5.1 PERICRANIUM, SUTURE MESENCHYME AND DURA MATER

Analysis of the intrinsic cytokine expression profile of suture mesenchyme suggested that it
reacts to instruction from an external source (Spector et al., 2000), however mesenchymal
cells in the suture have been shown to produce osteoinductive factors $Mx1$, $Mx2$, $Bmp4$ (Kim et al., 1998), $Twist1$ and $Fgfr2$ (Hollevillec et al., 2007) suggesting fusion may be a self perpetuating process. The pericranium was eliminated as a major contributor of these factors in suture fusion by experiments demonstrating that removal from rat calvaria did not affect suture fate (Moss, 1960). This was verified in fetal and neonatal sutures of rats (Opperman et al., 1994).

The dura mater however, has proven to be an important player in the process of suture maintenance and fusion. The dura mater is a highly enervated, thick collagenous membrane surrounding the brain. Its location in relation to the suture can be seen in Figure 1.4b. A pivotal study cementing the dura mater’s role in paracrine instruction of suture fate used a silicone membrane interposed between the dura mater and cranial sutures of rats. This interruption to the flow of hormones and cytokines released by the dura resulted in significantly delayed PF suture fusion (Roth et al., 1997). Research published by Levine in 1996 also elegantly demonstrated the importance of the dura mater in the maintenance of suture patency by performing an experiment that involved the surgical interchange of the PF (normally fused within 50 days) and sagittal sutures (normally remain patent), while leaving dura mater untouched. By replacing the sutures in either the normal orientation, or swapping the location of the PF and sagittal sutures the experiment resulted in the PF remaining patent while the sagittal fused in the samples that had been swapped (Bradley et al., 1996) demonstrating the role of the dura mater in suture maintenance. Closer analysis of the role of the dura mater has revealed an ability to secrete osteoinductive factors such as TGF-$\beta$s and FGFs (Roth et al., 1997) which may play a role in this control of the osteogenic fronts. Significant evidence has suggested that the expression of $TGF-\beta$s by the dura may be
important, particularly due to the finding that the expression of TGF-βs is different in dura underlying fused and unfused sutures (Kwan et al., 2008) and that the expression pattern of TGF-βs has a pro-osteogenic influence (Slater et al., 2009). TGF-β2 is upregulated in the dura of synostosed sutures (Poisson et al., 2004). Silencing technology has also shown the importance of TGF-β1 in suture maintenance in a dura culture model (Gosain et al., 2009). The possible effects of genetic mutation that may allow for this aberrant cell behaviour are discussed in Section 1.7.

1.5.2 SUTURE FUSION

The process of fusion has been extensively studied, yet is still not well understood. As mentioned previously, apart from the metopic suture, sutures in a human skull do not begin to fuse until middle age and may remain patent for life. Using the mouse PF suture as a model, it has been demonstrated that sutures fuse in an endocranial to ectocranial and anterior to posterior direction (Bradley et al., 1996) and it is assumed that this reflects the process that occurs in the human metopic suture (Warren and Longaker, 2001a). It is important to note, however, that this PF model follows a physiological not a pathological process. This means that its usefulness may be limited to a study of natural fusion, and for studies of craniosynostosis, the genetically modified mouse models may be superior.

The term fusion refers to an area of a suture in which the bony osteogenic fronts have met across the suture causing obliteration. The main event that allows the fusion of the osteogenic fronts across a suture is the change in behaviour of the suture cells, from the normal
proliferative state to one of differentiation. Therefore, the preosteoblast cells are allowed to differentiate into osteoblasts, building up the bone. The increase in cells with the ability to mineralise allows the osteogenic front to invade the suture space and exacerbate the process. It is also believed that apoptosis plays a role in the maintenance of suture patency and that dysregulation of apoptosis may allow suture fusion (Furtwangler et al., 1985).

1.6 ENVIRONMENTAL CONTRIBUTORS TO CRANIOSYNOSTOSIS

Environmental factors have been implicated in causing craniosynostosis but are difficult to analyse. Foetal positioning has been linked to metopic, sagittal and coronal craniosynostosis in humans (Graham et al., 1980). Other research has also reported a link between head constraint and suture fusion (Higginbottom et al., 1980). Mice that experienced forced long-term pregnancies, resulting in crowding in the womb, had fused sutures in their offspring (Furtwangler et al., 1985). Similarly, application of an adhesive to the coronal suture of rabbits also resulted in fusion of the suture (Persson et al., 1978) suggesting that craniosynostosis may be a consequence of physical force on the skull and sutures. Intrauterine head constraint has been shown to induce increased expression of osteogenic growth factors (Kirschner et al., 2002) and cause suture fusion in mice (Smartt et al., 1995). A recent study has complicated the picture however with, rather than high birthweight and long-term pregnancies producing craniosynostosis, a link was found between premature, low birthweight and craniosynostosis (Sanchez-Lara et al., 2010). This epidemiological study also showed a greater likelihood of metopic craniosynostosis being linked to low birth weight and pre-term delivery.
Non-syndromic craniosynostosis has also been reported in children where the mother has been exposed to sodium valproate (commonly used in the treatment of epilepsy) (Assencio-Ferreira et al., 2001, Singh et al., 2010), chemotherapy (Artlich et al., 1994), cocaine (Beeram et al., 1993), maternal smoking (Alderman et al., 1994), fertility treatments (Singh et al., 2010) and other teratogens. A recent study suggests that environmental effects may be suture dependent with a large scale study showing no correlation between folate supplementation and coronal, sagittal or lambdoid synostosis, but a positive correlation was seen with metopic synostosis (Carmichael et al., 2010). The difference in behaviour between the sutures may reflect the different developmental origins of these sutures and warrant further investigation in animal models.

Significantly, retinoic acid (RA) is implicated as a craniosynostosis causing teratogen, with epidemiological evidence linking RA exposure and craniosynostosis in humans (Gardner et al., 1998). Primate studies also show that coronal suture synostosis can result from RA overexposure in pregnancy (Yip et al., 1980). With the great number of possible craniosynostosis causing teratogens the question remains whether these factors are causing the craniosynostosis or merely exaggerating its effect. If these factors were a direct cause of craniosynostosis, the condition would surely be more common. It is likely therefore, that these cases of craniosynostosis occur as a combination between a genetic predisposition and environmental factors.
1.7 GENETIC CONTRIBUTORS TO CRANIOSYNOSTOSIS

Historically it was theorised that elucidation of the genetic cause of craniosynostosis would lead to a ‘silver bullet’ for its treatment (Cohen, 1980). Unfortunately, as more research has been performed it has become clear that craniosynostosis is the result of a myriad different mutations and that the picture is more complex than initially thought. There are continuing problems with the classification of different syndromes and their relevant genotypes. Some patients with the same genetic mutation have greatly differing phenotypes, while other patients who present with the same phenotype have a differing genotype. This indicates that there are numerous genetic contributors and modifiers of craniosynostosis.

1.7.1 FIBROBLAST GROWTH FACTOR RECEPTORS (FGFR)

Fibroblast growth factor receptors are a family of proteins (FGFR1, 2, 3 and 4) vital for diverse processes in human and animal development. Binding of a ligand (fibroblast growth factor; FGF) to its receptor causes activation of tyrosine kinase and a cascade of downstream signalling molecules. FGFR signalling is important in diverse biological processes including wound healing (Werner et al., 1994), angiogenesis (Deindl, 2003) and embryonic development (Niswander et al., 1993). In particular, FGFR signalling has been shown to have significant roles during skeletogenesis. Over 50 FGFR mutations with craniosynostosis as a primary clinical outcome have been identified. Many of these are missense mutations (Wilkie, 1997) and generally involve gain of function dominant mutations (Marie et al., 2005). The locations of some of the FGFR mutations can be seen in Figure 1.6. As the multiple mutations in the
Figure 1.6: Location of some of the common craniosynostosis related mutations in FGFRs (A), and TWIST (B). Adapted from Wilkie (Wilkie, 1997) and (Morriss-Kay and Wilkie, 2005).
FGFRs can be complex, they are divided into their individual isotypes to avoid confusion. A list of some common FGFR mutations is given in Table 1.3. Currently, there are no known craniosynostosis causing mutations of FGFR4.

1.7.1.1 FGFR1
Although very rare, the most well known FGFR1 mutation, a Pro252Arg substitution, is associated with Pfeiffer syndrome (Muenke et al., 1997). When introduced into osteoblast cells in culture this mutation increases proliferation and differentiation (Zhou et al., 2000) modelling the events occurring in suture fusion. Mutation of FGFR1 has also been documented in cases of non-syndromic trigonocephaly (Kress et al., 2000, Wu et al., 1996). Evidence of families with Pfeiffer syndrome related FGFR1 mutations but no premature suture fusion has raised questions about the direct role of FGFR1 in craniosynostosis (Hackett and Rowe, 2006) and screening has eliminated FGFR1 mutation as a causative agent in metopic craniosynostosis (Jehee et al., 2006).

1.7.1.2 FGFR2
FGFR2 mutations are the most common mutations in craniosynostosis, currently over 40 have been recognised. This significant influence may reflect the widespread expression of FGFR2 with expression detected in the osteogenic fronts, dura mater and suture mesenchyme. The FGFR2 mutations are more often found in cases of Pfeiffer (Oldridge et al., 1995, Lee et al., 2010) and Crouzon syndromic craniosynostosis (Reardon et al., 1994) however they have also been associated with other syndromes. There are also examples of a specific FGFR2 mutation being present in more than one different syndrome (Rutland et al., 1995, Piccione et al., 2009). Recently FGFR2 mutations have been found in Beare-Stevenson syndrome.
**Table 1.3: FGFR mutations associated with craniosynostosis**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Mutation</th>
<th>Syndrome</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>FGFR1</td>
<td>P 252 R</td>
<td>Pfeiffer</td>
<td>Meunke 1994</td>
</tr>
<tr>
<td></td>
<td>I 300 T</td>
<td>Non Syndromic</td>
<td>Kress 2000</td>
</tr>
<tr>
<td>FGFR2</td>
<td>A 344 P</td>
<td>Pfeiffer</td>
<td>Shotelersuk 2001</td>
</tr>
<tr>
<td></td>
<td>A 344 G</td>
<td>Crouzon</td>
<td>Gorry 1995</td>
</tr>
<tr>
<td></td>
<td>C 273 F</td>
<td>Pfeiffer</td>
<td>Oldridge 1995</td>
</tr>
<tr>
<td></td>
<td>C 278 L</td>
<td>Pfeiffer</td>
<td>Lee 2010</td>
</tr>
<tr>
<td></td>
<td>C 342 G</td>
<td>Pfeiffer</td>
<td>Cornejo-Roldan 1999</td>
</tr>
<tr>
<td></td>
<td>C 342 S</td>
<td>Pfeiffer</td>
<td>Addor 1997</td>
</tr>
<tr>
<td></td>
<td>C 342 R</td>
<td>Crouzon</td>
<td>Reardon 1994</td>
</tr>
<tr>
<td></td>
<td>C 342 Y</td>
<td>Crouzon</td>
<td>Reardon 1994</td>
</tr>
<tr>
<td></td>
<td>D 336 G</td>
<td>Crouzon</td>
<td>Gorley 2010</td>
</tr>
<tr>
<td></td>
<td>F 276 V</td>
<td>Pfeiffer</td>
<td>Cornejo-Roldan 1999</td>
</tr>
<tr>
<td></td>
<td>G 338 E</td>
<td>-</td>
<td>Pulley 1996</td>
</tr>
<tr>
<td></td>
<td>G 384 R</td>
<td>-</td>
<td>Pulley 1996</td>
</tr>
<tr>
<td></td>
<td>I 288 M</td>
<td>Pfeiffer</td>
<td>Cornejo-Roldan 1999</td>
</tr>
<tr>
<td></td>
<td>P 253 R</td>
<td>Apert</td>
<td>Wilkie 1995</td>
</tr>
<tr>
<td></td>
<td>Q 289 P</td>
<td>Crouzon</td>
<td>Gorry 1995</td>
</tr>
<tr>
<td></td>
<td>S 252 W</td>
<td>Apert</td>
<td>Wilkie 1995</td>
</tr>
<tr>
<td></td>
<td>S 267 P</td>
<td>Pfeiffer</td>
<td>Cornejo-Roldan 1999</td>
</tr>
<tr>
<td></td>
<td>S 351 C</td>
<td>Antley-Bixley</td>
<td>Chun 1998</td>
</tr>
<tr>
<td></td>
<td>S 354 C</td>
<td>Crouzon</td>
<td>Okajima 1999</td>
</tr>
<tr>
<td></td>
<td>S 372 C</td>
<td>Beare-Stevenson</td>
<td>Przyplepa 1996</td>
</tr>
<tr>
<td></td>
<td>W 290 C</td>
<td>Pfeiffer</td>
<td>Tartaglia 1997</td>
</tr>
<tr>
<td></td>
<td>Y 281 C</td>
<td>Crouzon</td>
<td>Tsai 2001</td>
</tr>
<tr>
<td></td>
<td>Y 340 C</td>
<td>Pfeiffer</td>
<td>Cornejo-Roldan 1999</td>
</tr>
<tr>
<td></td>
<td>Y 340 H</td>
<td>Crouzon</td>
<td>Reardon 1994</td>
</tr>
<tr>
<td></td>
<td>Y 375 C</td>
<td>Beare-Stevenson</td>
<td>Przyplepa 1996</td>
</tr>
<tr>
<td>FGFR3</td>
<td>A 391 E</td>
<td>Crouzon</td>
<td>Meyers 1995</td>
</tr>
<tr>
<td></td>
<td>G 380 R</td>
<td>Achondroplasia (Non-syndromic)</td>
<td>Georgoulis 2011</td>
</tr>
<tr>
<td></td>
<td>P 250 R</td>
<td>Muenke</td>
<td>Moloney 1997</td>
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</tbody>
</table>
(Przylepa et al., 1996, Eun et al., 2007), Apert syndrome (Wilkie et al., 1995, Girisha et al., 2006) and Saethre-Chotzen syndrome (Freitas et al., 2006). In addition to point mutations, there has also been identification of an FGFR2 mutation involving a large (1.93kb) deletion of the gene in a case of Apert syndrome (Bochukova et al., 2009). Interestingly, unlike FGFR1 and 3, FGFR2 has yet to be implicated in non-syndromic craniosynostosis. This may reflect a greater role for FGFR2 in development as the mutations seen have greater whole body effects.

1.7.1.3 FGFR3

FGFR3 mutations are commonly associated with diseases involving skeletal abnormalities. Mutations of FGFR3 have been recorded in cases of Crouzon syndrome (Wang Jabs et al., 1994), Meunke syndrome (Muenke et al., 1997) and non-syndromic craniosynostosis (Moloney et al., 1997, Williams et al., 1997). The FGFR3 mutation that causes Muenke syndrome is the most well characterised and common, with an original study identifying over 60 individuals with the P250R mutation (Muenke et al., 1997). This mutation has been linked with good clinical outcomes after surgery with a decreased need for repeat surgery (Ridgway et al., 2011). This P250R FGFR3 mutation has also been linked to multiple cases of Saethre-Chotzen syndrome (Paznekas et al., 1998). With the same mutation being linked to two distinct syndromes, the questions must be asked whether this is a mistake in the naming and identification of the different craniosynostosis syndromes, or does it reflect the great phenotypic variance that can result from a genetic mutation? Recently, multiple suture synostosis has been reported in association with FGFR3-related achondroplasia (Georgoulis et al., 2011). Although this patient has a bone growth syndrome, the lack of the recognised craniosynostosis syndrome means this mutation is now associated with non-syndromic craniosynostosis.
1.7.2  TWIST1

The *TWIST1* gene was first associated with craniosynostosis when genetic mapping showed a

correlation to a genetic locus previously identified as a candidate region in Saethre-Chotzen

syndrome (human chromosome 7p21)(el Ghouzzi et al., 1997, Brueton et al., 1992). Despite

this strong association, there are cases of Saethre-Chotzen syndrome that exhibit

craniosynostosis but do not have *TWIST1* mutation (Sahlin et al., 2009).

The fact that craniosynostosis syndromes can be attributed to such a range of mutations speaks

for the difficulty experienced by researchers looking for a cause. Not only have multiple

genes been implicated, but often multiple different mutations at different sites in the gene are

involved. Additionally, as discussed in section 1.6, the majority of the cases seen still present

without a recognised mutation. It is therefore likely that a combination of events are occurring

that are allowing the premature fusion of sutures. Analysis of the downstream signalling

effects that occur as a result of these mutations may assist in pinpointing a role for these genes.

1.8  SIGNALLING EFFECTS OF GENETIC MUTATIONS

While a range of genetic mutations have been identified in craniosynostosis, the downstream

effects of these mutations and how they may cause premature suture fusion is still poorly

understood.

FGFR3 is most commonly associated with achondroplasia, a condition displaying significant
bone developmental defects. Mice with Fgfr3 deletions have shown significant bone growth defects and displayed osteopenia, mimicking this human phenotype (Valverde-Franco et al., 2003). The reason for the bone growth defect in patients with FGFR3 mutation may be attributed to the active form of FGFR3 requiring homodimers. As a heterozygous dominant mutation, it has been shown that when mutated, the FGFR3 can sequester the native form and heighten the effect of the mutation (Alman et al., 2011). In this dimerised form, FGFR3 may be able to regulate endochondral ossification (Koch, 2010). The P250R mutation involved with FGFR3-related craniosynostosis is believed to result in constitutive expression and promote pro-chondrogenic gene expression profiles (Schibler et al., 2009), identifying a possible link with FGFR3 and cartilage formation in craniosynostosis.

Like the craniosynostosis-related FGFR3 mutations, FGFR2 mutations are gain of function mutations resulting in the receptors becoming constitutively active. FGFR2 has been shown to be a positive regulator of bone growth, with mice defective in Fgfr2 displaying decreased bone growth and dwarfism (Eswarakumar et al., 2002). Interestingly, different Fgfr2 mutations have been shown to have different effects, with a Crouzon mutation promoting proliferation in osteoblast cells and an Apert mutation promoting differentiation (Ang et al., 2010). How these different influences both result in the outcome of suture fusion is unclear. Apert syndrome Fgfr2 mutation may elicit this response through activation of p38 and the ERK1/2 signaling pathway (Miraoui et al., 2009, Wang et al., 2010). FGFR2 has multiple sub-types and it has been shown that during episodes of craniosynostosis, gene expression changes and the loss of one subtype may result in the compensatory expression of another and this may be the causative factor in FGFR2-related craniosynostosis (Veistinen et al., 2009).
*FGFR1* may play multiple roles in bone growth with expression in both osteoblasts and osteoclasts (Kawaguchi et al., 2004). *FGFR1* deficient cultured osteoblasts have shown a decreased capacity for differentiation and mineralisation (Jacob et al., 2006). These observations led to a conclusion that the activating mutations seen in craniosynostosis would lead to an increase in bone formation and this is relevant to what is seen at the osteogenic fronts in craniosynostosis.

TWIST1 protein is a transcription factor containing a helix-loop-helix protein interaction domain and a DNA binding domain (Gripp et al., 2000). Loss of function mutations are thought to reduce the efficiency of the DNA binding domain within the protein (El Ghouzzi et al., 2001). TWIST1 is involved in normal craniofacial development (Yu et al., 2008, Bildsoe et al., 2009) and may control osteogenesis by determination of cell fate. Reduction of *Twist1* has also been shown to increase the osteogenic differentiation of human and mouse mesenchymal cells (Miraoui and Marie, 2010). *Twist1* expression can promote chondrogenesis (Dong, 2007) a process recently linked to craniosynostosis, and decrease osteoblast differentiation (Hayashi, 2007, Isenmann, 2009). The resultant endochondral ossification has been linked with Twist1-related craniosynostosis (Behr et al., 2011).

In addition to the possible effects of individual mutations, there is evidence that FGFR1, 2, and 3, MSX2 and TWIST may be interacting in similar signalling pathways, increasing the consequence of any mutations in bone growth. In a mouse model of Saethre-Chotzen syndrome, mice with haploinsufficiency of *Twist1* displayed not only sutural fusion, but also had decreased expression of *Fgfr2* (Rice et al., 2000) and interactions between TWIST and the *FGFR2* promoter have been demonstrated in osteoblasts, implicating a common signalling
pathway (Guenou et al., 2005). Evidence has also been presented that BMPs, vital cytokines in bone growth and differentiation, may be under the influence of the FGF signalling pathway (Ribes et al., 2006). MSX2, a homeobox protein vital in development of the vertebral column and craniofacial development has also been shown to be under the influence of FGFR signalling pathways (Ignelzi et al., 2003).

1.9 MOUSE MODELS OF CRANIOSYNOSTOSIS

Mouse models of disease provide a useful tool for medical research. Multiple mouse models have now been developed for craniosynostosis, mainly mimicking specific craniosynostosis syndromes by using genetic manipulation. The FGFR family have been a focus in the development of these mouse models, reflecting their prominence in known craniosynostosis causing mutations. Mice with a P250R substitution in the Fgfr1 gene display premature fusion of calvarial sutures similar to that seen in Pfeiffer syndrome and these mice have been useful in demonstrating not only the importance of FGFR1 in Pfeiffer syndrome, but also in indicating that it may do this through increased differentiation of preosteoblastic cells (Zhou et al., 2000).

Fgfr2 null mice are embryonic lethal (Zhao et al., 2004) but introduction of the heterozygous Fgfr2 mutations found in human craniosynostosis have proven successful for studies of craniosynostosis. In one example, where introduction of an S252W mutation modelled Apert syndrome in humans (Alappat et al., 2003) severe coronal suture fusion was one of the most marked features of these mice and further examination demonstrated a decrease in the
numbers of mesenchymal cells in the coronal suture and increased expression of Bax, a protein involved in apoptosis. Mice with this mutation have also been used to show changes in cartilage development, as well as abnormalities in the heart, brain, thymus, lungs and intestines. Another mouse model of Apert syndrome, where a P253R mutation has been introduced to the *Fgfr2* gene, displayed shortened skull and malformed mandibles (Du et al., 2010). A phenotype likened to Crouzon and Pfeiffer syndromes was achieved by the substitution of C342Y in *Fgfr2* (Eswarakumar et al., 2002). In addition to the suture fusion seen in these mice, they also displayed high numbers of proliferating osteoprogenitor cells in the sutures and in long bones. A more recent study which utilised *Fgfr2* mutant mice also reported a high rate of coronal and sagittal fusion via microCT analysis (Perlyn et al., 2006). The number of phenotypes seen in *Fgfr2* mutant mice highlights the diversity of its functions, and the likelihood of common pathways with multiple biological processes. A recent study has shown that for an effective study of particular craniosynostosis syndromes, identification of the affected gene alone is not enough. In a comparison between the *Fgfr2*<sup>+/S252W</sup> mice and *Fgfr2*<sup>+/P253R</sup> mice, differences were detected between craniofacial phenotypes including changes in the hard palate and severity of malformation (Martinez-Abadias et al., 2010).

*Fgfr3* mutant mice have also been shown to have skeletal malformations. Mice with the G380R mutation in the *Fgfr3* gene develop achondroplasia, a form of dwarfism, reflecting what is seen in human patients (Wang et al., 1999b). These mice also display craniofacial malformations, in particular smaller calvaria and facial bones and deformed cranial base (Segev et al., 2000). However, these mice have not been assessed for calvarial suture fusion. Introduction of the P244R mutation into *Fgfr3* resulted in a mouse with a rounded skull and shortened, asymmetric snout (Twigg et al., 2009). These mice also experienced hearing loss,
attributed to changes in FGF signalling (Mansour et al., 2009). The variable penetrance of the mutation and the lack of craniosynostosis (present in less than 1% of affected mice) make it a questionable model for Muenke syndrome.

Heterozygous null *Twist1* mice mimic the mutation seen in the human Saethre-Chotzen syndrome (Chen and Behringer, 1995). These mice display fusions of the coronal suture, craniofacial abnormalities and polydactyly of the hind feet and are considered a suitable model for the human syndrome (Carver et al., 2002). Early reports of coronal sutures fusion in these mice identified fusion at 30 days of age, although this has recently been challenged with new reports of fusion in these mice occurring much earlier (Behr et al., 2011).

When a Pro to His mutation is introduced to the *msx2* gene in mice, premature fusion of sutures ensued (Liu et al., 1995) and MSX2 overexpression by a similar P148H mutation also caused severe craniosynostosis and cleft palate (Winograd et al., 2001). This mutation is analogous to the mutation that is detected in human Boston-type craniosynostosis syndrome (Liu et al., 1995).

Despite the extensive study of craniosynostosis using animal models elucidating some major contributors to the process of suture fusion, there is still a need for a model that can better mimic the events that occur in vivo in humans. Unfortunately there is currently no model that can truly emulate this human process, however, using a combination of human tissue samples, human suture-derived cells and animal models may give a more specific insight to what is occurring in craniosynostosis patients.
1.10 THE ROLE OF RETINOIC ACID SIGNALLING IN OSTEOGENESIS AND CRANIOSYNOSTOSIS

Retinoids, the metabolic end products of the breakdown of dietary vitamin A have roles in multiple vital biological processes. Manifestations of vitamin A deficiency include visual impairment or blindness (Underwood and Arthur, 1996, Jordan et al., 2008), increased rate of viral infection (Ross, 1996), dry or irritated skin (McLaren, 1956), problems with adult brain function (Goodman, 2006), lactation problems (Ajans et al., 1965) and in extreme cases to death (Rahmathullah et al., 1990). Alternatively, when present at high levels, vitamin A is a potent teratogen causing birth defects (Lammer et al., 1985), hair loss, skin discolouration, nausea (Bergen and Roels, 1965) and again in extreme cases can be toxic (Hathcock et al., 1990). However, at no time are retinoids arguably more important that during embryogenesis.

The signalling pathway of RA is complex, and RA can act as a ligand for a number of distinct RAR or RXR isoforms (nuclear transcription factors) (Figure 1.7), whose activation regulate proliferation and differentiation. The signalling pathway of Vitamin A begins with dietary retinol. After uptake of dietary retinol by intestinal epithelial cells, retinol typically interacts with CRBP2 (cellular retinol binding protein 2). Most retinol is converted to retinyl esters which is exported in chylomicrons and is taken up by the liver for storage (Wongsiriroj et al., 2008). Subsequently, retinyl ester is converted to retinol and secretion bound to RBP4 (retinol binding protein 4). The RBP4-retinol, or holo-RBP4, binds to its cellular receptor STRA6 on the target cells. Once transferred to the target cells, retinol can be reversibly metabolised to RA or stored, a process controlled by dehydrogenases (Bushue and Wan, 2010). Depending on its form, (RA or retinol) storage is facilitated in the cell by CRBPs (cellular retinol binding
Figure 1.7: Possible role of RBP4 in intracellular retinol signalling and storage. Adapted from (Gutierrez-Mazariegos et al., 2011). This diagram shows the classic intracellular metabolic pathway for the conversion of retinol to RA and a potential role for RBP4.
proteins) and CRABPs (cellular retinoic acid binding proteins). RA is able to regulate cell growth by its unique ability to enter the nucleus of target cells and with the aid of nuclear receptors bind to target genes (Duester, 2008).

1.10.1 RETINOIC ACID SIGNALLING INTERACTIONS WITH BONE GROWTH PATHWAYS

In addition to the complications described above, retinoic acid (RA), a metabolite of vitamin A, has been shown to have a significant influence on bone growth. Administration of RA to osteoprogenitor cells in vitro has been shown to halt proliferation, promote differentiation, upregulate BMPs (Helvering et al., 2000) and alter FGFR expression profiles (Song et al., 2005). Additionally, the introduction of RA to human osteoblast cell culture is able to significantly increase the expression of osteocalcin, suggesting a direction towards mature mineralising cells (Oliva et al., 1993). RA has also been shown to drive differentiation of adipose-derived stromal cells to express bone markers ALP, OC and Runx2 and form bone nodules (Wan et al., 2006). A similar effect has been seen in osteoprogenitor cells where the addition of RA can promote osteogenic gene expression (Roberts et al., 2011).

However, data also suggests an argument for a role of RA in bone degradation. Patients with ankylosing spondylitis, a condition involving fusion of bones, particularly in the spine, have a decreased level of circulating retinol (O'Shea et al., 2007). Osteoporosis has been seen as a side effect of RA treatment of skin conditions and has led to mouse models of the disease (Wu et al., 1996). In rats, oral administration of RA resulted in a high incidence of spontaneous
fractures and a significant decrease in bone density and mineral content. RA also gave a
decrease in bone density and mineral content but to a lesser extent than all-trans-RA. Studies
have also reported limb developmental abnormalities in rodents exposed to high doses of
vitamin A during development (Kochhar, 1973, Nakamura et al., 2009). The increase in bone
resorption may be attributed, in part, to an increase in RANKL and therefore osteoclast
differentiation (Conaway et al., 2011) or an increase in osteoclast progenitor proliferation (Hu
et al., 2010).

Contrary to both schools of thought, some data have also shown no correlation between RA
and bone mineral density at all (Wattanapenpaiboon et al., 2003). A possible explanation for
this controversy is that the dose may have a significant impact, with some studies
demonstrating differing cellular responses to RA depending on both the dose and length of
exposure to the teratogen (Togari et al., 1991). Alternatively, it may be possible that RA
exhibits site-specific functions, as a majority of the pro-osteogenic data comes from the
craniofacial skeleton, rather than trabecular bone (James et al., 2010a).

How RA exerts these varied effects of bone growth remains unknown although research has
shown many potential interactions between RA and bone growth pathways. In particular,
BMPs play an important role in bone growth and it has been hypothesised that RA and BMP
signalling pathways may interact (Li et al., 2003, Skillington et al., 2002). BMPs decrease the
availability of RA and members of the RA signalling pathway are significantly downregulated
in response to BMP4 (Hoffman et al., 2006). Additionally, a link has been made between RA,
sonic hedgehog (Shh) and FGF8 in craniofacial and limb development. In several chick
models of limb development it has been shown that RA is able to induce the expression of Shh
(Stratford et al., 1996, Helms et al., 1997) and suppress expression of indian hedgehog (Ihh) driving bone to a more ‘mature’ profile (Wu et al., 2002). In the craniofacial development of chicks RA has also been shown to regulate the expression of Shh and FGF8. Removal of RA results in malformation in the frontonasal region in chicks but this phenotype could be rescued by reintroduction of RA to the area (Schneider et al., 2001).

In a recent microarray study from this laboratory, the expression of 18,000 genes was compared across 17 sutures isolated from 5 children with craniosynostosis, 4 with non-syndromic and 1 with syndromic (Apert) craniosynostosis (Coussens et al., 2007). This data, comparing fused (craniosynostotic), fusing and unfused human suture samples highlighted a number of novel genes with dysregulated expression. In particular RBP4, a retinol binding protein, was downregulated 37 fold as sutures fused. RBP4 protein was localised in suture cells at the osteogenic front and in cells at the bone surface of unfused sutures (Coussens et al., 2007). RBP4 has an important role in binding retinol, the precursor to RA of the RA signalling pathway. This down regulation of RBP4 in craniosynostosis led to the suggestion that changes in the RBP4/retinol-RA axis may have a major role in regulating osteogenesis and suture fusion in craniosynostosis in humans (Coussens et al., 2007).

### 1.10.2 RA AND CRANIOGENESIS

*In vitro* and *in vivo* experiments have demonstrated RA to be a possible craniosynostosis causing teratogen and to be involved in many developmental craniofacial anomalies. RA has the ability to induce facial clefting when introduced into the face of developing chicks.
(Richman, 1992). This phenomenon whereby increased vitamin A causes cleft palate has also been reported in rats (Lorente and Miller, 1978), rabbits (DiGiacomo et al., 1992), mice (Newall and Edwards, 1981, Yasuda et al., 1986), hamster (Willhite, 1986) and cats (Freytag et al., 2003). RA may control craniogenesis by control of chondrocytes proliferation and differentiation at the cranial base (Kwon et al., 2011), inhibition of the migration of cranial neural crest cells, or altering the proliferation and differentiation of mesenchymal cells (Sulik et al., 1988).

Although most research has been performed in avian or rodent models, exposure of pregnant female rhesus monkeys to large doses of RA resulted in the birth of offspring with fused cranial sutures (Hendrickx et al., 1980). Pigtail monkeys have also displayed development of craniofacial anomalies in response to high dose maternal vitamin A (Newell-Morris et al., 1980). Epidemiological data suggests a similar trend in humans exposed to high levels of RA during pregnancy (Gardner et al., 1998, Lammer et al., 1985). Mesenchymal cells isolated from the sutures of mice have shown enhanced osteogenic differentiation in response to RA (James et al., 2010a).

This research has given a clear indication of an important role for RA during craniogenesis. However, despite the clear links between RA and craniosynostosis, there has not yet been a thorough analysis of the potential role of RA in the suture mesenchyme. Given its importance in the regulation of bone growth in other areas of the skeleton, it is likely that it may display a level of control of the cells at the osteogenic fronts of the suture.
1.10.3 RETINOL BINDING PROTEINS

As diet is the only source for vitamin A, retinols must be able to be transported in the blood to and from the liver, small intestine and appropriate organs and tissues. Due to their hydrophobic nature, retinols and retinoids require protein transporters in the body and this is provided by retinoid binding proteins (RBPs). Although there are a variety of proteins that bind retinol, they can be divided into two subsets; lipocalins, of which RBP4 is a member, and intracellular lipid-binding proteins (iLBPs) encompassing the cellular retinoid binding proteins (CRBPs) and cellular retinoic acid binding proteins (CRABPs) (Flower, 1996).

1.10.4 RBP4

Unlike other retinol binding proteins, RBP4 belongs to the lipocalin family. RBP4 is a small, secreted protein whose function as a carrier molecule is enabled by its distinct structure. RBP4 binds its ligand, retinol, within a β-barrel. The structure protects the ligand, with one end of the barrel blocked by the N-terminus of the protein and the other by the binding site of transthyretin (TTR), a molecule which assists in the movement of retinol in the blood (Goodman, 1980) (Figure 1.8).

In the past, the only proposed function of RBP4 was to assist in the transport of retinol between tissues and cells in the plasma (Goodman, 1980) with RBP4 levels correlating with retinol levels in the circulation of healthy subjects (Erikstrup et al., 2009). However, research is starting to question this dogma. As the precursor for retinoic acid, retinol is vital in a
Figure 1.8: Retinol bound bovine serum-RBP. Retinol is shown in red. From (Noy, 2000) molecule.
myriad of biological processes, including prenatal neuronal differentiation and outgrowth (Lane and Bailey, 2005) and adult brain function (Mey and McCaffery, 2004). Delivery of retinol to the brain is therefore vital, and RBP4 allows movement across the choroid plexus to the cells in the brain (Herbert et al., 1986). Interruption of this process may be involved in the neurological degeneration seen in Alzheimer’s disease (Goodman, 2006).

A role for RBP4 in regulating insulin levels and its potential involvement in type II diabetes has also been uncovered. Glut4 deficient mice, which display obesity and insulin resistance (Abel et al., 2001) have high levels of Rbp4 in serum and in adipocytes (Yang et al., 2005b). Exogenous addition of a synthetic retinoid called fenretinide improved the insulin sensitivity of these mice (Yang et al., 2005b). Human subjects have also shown a direct correlation between RBP4 levels and fasting insulin and body mass index (BMI) (Graham et al., 2006), with a link also being made between high RBP4 levels and high levels of adipose tissue (Cho et al., 2006, Kelly et al., 2010). RPB4 levels may also indicate risk for type 2 diabetes (Polonsky, 2006, Pittenger et al., 1999). Strengthening this link between obesity, diabetes and RBP4, studies have shown that significant weight loss and improvement in insulin sensitivity can decrease the levels of circulating RBP4 (Janke et al., 2006, Graham et al., 2006, Vitkova et al., 2007).

Additionally, RBP4 has been shown as an adipokine with the ability to alter activity of factors in muscle and liver vital to insulin dependent glucose uptake (Yang et al., 2005a). These studies suggest a more diverse role for RBP4 than merely as a carrier. Although generally believed to be only required for extracellular transport from the liver, RBP4 has been demonstrated to be expressed in multiple extra hepatic sites. Expression occurs in kidney,
brain, adipose tissue, cartilage (Soprano and Blaner, 1994) and it was recently shown that
RBP4 is expressed in human cranial sutures (Coussens, 2007). As RA has been demonstrated
as influencing cranial suture development (Hendrickx et al., 1980), and RBP4 has shown to be
dysregulated during fusion, this laboratory has proposed that, in the suture, RBP4 may
function intracellularly by regulating the availability of RA and that downregulation of RBP4
leads to excessive production of RA and excessive bone growth.

1.10.4.1 An Rbp4 knockout mouse model

A Rbp4\(^{-/-}\) mouse has provided a model for further analysis of the role of RBP4. By insertion
of PMC1neo (a neomycin resistance cassette) it was shown that the mutant Rbp4 gene now
encoded a 28 kDa protein which had no sequence or functional similarity to the wildtype Rbp4
molecule (Quadro et al., 1999). These mice, although appearing phenotypically normal,
cannot access hepatic retinol stores and have lower levels of circulating retinol. When
maintained on a normal diet, a small amount of residual serum retinol can be measured in
Rbp4\(^{-/-}\) mice, but this was abolished when the animals were placed on a low vitamin A diet.
However, it was shown that hepatic stores of retinol in the Rbp4\(^{-/-}\) were comparable to WT
counterparts, demonstrating that although these mice lack the ability to transport retinol
(attributed to the loss of Rbp4) they are still able to accumulate hepatic stores.

This lack of Rbp4 and hence serum retinol leads to multiple developmental abnormalities in
these mice. These abnormalities include difficulties with vision, cardiac growth abnormalities
(Wendler et al., 2003), retarded foetal growth and defective organogenesis (Quadro et al.,
2005). However, these abnormalities are often not detectable until mice are maintained on a
low vitamin A diet, and it appears that a lot of these defects can be rescued if mice are moved to a high vitamin A diet (Quadro et al., 2002). In regards to skeletal development, not until mothers were maintained on a vitamin A deficient diet were skeletal abnormalities seen in the offspring. At this point, retarded ossification and severe craniofacial malformations were evident and embryos died in utero (Quadro et al., 2005). The craniofacial anomalies included loss of midfacial and parietal bone. These mice lacked nasal capsule, septum and supraoccipital bone and had decreased or malformed parietal, interparietal and exoccipital bones. A link was made between the areas affected and their origin from the frontonasal mesectoderm suggesting that this was the region where the abolition of Rbp4 had the greatest effect. Other skeletal sites affected were a malformed sternum and decreased size and fusion of some cervical vertebrae. This is not surprising as the mesectoderm has been shown to be a major site of RA action in the developing fetus of mice (Levenson et al., 1998).

1.10.5 STRA6

STRA6 (stimulated by retinoic acid gene homolog 6) is the cellular receptor for RBP4, allowing bi-directional transport of retinol into and out of cells (Kawaguchi et al., 2007). STRA6 expression has been identified in a number of tissues, but has not previously been identified in skeletal or cranial tissue. An increase in expression has been seen in colon cancer (Szeto et al., 2001) and, like RBP4, a link has recently been made between STRA6 expression and Type 2 diabetes (Nair et al., 2010). Mutation of STRA6 results in a syndrome referred to as Matthew-Wood syndrome or PDAC (pulmonary hypoplasia/agenesis, diaphragmatic hernia/eventration, anophthalmia/microphthalmia, and cardiac defect) (Chiyat et al., 2007).
Numerous different mutations in \textit{STRA6} have been identified in this syndrome, and this may explain some of the large phenotypic variation seen in patients (Chassaing et al., 2009). Although not thoroughly categorised, patients with Matthew-Wood syndrome do present with craniofacial, in particular orbital, malformation. The seemingly large discrepancy in phenotype between mutation of RBP4 in mice and STRA6 in humans has been thought to highlight the fact that STRA6 may potentially play more roles than simply as a receptor for RBP4. A reduction in Stra6 in a zebrafish model resulted in significant defects, however the fact that a healthy phenotype was rescued with a decrease in RBP4 further strengthens their biological relationship (Isken et al., 2008).

\textbf{1.10.6 CRBPs}

The role of retinol storage inside target cells is generally performed by CRBPs. There are 3 different CRBPs, CRBP1, CRBP2 and CRBP3. The different CRBPs have been shown to have differential expression, with CRBP1 and 3 displaying widespread tissue expression in both embryonic and adult tissue whereas CRBP2 expression is restricted to the intestine (Albalat, 2009). In particular, CRBP1 is expressed in the adult cerebellum where it is thought to be involved in plasticity (Parenti and Cicirata, 2004), in stellate cells in the liver (Lepreux et al., 2004) and in the endometrium (Orlandi et al., 2004). CRBP1 has been shown to be downregulated in cancer, in particular breast cancer (Farias et al., 2005a) and this may allow for tumour progression by decreasing cell differentiation (Farias et al., 2005b). Conversely, an increase in CRBP1 expression has been suggested as a marker for long-term tumour survival in laryngeal carcinomas (Peralta et al., 2010). CRBP1 and 2 knockout mice have both shown
to be viable when raised on a normal vitamin A diet (Ghyselinck et al., 1999, Zhang et al., 2002).

CRBP1 has been shown to be a substrate carrier in the metabolism of retinol to retinoic acid (Posch et al., 1991). This role may explain its widespread expression through tissue, as RA is required at multiple sites during development. However, it should be noted that CRBP1 expression does not always coincide with tissues that synthesise retinoic acid (Matt et al., 2005).

Recently, a link has also been made between CRBP1 and adipogenesis, highlighting the possibility of overlapping roles of CBRP1 and RBP4. In that study, CRBP1 expression was localised to adipose tissue, where levels positively correlated with the level of obesity and insulin insensitivity (Zizola et al., 2010).
1.11 HYPOTHESIS AND RESEARCH AIMS

As described above, RA plays a pivotal role in osteogenesis and when present at high levels has been shown to induce craniosynostosis in animal models. Based on this information and the data showing dramatic downregulation of RBP4 during human suture fusion, our laboratory has hypothesised that RBP4 may be acting as a storage molecule in sutures and that, by sequestering retinol, RBP4 may regulate the availability of suture RA and thereby regulate osteogenesis and cranial suture patency.

To investigate this hypothesis, the following specific aims are proposed:

1. To investigate the expression of RBP4 in suture fusion in a natural mouse model of suture fusion and in a genetic mouse model of Saethre-Chotzen craniosynostosis
2. To investigate the subcellular localisation of RBP4 in cell culture models
3. To generate a method of overexpression of RBP4 in human and mouse cells and tissue
Chapter 2

Materials and Methods
2.1 MATERIALS

2.1.1 GENERAL SOLUTIONS AND CHEMICALS

Chloroform, isopropanol and methanol were purchased from APS Ajax Finechem, Auburn, NSW, Australia. Absolute ethanol was purchased from ACE Chemical Company, Camden Park, SA, Australia. Glycerol was purchased from Merck (Darmstadt, Germany). MilliQ water was used in most experimental procedures, however in RNA protocols diethyl pyrocarbonate (DEPC) water was made by adding 0.1% v/v DEPC (Sigma-Aldrich, St Louis, MO, USA), incubating at room temperature (RT) overnight, then autoclaving.

Tris used in stock buffers was purchased from Sigma-Aldrich. Ethylenediaminetetraacetic acid (EDTA) and sodium chloride (NaCl) were from APS Ajax Finechem. Stock solution of 20x phosphate buffered saline (PBS) was made as described (Sambrook and Russell, 2001) and diluted to 1x for use in experiments. Tris buffered saline (TBS) was made as described in (Sambrook and Russell, 2001). TTBS was made by adding 0.5% v/v Tween20 (Sigma-Aldrich) to TBS. Sodium dodecyl sulphate (SDS) was purchased in tablet form from Medicago, Uppsala, Sweden.

Unless otherwise specified, all chemicals were of analytical grade.
2.2 METHODS

Research involving human tissue was approved by the Children, Youth and Women’s Health Service Research Ethics Committee. All animal experiments and animal breeding were approved by the Children, Youth and Women’s Health Service Animal Ethics Committee. DNA manipulations involving genetically modified organisms were approved by the Children, Youth and Women’s Health Service Biohazard Committee and by the Office of the Gene Technology Regulator.

2.2.1 ANIMAL STUDIES

2.2.1.1 Animal husbandry

*Twist1* heterozygous knockout mice (*Twist1<sup>+/−</sup>*) were generated by targeted gene deletion of a segment of the *Twist1* gene (Chen et al., 2007) and sourced from Patrick Tam and David Loebel, Children’s Medical Research Institute, Sydney, Australia. These mice were from a mixed C57BL/6 x 129S background. Wild-type litter mates were used as controls. C57BL/6 mice were sourced from the University of Adelaide. Animals were housed at the Children’s, Youth and Women’s Health Service Animal Facility for the entirety of the animal trial and had access to water and “meat free rat and mouse diet” (Specialty Feeds, Glen Forrest, WA, Australia) *ad libitum*. 
2.2.1.2 Animal genotyping

_Twist1+/−_ animals were genotyped by collection of tail snips at the time of weaning or surgery. DNA was extracted from 0.5 cm of tail using a QIAGEN Blood and Tissue kit as per manufacturer’s instructions. Genotyping reactions contained 100 ng of DNA, 2 mM MgCl₂, 1 µl 10x PCR buffer (Applied Biosystems, New Jersey, USA), 0.1 µl 5 mM dNTPs, 5.625 nM forward and reverse primers, 0.1 µl AmpliTaqGold polymerase (Applied Biosystems) and H₂O. Genotyping PCR reactions were run on a GeneAmp PCR system 9700 (California, USA). PCR products were checked on a 1.5% agarose gel, run for 30 minutes at 110V. Genotype was confirmed by the presence or absence of the ‘deleted allele’ band. Primers and conditions can be found in Table 2.1.

2.2.1.3 Sample collection and preparation

 Appropriately aged animals were euthanised using CO₂ asphyxiation and cervical dislocation. The cranial vault was dissected from animals and either placed intact in 10% buffered formalin in preparation for microCT and histological analysis or the individual sutures were trimmed as much as possible (leaving no more than 1 mm of bone either side of the suture) and frozen in liquid nitrogen (LN2) for RNA extraction (Figure 2.1).

Samples in 10% buffered formalin were used for microCT scans using a SkyScan X-ray Micro CT Scanner 1072. Scan conditions were 35x magnification, 8.93 µm pixel, Xray source 50 kV/191 µA, 15 random movement, flat field correction on, gain of 1, 180° rotation with 0.68° steps, no filter and exposure 2.2 seconds. Reconstruction conditions were ring artefact 12, beam hardening 50% and threshold 52-220. 3D models of microCT scans were created using CTvol v.1.9.4.1 and CTan v.1.5.0.2 (SkyScan, Aartselaar, Belgium). Once these samples had
Table 2.1 PCR primers used for genotyping and sequencing

<table>
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<tr>
<th>Target</th>
<th>Sequence 5’-3’</th>
<th>Annealing Temp</th>
<th>Amplicon size</th>
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<tr>
<td>F</td>
<td>AGCGGT CATAGAAAACAGCC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R</td>
<td>CCGGATCT ATTTTGCATTTTACCATGGGTCAG</td>
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<td>R</td>
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<td></td>
</tr>
<tr>
<td>R</td>
<td>AGATCCATAGCATAGCTCTACAAAGGGTTTCTTTTC</td>
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<td>F</td>
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<tr>
<td>R</td>
<td>GCGCGCAATTAACCCTCCTCA</td>
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Figure 2.1 Calvaria and suture dissection diagram. Dotted lines indicate where incisions were made. Skull diagram was adapted from The Jackson Laboratory (www.jax.org)
been scanned they were moved to 75% ethanol and processed into paraffin for sectioning. Sections were cut at 4 µm thickness and baked overnight at 37°C. H&E staining was used to confirm microCT results. The H&E staining protocol is described in section 2.2.4.2.

2.2.2 GENE EXPRESSION ANALYSIS

2.2.2.1 RNA extraction

RNA was extracted from both cells and tissues using Trizol (Invitrogen, Carlsbad, California, USA) as per manufacturer’s instructions. Some optimisation of tissue homogenisation and RNA precipitation were performed for murine suture tissue and are outlined here. For RNA extraction from cell culture, media was removed and cells washed in 1x PBS. Cells were completely covered in Trizol; 1 ml for a 12 well plate, 500 µl for a 24 well plate. For RNA extracted from mouse tissue, samples were prepared by initial mincing with scissors and then treatment with a Heidolph DIAX600 homogeniser (Heidolph Instruments, Schwabach, Germany) until all lumps of tissue had been removed. For RNA extracted from bone and suture tissue, samples were placed into a clean eppendorf tube and approximately 1 ml of LN2 added. Bone was then crushed with a pre-cooled pestle until powdered. 1 ml of Trizol was used per 0.5 g of tissue.

Alterations to the Trizol manufacturer’s protocol were as follows. For mouse bone/suture samples, 5 ng of glycogen (Ambion, Austin, TX, USA) was added to isopropanol to aid in RNA precipitation. RNA precipitations were performed O/N at -20°C, rather than at RT. After O/N incubation, samples were centrifuged at 17,000 g, 4°C for 1 hour. The supernatant
was discarded and RNA pellets were washed in 75% ethanol, air dried for 1 minute and resuspended in DEPC water. A ten minute incubation at 60°C aided resuspension. All RNAs were stored at -80°C after extraction. RNA quality was checked on a 1% agarose gel and concentrations quantified using a NanoVue spectrophotometer (GE healthcare, Pittsburgh, PA, USA) (Figure 2.2).

2.2.2.2 DNAse treatment and cDNA synthesis
RNA samples were DNAse treated to eliminate any contaminating DNA by use of a DNAse I (RNAse free) enzyme (New England Biolabs, Ipswich, MA, USA). DNAse treatment was performed as per manufacturer’s protocol with provided buffers. In the case of samples of low RNA concentration, rather than treating 10 µg of RNA, the maximum available volume of RNA for the reaction was used. As RNA concentrations of mouse suture samples were minimal, they were excluded from DNAse treatment, as the extra handling posed too high a risk for loss and degradation of the small amount of RNA present.

cDNA was synthesised using a Superscript III First Strand Synthesis kit (Invitrogen). Synthesis was performed as per manufacturer’s instructions. Reactions were set up with 2 µg RNA with DEPC water to a volume of 8 µl. For suture samples with low RNA yields, 2 µg RNA was not always available. In these cases, 8 µl of neat RNA was added to the cDNA reaction, to maximise the amount of cDNA produced.

2.2.2.3 Real Time Quantitative Polymerase Chain Reaction (RT-qPCR)
RT-qPCR reactions were set up to analyse changes in gene expression. All primers used for RT-qPCR (Table 2.2) were purchased from Geneworks, Adelaide, Australia. PCR reactions
Figure 2.2 Representative examples of RNA collected from mouse suture and cell culture and electrophoresed on a 1% agarose gel at 110V for 30 minutes. A) Examples of good quality RNA, showing 2 distinct bands at approximately 5kb (28S) and 2kb (18S). B) An example of poor quality, degraded RNA.
Table 2.2 Primers used for RT-qPCR analysis

<table>
<thead>
<tr>
<th>Target</th>
<th>Sequence 5’-3’</th>
<th>Annealing Temp</th>
<th>Amplicon size</th>
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<td>eYFP</td>
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<td>CypA</td>
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were made to final concentrations as follows. 2 µl of cDNA, 100x SYBR green (Abgene, Epsom, UK), 1x AmpliTaq PCR Buffer (Applied Biosystems), 2 mM MgCl₂ (Applied Biosystems), 0.4 mM dNTP (Invitrogen), 5.625 µM primers (forward and reverse), 1 Unit AmpliTaqGold DNA polymerase (Applied Biosystems) and H₂O to a volume of 25 µl. Reactions were vortexed and centrifuged at each step. RT-qPCR reactions were run on a Corbett Rotorgene 6000 Real Time cycler (QIAGEN), with an initial 95°C step to activate the DNA polymerase enzyme, then 30-40 cycles of denaturation (95°C for 40 seconds), annealing (30 seconds at appropriate temperature (Table 2.2) and elongation (72°C for 40 seconds). Data was analysed using the DeltaCT method (Livak and Schmittgen, 2001).

2.2.3 PROTEIN ANALYSIS

2.2.3.1 Protein extraction

Protein was collected from tissue samples and cell culture using RIPA buffer. The RIPA buffer consisted of 50 mM TrisHCl pH 7.4, 150 mM NaCl, 0.1% SDS, 1 mM EDTA, 1% TritonX-100. To 10 ml of the RIPA buffer, 1 protease inhibitor cocktail tablet (Roche, Basel, Switzerland) was added. To extract tissue, samples were homogenised and sonicated (using ultrasonic probe) in RIPA buffer (100 µl per 0.5 g of tissue). Cell samples were immersed in RIPA buffer (200 µl on a 60% confluent 6 well plate). Extracted protein was stored in the RIPA buffer at -80°C until use.

2.2.3.2 Protein quantification

Protein quantification was performed using a Pierce BCA protein assay kit (ThermoFischer,
Rockford, IL, USA) as per manufacturer’s instructions. A standard curve of known concentrations was created using dilutions of bovine serum albumin (BSA) provided in the BCA kit. Concentrations of unknown samples were then extrapolated from this curve, giving a protein concentration in µg/ml.

2.2.3.3 Western blotting

Protein from supernatant, tissue and cells was separated by western blot using Mini-PROTEAN Tetra Electrophoresis system from Bio-Rad (Hercules, CA, USA). Protein was separated on a 10% SDS-PAGE gel, with a 4% upper gel to aid separation of proteins. 20-40 ng of protein was denatured at 100°C for 5 minutes and loaded onto the SDS-PAGE gel, for each individual experiment the total volume of protein loaded was consistent. Gels were run at 100V for approximately 2 hours with Kaleidoscope Precision Plus Protein Standard ladder (Bio-Rad) used as a visual guide to separation. Gels were run in SDS-running buffer (25 mM Tris, 200 mM glycine, 0.1% SDS). Proteins were then transferred to Amersham Hybond-P PVDF membrane (GE Healthcare) in transfer buffer (25 mM Tris, 200 mM glycine, 20% methanol) at 100V for 1 hour. The membrane was blocked in 12% skim milk in TTBS for 40 minutes and washes were performed in TTBS or TBS. Primary antibody incubation was done O/N at 4°C. HRP-conjugated secondary antibodies (Table 2.3) were used at 1:2000 dilution at RT for 3 hours. Protein bands were detected using Super Signal West Femto Kit (ThermoFisher) and visualised on a G-Box BioImaging system using GeneSnap software version 6.08.04 (Syngene, Cambridge, UK).

2.2.3.4 Membrane stripping and reprobing

To strip membranes and reprobe with loading control or other antibody, membranes were
<table>
<thead>
<tr>
<th>Antibody</th>
<th>Raised in</th>
<th>Supplier</th>
<th>Working concentration</th>
<th>Primary/Secondary</th>
<th>Conjugate</th>
</tr>
</thead>
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<td>Rabbit</td>
<td>Dako</td>
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<td>-</td>
</tr>
<tr>
<td>Phalloidin (anti-actin)</td>
<td>(Purified from) <em>Amanita phalloides</em> mushroom</td>
<td>Invitrogen</td>
<td>1:200</td>
<td>-</td>
<td>FITC</td>
</tr>
<tr>
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<td>Mouse</td>
<td>Sigma-Aldrich</td>
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<td>-</td>
</tr>
<tr>
<td>Rab5 (endosome marker)</td>
<td>Rabbit</td>
<td>Cell Signalling Technology</td>
<td>1:100</td>
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<td>-</td>
</tr>
<tr>
<td>Calnexin (ER marker)</td>
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<td>1:200</td>
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<td>-</td>
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<tr>
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<td>1:2000</td>
<td>Secondary</td>
<td>AlexaFluor 488</td>
</tr>
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</table>
incubated in stripping buffer (2% SDS, 100 mM β-mercaptoethanol [Sigma-Aldrich], 62.4 mM Tris, pH 6.7) for 15 minutes. They were then washed in TBS and blocked in 12% skim milk and reprobed with new primary and secondary antibodies as described above.

2.2.4 HISTOLOGY

2.2.4.1 Sample preparation and sectioning
Mouse tissue samples collected during experiments were processed for histology and immunohistochemistry as follows. Samples were placed in 4% paraformaldehyde O/N at RT followed by 75% ethanol O/N at RT. Bone samples were then decalcified for 1-3 days, depending on size and age, in 5% EDTA. When decalcified, the fixed samples were placed in cassettes and processed in a VIP-200 fluid transfer tissue processor (Leica, Wetzlar, Germany) and embedded in paraffin wax. Sections were cut using a Leica microtome at 4 µm thickness and mounted onto pre-cleaned Menzel-Glazer microscope slides (Braunschweig, Germany) for H&E staining. Tissue sections on slides were dried at 37°C O/N.

2.2.4.2 Haematoxylin and Eosin (H&E) staining
Sections were dewaxed and rehydrated with the following: 2 changes of xylene, 2 changes of 100% ethanol, 70% ethanol and 30% ethanol for 2 minutes each followed by water for 4 minutes. A six minute immersion in Lillie Mayer’s Haematoxylin (0.625% haematoxylin, 5% aluminium ammonium sulphate, 30% glycerol, 0.2% acetic acid) (Surgipath, Richmond, IL, USA) was followed by 15 seconds in 5% sodium bicarbonate solution. Sections were then dipped 8 times in 0.25% acid alcohol (HCl and 100% ethanol) and placed in sodium
bicarbonate solution with water rinses between these steps. After a 2 minute immersion in Eosin Y (1% eosin, 1% phlosine, 40% acetic acid in 95% ethanol: Surgipath) sections were again rinsed in water before being dehydrated in 70% ethanol followed by 2 changes of 100% ethanol. Finally, samples were incubated in 2 changes of xylene for 2 minutes and mounted in DEPX mounting media (Ajax Finechem).

2.2.5 POSITIVE CONTROL TISSUES

Positive control tissues were used in all experiments. For RT-qPCR experiments, mouse liver tissue was collected and processed as described in 2.2.2.1 and 2.2.2.2 to make cDNA. Liver was also used as a positive control in histology experiments and was processed as described 2.2.4

2.2.6 CELL CULTURE

2.2.6.1 General cell culture maintenance and solutions

Unless stated otherwise, all cells were grown in Dulbecco’s Modified Eagle Medium (DMEM) (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Sigma-Aldrich), 5% penicillin/streptomycin (Sigma-Aldrich) and 5% antibiotic:antimycotic (Sigma-Aldrich) at 37°C in 5% CO₂. MC3T3-E1 M cells were sourced from Dr. Maya Kansara (Ian Potter Foundation Centre for Cancer Genetics and Preventative Medicine, Peter MacCallum Cancer Centre, Melbourne, Victoria, Australia). MC3T3-E1 S cells were a gift
from Dr. Michael Cunningham (Department of Oral Biology, University of Washington, Seattle, WA, USA). MC3T3-E1 subclone 4 cells were sourced from American Type Culture Collection (ATCC) (Manassas, VA, USA) and grown in alpha minimal essential media (αMEM) with 10% FBS, 5% pen/strep. NIH3T3 cells were a gift from A/Prof Don Anson (Gene Technology Unit, Women’s and Children’s Hospital, North Adelaide, SA, Australia) and HEK293T cells were a gift from Dr. Cheryl Shoubridge (Department of Molecular and Genetic Pathology, SA Pathology, SA, Australia). Huh7 cells used as RBP4 controls were a gift from Prof Eric Gowans, WCHRI, Adelaide, SA, Australia.

All cells were passaged using trypsin-EDTA from Invitrogen. Briefly, media was removed from the cells and cells washed in sterile PBS to remove any traces of media. Trypsin (1x) was then added to just cover cells (eg 1ml in a T75 flask) and the flask placed at 37°C for 5 minutes. Media was then added to quench the trypsin (3:1 media:trypsin) and the media/cell cocktail removed to a new sterile 50 ml falcon tube and centrifuged at 1200g to pellet cells and remove trypsin. After trypsin was removed, cells were resuspended in fresh media and moved to a new culture flask. MC3T3-E1, HEK293T, Huh7 and NIH3T3 cells were split at 1 in 10. Primary cells of human and mouse origin were split 1 in 3.

### 2.2.6.2 Primary cell isolations

#### 2.2.6.2.1 Human cells

Human primary suture cells were isolated and cultures established from samples excised during corrective surgery by A/Prof Peter Anderson (Australian Craniofacial Unit, Adelaide, SA, Australia). Cells were isolated as described previously (Marie, 2003, Coussens et al., 2007). Maintenance of human primary cells was performed by multiple laboratory members.
AC115 cells were isolated from a patient with non-syndromic unicoronal craniosynostosis. AC124 cells were from a patient with non-syndromic metopic craniosynostosis and AC125 cells were from a patient with non syndromic sagittal synostosis. All patients were tested for known craniosynostosis causing mutations and were negative.

2.2.6.2.2 Mouse cells

Calvarial (parietal bone) and suture (PF, sagittal, coronal and lambdoid) primary cells were isolated by 3 different methods. All methods involved initial euthanasia of animals and dissection of suture and bone as described in Section 2.2.1.3. This process was performed in an Infinity class II biosafety cabinet (Esco, Hatboro, PA, USA) and samples washed in sterile PBS (Invitrogen) and DMEM during dissections in an attempt to maintain sterile conditions. For bone chip outgrowth (BCO) isolations samples were then homogenised into small pieces (approx 1mm²) and placed into the bottom of sterile 24 well plates (Nunc, Roskilde, Denmark) and allowed to briefly air dry to stick the sample to the bottom of the well. 0.5 ml of DMEM was then added to each well and cells incubated. Media was changed every 2-3 days and, when confluent, the cells passaged to a larger well/flask and the bone chip left to allow more cells to grow and migrate out of the chip.

Full digestion (FD) and partial digestion (PD) methods were based on methods described in ‘Bone Research Protocols’ (Helfrich and Ralston, 2003). Briefly, FD involved digestion of the bone/suture chips in collagenase solution (2 mg/ml collagenase II in DMEM):(Sigma) twice for 30 minutes at 37°C, followed by trypsin solution (0.25% trypsin EDTA in PBS) for 30 minutes at 37°C. Bone or suture fragments were then placed in 24 well plates and allowed to dry briefly before adding media and incubating. Media was changed every 2-3 days and,
when confluent, cells were passaged and new media added to the bone fragment to allow for outgrowth of more cells.

The FD protocol involved incubation of the suture/bone in digestion solution (0.25% Trypsin EDTA and 0.64 mg/ml collagenase II in DMEM) for 20 minutes at 37°C. Digestion solution was collected, as this was the fraction containing cells. This was repeated 3 times and digestion solution was centrifuged at 1200g to pellet cells and remove solution. Cells were resuspended in media and plated into 24 well plates. Digested bone remnants were discarded.

2.2.6.3 Osteogenesis assays

Osteogenesis assays were performed to test the mineralisation ability of cells in vitro. Osteogenesis assays involved plating $2.5 \times 10^4$ cells per cm$^2$ in a tissue culture plate. These cells were then maintained until confluent. When confluent, cells were induced using osteogenic media (OG), OG + dexamethasone (Dex) or DMEM + retinoic acid (RA). OG media was DMEM with 50 μM L-ascorbic acid phosphate magnesium salt (Sigma-Aldrich) and 5 mM glycerol-2-phosphate disodium salt (Wako, Germany); Dex was OG media with 100 μM dexamethasone (Sigma) and RA was DMEM media with 10 μM retinoic acid (Sigma-Aldrich). The first day of addition of induction media was considered day 0 and samples were collected at timepoints following that.

2.2.6.3.1 Alizarin Red S staining

Alizarin Red S staining was performed to visualise mineralisation. Media was removed and cells washed with PBS. Cells were then fixed using ice cold 70% ethanol for 10 minutes and washed again in PBS. To stain, cells were submerged in 0.1% Alizarin Red S (Sigma Aldrich)
in H₂O for 10 minutes. Alizarin Red S was then removed and cells washed in water. Cells were allowed to dry and images captured using a Nikon TE2000 inverted microscope and Nikon D40X camera.

2.2.6.3.1.1 **Alizarin Red S destain**

Levels of Alizarin Red S staining was quantified using a destain protocol. Wells that had been previously stained and dried were covered with a solution of freshly made 1:1 acetic acid:methanol. The solution was left on cells for 30 minutes at RT. The solution that had drawn out the yellow of the Alizarin Red S stain was then pipetted into a 96 well plate and measured in a Sunrise Touchscreen Colour Plate Reader at 450 nm using Magellan software (Tecan, Mannendorf, Switzerland). Absorbance readings were normalised to cell number.

2.2.6.3.2 **Protein and RNA collection**

RNA and protein samples were collected as described in Section 2.2.2.1 and 2.2.3.1.

2.2.6.4 **Cycloheximide treatment**

Human suture cells were plated on 70% ethanol sterilised coverslips at 1.5 x 10⁴ cells per cm² to be subconfluent. After settling overnight, they were treated with 10 mM cycloheximide (Sigma) in DMEM for 1 hour. Cells were then washed in 3 times 1x PBS for 5 minutes to remove cycloheximide. At desired timepoints, cells were fixed in 4% PFA for 30 minutes. Immunocytochemistry was performed as described in 2.2.7.
2.2.7 IMMUNOCYTOCHEMISTRY

Immunocytochemistry was performed as follows. Cells were grown on glass coverslips and when appropriate fixed in 4% PFA for 30 minutes. Cells were then washed 3 times in 1x PBS. Cells were permeabilised in 0.1% TritonX (Sigma) in PBS for 1 hour followed by a further 3 washes in PBS. Cells were then blocked for 1 hour in 5% serum from the animal in which the secondary antibody was raised; either normal rabbit serum (Dako), normal goat serum (Invitrogen) or normal horse serum (Santa Cruz). Primary antibodies were diluted in 5% serum (concentrations listed in Table 2.3) and cells incubated O/N at 4°C. After 3 washes in PBS, secondary antibody was added and incubated at RT for 1hr. Cells were then washed 3 times in PBS for 5 minutes and mounted in ProLong Gold Antifade (with DAPI) (Invitrogen). Slides were stored at -20°C. Images were captured using a Leica Leitz DMRB fluorescence microscope (Wetzlar, Germany) and Olympus DP72 camera (Center Valley, PA, USA). Images were processed using Cell^F Image Processing software (Olympus).

2.2.8 LENTIVIRAL PREPARATION

2.2.8.1 General solutions and reagents

Bacterial cultures were grown in Luria-Bertani (LB) broth and on LB agar. LB was made as described (Sambrook and Russell, 2001). Chloramphenicol and Ampicillin (Invitrogen) were used at a final concentration of 30 µg/ml and 50 µg/ml respectively. Competent DH5α E. coli for transformations were purchased from Invitrogen.
2.2.8.2 DNA preparations

2.2.8.2.1 Vector construction and production

Helper vectors, pcDNA3.1Tat (Tat), phCMV-G (G), phCMV-gagpol (gagpol) and phCMV-Revm/whv (Rev) were a gift from A/Prof Don Anson, as was the positive control vector pHIV-1SDmEF1a-eYFPv2 (eYFP) (Koldej et al., 2005). The overexpression vector for RBP4 pHIV-1SDmEF1a-hRBP4 was produced as follows. Human RBP4 (hRBP4) was amplified by PCR from a template purchased from GeneCopoeia (Rockville, USA) to be flanked by a 5’ KpnI and ClaI restriction enzyme site (5’ ATG GTA CCA TCG AT A CCG GTC GCC ACC ATG AAG T GG GTG TGG GCG 3’) and a 3’ NdeI restriction enzyme site (5’ AGA TCC ATA GCA TAT GCT A CTA CAA AAG GTT TCT TTC 3’). This PCR was performed at an annealing temperature of 60°C with an elongation time of 2 minutes.

Empty pHIV-1SDmEF1a vector and purified hRBP4 PCR product were digested with NdeI and ClaI to prepare the ends for ligation. Digestion was performed for 1hr at 37°C. Ligation was performed with T4 DNA ligase (New England Biolabs) as per manufacturer’s instructions. Ligated DNA was then transformed via a 42°C heatshock for 45 seconds and then 2 minute incubation on ice into DH1α cells under selection.

2.2.8.2.2 DNA production and purification

Vectors were transformed into competent DH1α E.coli cells and were purified using QIAGEN miniprep and midiprep kits (Qiagen) as per manufacturer’s instructions. Briefly, O/N cultures were grown in LB broth at 37°C with gentle shaking. After this, bacteria were pelleted and lysed. Purification using the midiprep kits allowed for larger scale DNA preparation. For a miniprep of DNA, 3 mls of culture was grown and for a midiprep, 50 mls of culture was
2.2.8.2.3 Product size and sequence verification

After purification, DNA was verified by size of digestion products as follows. Restriction enzymes NotI, BamHI, NdeI, ClaI and XhoI were all purchased from New England Bioloabs. Enzyme digestions were set up with 1 μg DNA, 1x NEB reaction buffer and 1 unit of restriction enzyme. NotI and BamHI were digested with NEB reaction buffer 3 and NdeI, ClaI and XhoI were digested with NEB reaction buffer 4. All reactions, apart from NdeI, were also set up with 100 μg/ml bovine serum albumin (BSA). Digestion reactions were incubated for 1hr at 37°C. Products were then separated on a 1% agarose gel containing 0.05 μl/ml of 10,000X GelRed Nucleic acid stain (Biotum, Hayward, CA, USA). Products were then visualised using a GBox (Syngene).

The RBP4 sequence was verified by sequence analysis. Reactions were set up with 12 μl water, 3 μl 5x sequencing buffer, 1 μl 10 μM primer, 1 μl BigDye 3.1 and 1 μl DNA. This was run with an initial step of 94°C for 5 minutes followed by 30 cycles of 96°C for 10 seconds, 50°C for 5 seconds and 60°C for 4 minutes. DNA was then precipitated using 10% 3 M sodium acetate in ethanol for 1 hr and pelleted with a 1 hour centrifugation at 15,000g. Supernatant was then removed and pellets allowed to air dry. This product was then sent to IMVS Molecular Pathology Sequencing, Adelaide, SA, Australia.

2.2.8.3 Lentivirus Production

2.2.8.3.1. Cell culture

Lentivirus was produced in HEK293T cells as described in Kodlej (Koldej et al., 2005). Virus
was tested on an NIH3T3 cell line, WT mouse coronal primary cells, the MC3T3-E1 subclone 4 cell line and human primary coronal suture cells. All cells were maintained in DMEM with 10% FBS and 2% pen/strep. Cells were incubated at 37 °C and 5% CO₂.

2.2.8.3.2 Transfection of viral vectors and collection of virus

HEK293T cells were plated at 80-90% confluence in a 6 well cell culture dish in DMEM with 10% FBS (antibiotic-free). Vectors were transfected into HEK293T cells using lipofectamine 2000 (Invitrogen). 10 µl of lipofectamine (in 200 µl Optimem media) was used per well. The 4 helper vectors that are required for structural assembly of the virus (Koldej et al., 2005), plus the hRBP4 or eYFP expression vectors were transfected at the following concentrations; RBP4/eYFP, 7.5 µg; G, 375 ng; Tat, 150 ng; gagpol, 750 ng; Rev, 150 ng. Virus was also made without the expression vector (4 helper vectors only) as a negative control to ensure that any changes seen in cells were due to expression changes caused by the expression vector, not a reaction to the process of lentiviral infection. Cells were left to incubate and media changed after 16 hours. After an additional 48 hours, supernatant was collected and filter-sterilised through a 0.22 µm filter, aliquoted and stored at -80º.

2.2.8.3.3 Infection of cell culture

Cells were infected with 300 µl of lentiviral supernatant and polybrene (Sigma) at a final concentration of 8 µg/ml. Cells were left with supernatant overnight before replacing with normal media. After 48 hours, infection efficiency was visualised by immunocytochemistry (Section 2.2.7) and PCR (Section 2.2.2.3) for RBP4 infected cells and by flow cytometry and fluorescence for eYFP infected cells. eYFP was visualised 48 hours after infection on a Nikon TE2000 inverted microscope. Cells were prepared for flow cytometry analysis by trypsinising
and resuspending approximately 10,000 cells in FACSfix (2% PFA in PBS). FACS was run on a BD FacsAria III cell sorter and analysis performed on FACS Diva software (BD biosciences, San Jose, CA, USA). Osteogenesis assays were performed as described in Section 2.2.6.3.

2.3 STATISTICAL ANALYSIS

Statistical analysis was performed using either a one or two-tailed student’s unpaired t test where appropriate. Significant difference was considered to be a value of p<0.05. Analysis was performed using Microsoft Excel for Windows and Graphpad Prism 5 for Windows.
Chapter 3

Analysis of suture fusion and gene expression in wildtype and Twist1 heterozygous knockout mice; a model of Saethre-Chotzen syndrome.
3.1 INTRODUCTION

To fully identify the aetiology of complex disorders such as craniosynostosis, an understanding of the normal processes involved must be achieved. The process of natural suture fusion that occurs in humans is still poorly understood. Elucidation of this process may assist in comprehending the premature fusion seen in craniosynostosis.

The rodent posterior frontal (PF) suture is often used as a model of the natural fusion process as it is known to fuse early in the life of a mouse, unlike other sutures which remain patent (Bradley et al., 1996, Sahar et al., 2010). Although not present in humans, the PF suture in rodents is likened to that of the metopic suture in humans, which also fuses early in life (Bradley et al., 1997, Song et al., 2004b). This fusion of the PF suture was identified in early suture research from Moss, showing the PF fusion of rats (Moss, 1962). Previous research has suggested a distinct pattern of PF suture fusion, being in an anterior to posterior direction, and occurring between 25 and 45 days of age (Bradley et al., 1996, Wadhwa et al., 2007). However recently, there has been evidence to suggest that the fusion is not necessarily this organised in its behaviour (Stadler et al., 2006). Although also reporting patent PF sutures at day 22, and fusion by day 45, closer analysis has shown that while the PF suture may appear fully fused there are often regions along the suture that have not fused (Stadler et al., 2006, Gosain et al., 2011). This mimics the behaviour of sutures in humans with craniosynostosis which can fuse unpredictably and sporadically along their length (personal communication, A/Prof Peter Anderson). In contrast, the other sutures in the mouse skull remain patent for the duration of the life of the mouse. Examples can be seen in mice 250 days old which have patent coronal sutures (Bradley et al., 1996). This provides an internal patent control for
studies looking at the fusion of sutures in rodent models.

A majority of this research has been performed in CD-1 mice, although C57BL/6, CF-1 mice and rats have been analysed on occasion (Bradley et al., 1996, Greenwald et al., 2001, Warren et al., 2008, Slater et al., 2009). The Twist1 heterozygous mice used in the present study were developed on a C57BL/6 background. It is known that animals from varying genetic backgrounds, in particular mice that have been repeatedly inbred, display differing rates of growth and development (Howard, 1941), (Council, 1995).

Most of this research into fusion of the PF suture has been histologically based. More recently however, the introduction of computerised scanning technology has allowed for high resolution images of bone growth without the need for invasive procedures such as sectioning of tissues. Computed tomography (CT) scans have been used as a non-invasive diagnostic tool for patients with craniosynostosis for many years now. They allow detailed 3D images to be created, assisting in the diagnosis of abnormal bone conditions and allowing greater planning for any surgical interventions required. These 3D CT scans have been shown to be a valuable and reliable method for analysing suture morphology (Vannier et al., 1989, Furuya et al., 1984, Warren et al., 2008).

MicroCT works on the same principles as large scale CT scanning, with the difference being that microCT scans are performed on smaller samples (micrometre scale). Initially developed for scanning for faults in ceramic materials there is now a focus on its use for analysis of defects and growth in bone. Commonly used in the study of trabecular bone, there have also been studies utilising the technique for analysis of suture and calvarial growth and defects.
In this study a mouse model of the Saethre-Chotzen craniosynostosis syndrome was used. These mice were developed by targeted disruption of the Twist1 gene (Chen et al., 2007) and selective breeding with an X linked CMV-Cre transgene (Bildsoe et al., 2009). More information about the role of TWIST1 in human craniosynostosis can be found in Chapter 1 (section 1.7). The Twist1+/− mice have been reported to exhibit coronal suture fusion by day 30 (Carver et al., 2002), although recent research has questioned this timing (Behr et al., 2011).

In this chapter the fusion of PF, coronal and sagittal sutures in WT C57BL/6 mice, and Twist1+/− mice were compared. RT-qPCR analysis was then used to investigate changes in gene expression over the time of suture fusion and osteogenesis, focussing on Osteocalcin and Alkaline phosphatase as markers of osteoblast differentiation, and on Rbp4 and its receptor Stra6.
3.2 RESULTS

3.2.1 POSTERIOR FRONTAL FUSION BEGINS TO OCCUR AS EARLY AS DAY 10 IN C57BL/6 MICE

Initial microCT analysis of the PF sutures demonstrated PF suture fusion as early as day 10 in WT C57BL/6 mice (Figure 3.1). This was confirmed by H&E analysis of the same samples. The fusion at day 10 appeared sporadic along the length of the suture, rather than contiguous in an anterior-posterior manner. By day 15 the majority of the length of the PF suture was fused and there appeared to be a trend of the posterior section of the suture displaying a higher level of fusion than the anterior sections. As seen in the histological photomicrographs, fusion occurred at the endocranial surface whereas at day 15 the ectocranial surface was still open in all mice (Figure 3.2).

3.2.2 β-ACTIN IS A SUITABLE REFERENCE GENE

Three genes were tested for their suitability as a reference gene for RT-qPCR analysis. For this, 3 groups of samples were analysed to assess variance in expression of these candidate genes. Hypoxanthine-guanine phosphoribosyltransferase (Hprt) had lower apparent expression in the samples tested, meaning higher Ct values and also had greater variance in expression in samples (Figure 3.3). Cyclophilin A (CypA) had higher expression than Hprt, however, in the suture samples there was a greater variation in Ct values. This indicated less stability. The most stable gene was β-actin. Expression of β-actin was higher in all 3 groups
Figure 3.1: Representative images displaying posterior frontal suture fusion in C57BL/6 mice. Representative microCT images from mice aged 5, 10 and 15 days and H&E histological images from the same mice. Red box on microCT image indicates approximate location of the fused H&E section and the green box indicates location of unfused section. N=3 mice per age group. Sections were cut on the coronal plane in an anterior to posterior direction, 20 sections per mouse were analysed. Microscopic images are 200x magnification.
Figure 3.2: High magnification micrograph images of murine suture samples displaying different cell types. A) H&E stained section representing the PF suture of a 15 day old mouse and its surrounding bone. Black lines indicate regions of bone. Dashed lines show region which has been magnified. B) A higher magnification image identifying the different cell types and cranial bone commonly seen in murine sutures.
Figure 3.3: Selection of a suitable reference gene for RT-qPCR analysis of gene expression. RT-qPCR was performed to test 3 possible reference genes. Samples tested comprised tissue (T), cells (C) and sutures (S). A) Melt curve analysis of RT-qPCR results. B) Graphical representation of Ct values from RT-qPCR. Tissue samples were liver, brain and kidney; cell samples were MC3T3-E1 subclone 4 samples from an osteogenesis assay and suture samples were samples from the PF, sagittal and lambdoid suture. cDNA for each group was produced using the same amount of RNA. N=8 per group.
of samples tested and showed the least variability, particularly in the suture samples (Figure 3.3b).

3.2.3 *TWIST1*± MICE EXPRESS LESS *TWIST1* THAN THEIR WT LITTERMATES

Genotyping of pups from *Twist1*± and C57BL/6 parents identified the presence of the WT *Twist1* gene in all mice (Figure 3.4a) and of the disrupted *Twist1* allele in *Twist1*± mice (Figure 3.4b). Despite the disruption of one allele in *Twist1*± mice, these mice still expressed native *Twist1* mRNA from the unaffected allele. The amount of *Twist1* mRNA detected in these mice however, was significantly less than that seen in WT littermates (Figure 3.4c).

3.2.4 *TWIST1*± MICE EXHIBIT CORONAL FUSION AS EARLY AS DAY 3

MicroCT scanning was used to assess suture fusion in *Twist1*± mice and their WT littermates. From the microCT scans, WT mice appeared to have patent sagittal sutures at all timepoints, as did the *Twist1*± mice (Figure 3.5). There was some intermittent fusion in the PF suture of WT mice at day 10. In the *Twist1*± mice PF fusion also occurred in some of the mice at day 10. The main difference observed by microCT scans between WT and *Twist1*± mice was the coronal suture fusion in the *Twist1*± mice. This appeared to begin at day 3 and by day 10 the coronal suture in these mice was completely obliterated. In day 10 microCT scans of WT mice the coronal sutures appear to be fusing but histology confirms that although experiencing ‘contraction’ they are not fused and they still display 2 distinct bone fronts (Figure 3.6).
Figure 3.4: *Twist1* expression and genotyping of *Twist1*<sup>−/−</sup> mice and their WT littermates. Genotyping PCR reactions using tail snip DNA were run for 35 cycles at 55°C for 15 weaners. PCR products were then visualised by gel electrophoresis. A) WT bands were approximately 600 bp when run on an agarose gel. B) Heterozygous *Twist1* knockout bands. Heterozygous mice were identified by the presence of an additional 110 bp band on the agarose gel. Green arrows indicate examples of WT mice and red arrows indicate *Twist1*<sup>+/−</sup> mice. M indicates 100 bp marker lane. C) *Twist1* gene expression from coronal and parietal bone measured by RT-qPCR. N=3 mice per group. Bars represent average + SEM. * indicates significantly different to WT. P<0.05.
Figure 3.5: Representative MicroCT images comparing WT and Twist1+/− mice sutures. Images are representative of n=5 mice per group. Yellow arrows indicate regions of fused suture. MicroCT scans conducted with the same threshold for day 3-10 mice, day 0 mice conducted with a lower threshold. PF, posterior frontal; C, coronal; S, sagittal and L, lambdoid. N=5.
Figure 3.6: Representative H&E staining of WT and Twist1+/- coronal sutures. Sagittal sections of coronal sutures were stained with H&E to confirm microCT images. Arrows indicate regions of bony fusion. Images captured at 200x magnification. All images shown with ectocranial surface at the top.
This histology also confirmed that Twist1\(^{+/−}\) mice exhibit coronal suture fusion as early as day 3. The PF sutures that appeared fused in day 10 microCT scans were confirmed to have begun fusion by histology. An example of this can be seen in day 10 WT PF histology (Figure 3.7). Day 0, 3 and 5 PF sutures were patent in both groups of mice, as were all examples of sagittal sutures from all timepoints in both experimental groups (Figure 3.8).

### 3.2.5 PARIETAL BONE THICKENS WITH AGE

When compared histologically, the thickness of parietal bone significantly increased from day 0 to day 10 (Figure 3.9). This increase was 2 fold. There was no difference in thickness of the parietal bone between WT and Twist1\(^{+/−}\) mice of the same age.

### 3.2.6 OC EXPRESSION INCREASES AS SUTURES AND BONE MATURE

After microCT and histological confirmation of suture fusion in Twist1\(^{+/−}\) mice, RT-qPCR was performed to assess gene expression changes during this suture fusion. In all suture types analysed, as well as in parietal bone, Oc expression increased over time (Figure 3.10). In all cases, by day 10, the sutures and bone expressed a significantly higher level of Oc than at day 0. In the sagittal suture, levels remained relatively unchanged until day 10 (Figure 3.10c). In the PF suture Oc expression increased steadily in WT mice, with a significantly higher expression in day 5 mice. By day 10, the Twist1\(^{+/−}\) mice had the same level of expression of
Figure 3.7: Representative H&E staining of WT and Twist1<sup>+/−</sup> posterior frontal sutures. Sagittal sections of posterior frontal sutures were stained with H&E to confirm microCT images. Arrow indicates regions of bony fusion. Images captured at 200x magnification. All images shown with ectocranial surface on top.
Figure 3.8: Representative H&E staining of WT and Twist1\(^{+/-}\) sagittal sutures. Coronal sections of sagittal sutures were stained with H&E to confirm microCT images. Images captured at 200x magnification. All images shown with ectocranial surface on top.
Figure 3.9: Changes in thickness of Parietal bone in mice. A) Representative H&E staining of WT and Twist1+/− Parietal bone. Coronal sections of parietal bone were stained with H&E to assess changes in bone thickness. Images captured at 200x magnification. B) Graphical representation of the change in parietal bone thickness. Average of 6 measurements at regular intervals along each section. N=5 mice per group. # represents significantly greater than Day 0 WT. P<0.05.
Figure 3.10: Relative osteocalcin (OC) expression in WT and Twist1^{+/−} sutures measured by RT-qPCR.  
A) Oc expression in posterior frontal suture.  
B) Oc expression in coronal suture.  
C) Oc expression in sagittal suture.  
D) Oc expression in parietal bone.  
All values presented are average + standard error of the mean and data analysed using the Delta CT method.  
Samples normalised to β-actin and control was day 0 WT sample.  
# indicates significantly different to day 0 WT value.  
* indicates significantly different to comparable WT value.  
N=5.  P<0.05.
Oc as the WT mice (Figure 3.10a). In both the coronal suture (Figure 3.10b) and parietal bone (Figure 3.10d) Oc increased steadily with age and there was no significant difference between the groups.

3.2.7 ALKALINE PHOSPHATASE EXPRESSION IN CORONAL SUTURES

Alp expression in the PF suture remained steady at days 0, 3 and 5 (Figure 3.11a). There was a decrease in Alp expression in both WT and Twist1+/− at day 10, but this was not significant. There was no significant difference between Alp expression in WT and Twist1+/− mice in the PF suture at any timepoint. In the coronal suture, Alp was unchanged at day 3, compared to day 0, but at day 5 and 10 expression increased (Figure 3.11b). This occurred in both WT and Twist1+/− mice. The increase seen in Twist1+/− mice was significant at days 5 and 10, however, the increase in the WT mice was not. Expression of Alp in the sagittal suture remains relatively unchanged over all timepoints other than in the Twist1+/− mice where there was a significant decrease at day 3 (Figure 3.11c). The expression recovered at day 5. There was no significant difference between WT and Twist1+/− Alp expression at any timepoint in the sagittal suture. Parietal bone Alp expression did not differ between WT and Twist1+/− at any timepoint nor was there any significant difference over time (Figure 3.11d).
Figure 3.11: Relative alkaline phosphatase (Alp) expression in WT and Twist1 +/- sutures measured by RT-qPCR.  
A) Alp expression in posterior frontal suture.  
B) Alp expression in coronal suture.  
C) Alp expression in sagittal suture.  
D) Alp expression in parietal bone.  
All values presented are average + standard error of the mean and data analysed using the Delta CT method.  
Samples normalised to β-actin and control was day 0 WT sample.  
# indicates significantly different to day 0 WT value.  N=5.  P<0.05.
3.2.8 STRA6 EXPRESSION INCREASED DURING OSTEOGENESIS

Stra6 expression in the PF suture only exhibited a significant increase at day 10 in Twist1\(^{+/-}\) mice, whereas WT mice exhibited a small but non-significant increase at day 10 (Figure 3.12a). The coronal suture exhibited a significant increase in both WT and Twist1\(^{+/-}\) mice at day 10 (Figure 3.12b). All other time points remain unchanged. Apart from a significant increase at day 3, Stra6 expression in the WT sagittal suture remained unchanged (Figure 3.12c). This differed from the Twist1\(^{+/-}\) sagittal suture which exhibited a steady significant increase of expression to day 10. There was no difference in Stra6 expression between WT and Twist1\(^{+/-}\) mice in parietal bone (Figure 3.12d). There was however a steadily increasing expression of Stra6 in both groups at all time points.

3.2.9 RBP4 DECREASES DURING OSTEOGENESIS

Rbp4 expression in the PF suture remained steady from day 0 to 5, but at day 10 there was a significant decrease in expression in both WT and Twist1\(^{+/-}\) animals (Figure 3.13a). There was no difference between the expression levels in WT and Twist1\(^{+/-}\) at any timepoint in the PF suture. Rbp4 expression in the coronal suture decreased significantly in Twist1\(^{+/-}\) mice during suture fusion from day 3 (Figure 3.13b). Twist1\(^{+/-}\) mice have significantly less Rbp4 than WT counterparts at days 3 and 5. Expression of Rbp4 in WT mice remained unchanged at days 3 and 5 but then exhibited a significant decrease in expression at day 10. Rbp4 expression remained unchanged in the sagittal sutures of WT mice, but in the Twist1\(^{+/-}\) mice there was a significant decrease at day 5 and 10 (Figure 3.13c). Rbp4 expression in parietal bone
Figure 3.12: Relative *Strab* expression in WT and *Twist1*+/− sutures measured by RT-qPCR. A) *Strab* expression in posterior frontal suture. B) *Strab* expression in coronal suture. C) *Strab* expression in sagittal suture. D) *Strab* expression in parietal bone. All values presented are average ± standard error of the mean and data analysed using the Delta CT method. Samples normalised to β-actin and control was day 0 WT sample. # indicates significantly different to day 0 WT value. * indicates significantly different to comparable WT value. N=5. P<0.05.
Figure 3.13: Relative $Rbp4$ expression in WT and $Twist1^{+/−}$ sutures measured by RT-qPCR. A) $Rbp4$ expression in posterior frontal suture. B) $Rbp4$ expression in coronal suture. C) $Rbp4$ expression in sagittal suture. D) $Rbp4$ expression in parietal bone. All values presented are average ± standard error of the mean and data analysed using the Delta CT method. Samples normalised to $β$-actin and control was day 0 WT sample. # indicates significantly different to day 0 WT value. * indicates significantly different to comparable WT value. N=5. P<0.05.
decreased in all samples over time (Figure 3.13d). There was no difference between the WT and Twist1+/− mice at any timepoint.

3.3 DISCUSSION

In this chapter suture fusion was assessed in WT mice and a mouse model of Saethre-Chotzen syndrome. Gene expression was also analysed to test if there was a link between suture fusion and Rbp4, and its receptor, Stra6.

The PF suture in mice is a naturally fusing suture making it a useful model of physiological suture fusion (Greenwald et al., 2000b). In this study, microCT analysis of the PF suture in C57BL/6 mice was able to detect regions of suture fusion, which were confirmed by histological analysis. Fusion in the PF suture was identified from 10 days of age in these mice. The timing of suture fusion differed from previous research, which has demonstrated fusion between the ages of 25 and 45 days in CD-1, CF-1 and C57BL/6 mice (Bradley et al., 1996, Greenwald et al., 2000b) and incomplete fusion at day 45 in another report on CD-1 mice (Recinos et al., 2004).

In the case of craniosynostosis research, it is also important to use a pathological model of suture fusion, mimicking the events that occur during premature fusion seen in humans. The heterozygous Twist1 knockout mice are a model of Saethre-Chotzen syndrome (Bourgeois et al., 1998) in which only one allele is affected (Paznekas et al., 1998). Initially these mice were earmarked as a suitable model for the syndrome due to hindlimb abnormalities and rapid
ossification of the parietal bones (Bourgeois et al., 1998). Further analysis confirmed their suitability as a model of Saethre-Chotzen by demonstrating the coronal suture fusion, similar to that seen in humans with the condition (Carver et al., 2002). In that study, the authors described incomplete fusion of coronal sutures by day 30 and asymmetry of the coronal suture. Fusion of the coronal suture in Twist1+/− mice occurred from day 3 in the present study. This differed from the previous study which identified coronal suture fusion in Twist1+/− mice after day 30, although they do make mention of the presence of fusing sutures in mice younger than 30 days (Carver et al., 2002). In WT mice the coronal suture is still patent at 250 days (Bradley et al., 1996).

In both the C57BL/6 WT and Twist1+/− mice used here, fusion of the relevant sutures was noted earlier than in previous studies (Bradley et al., 1996, Carver et al., 2002, Chen et al., 2007). The seemingly early onset of the fusion of the sutures seen in the mice used in the present study could be contributed by many factors. Premature fusion in both human and animal models has been attributed to differences in dietary teratogens (Gardner et al., 1998) and foetal head constraint (Higginbottom et al., 1980, Graham et al., 1980). The most likely cause of the early fusion seen in this study however, is the strain of the mice.

In the majority of research into PF suture fusion, groups have used CD-1 mice. On occasion, however, C57BL/6 mice have been used (Bradley et al., 1996). The fusion seen in the mice used in this study differed significantly from previous reports of PF suture fusion in C57BL/6 mice. To explain this difference, it must be considered that inbreeding of mice at different institutions can alter the phenotype (Beck et al., 2000). The C57BL/6 mice used in the present study were sourced from within Australia, whereas in the study by Bradley et al. (Bradley et
al., 1996) the C57BL/6 mice were from Maine, USA. Constant inbreeding of mice strains, as occurs in the majority of animal facilities, has been shown to result in changes in genomic copy number (Watkins-Chow and Pavan, 2008) and increased telomere length (Manning et al., 2002) in C57BL/6 mice. Inbreeding has also had an effect on behaviour in mice (Nevison et al., 2000, Koide et al., 2000), raising a question of repeatability of experimental procedures in different laboratories (Wahlsten et al., 2006). The mice used in the present study have been bred at a local facility independently from the original C57BL/6 populations for many generations. The chances for these changes in the strain in comparison to the mice used in other studies, therefore, are high.

It was also confirmed by both microCT and histology that the PF sutures of the C57BL/6 mice did not fuse in a progressive anterior to posterior fashion, with regions appearing to fuse randomly along the suture. This finding is consistent with the pattern of fusion that occurs in human sutures, rather than from one end to another and is supported by other recent research in an analysis of the PF suture in CD-1 mice (Stadler et al., 2006).

In the case of the Twist1+/− mice it is possible that breeding our genetically modified mice into a C57BL/6 background may have accelerated the fusion of sutures. It is well known that mouse genotype can affect how genetic manipulations manifest. Analysis of a mouse model for the craniofacial disorder, Treachers Collins syndrome, has demonstrated significant differences in the phenotype of genetically modified mice bred through different mouse strains (Dixon et al., 2004). In mice bred on a C57BL/6 background, embryonic lethality with neural tube defects, eye defects, exencephaly and absence of the parietal, frontal and interparietal bones has been reported. A similar phenotype was seen in mice on a CBA/Ca background.
However, when the mutation was on a DBA/1 or Balb/c background, the offspring were viable, with no evidence of the developmental abnormalities seen in the C57BL/6 and CBA/Ca mice. The Twist1+/− mice were bred into a C57BL/6 background in the present study to emulate the original background of the mice, which were developed in C57BL/6 blastocysts and bred through C57BL/6 females (Chen et al., 2007). The mice used in the study by Carver et al. were bred on a mixed C57BL/6 and 129S1 background, but there was no mention of the percentage of the background (Carver et al., 2002). It is possible that breeding the Twist1+/− mice onto a different strain background may have delayed the onset of coronal suture fusion.

A recent study has also provided insight into the process of coronal suture fusion in the Twist1+/− and supported out finding of coronal fusion prior to 30 days as reported in the original publication. Twist1+/− in the recent study displayed coronal suture fusion begun at day 9 and was attributed to the appearance of chondrocytes and the resultant endochondral ossification (Behr et al., 2011). The early fusion in this study when compared to the orginal was attributed to different experimental techniques, and in light of the results in the present study, it is likely that the analysis in this original study was not incorrect, but rather incomplete.

Selection of a housekeeping gene for gene expression analysis is an important factor to consider when looking for changes in gene expression, particularly if the changes being analysed are small. A variety of papers have shown that a housekeeping gene that may be suitable for one tissue may not be suitable for another (Dheda et al., 2004, Silver et al., 2006). In this study 3 candidate housekeeping genes were tested. CypA, Hprt and β-actin all showed good stability in samples from liver, brain, kidney and MC3T3-E1 cell culture. In the suture
samples, *Hprt* showed high variation in Ct values from different suture samples of the same concentration and was excluded. *CypA* had reproducible expression in suture samples, however reports of the interaction between *CypA* and retinoic acid in neuronal development raised concerns of its suitability as a reference gene in this study of RBP4 (Song et al., 2004a). Additionally, it has been shown that *CypA* may play a role in metabolosis in the liver, again showing a risk of interference with retinol signalling (Rhoads et al., 2003). *β-actin* was chosen as the most suitable reference gene as it showed the smallest Ct variation between samples.

Gene expression analysis of osteoblast marker osteocalcin (also known as BGLAP; bone GLA protein) showed an increase in all examples of sutures and bone measured here. It is important to note here that there are 2 *OC* genes, *OC1* and *OC2*, however the majority of published research does not distinguish between the two, suggesting that they may play similar roles (Yousfi et al., 2002, Ignelzi et al., 2003, Lin et al., 2007, Kapustin and Shanahan, 2011). The primers detected both isoforms. An increase in *Oc* during osteogenesis was expected as it has been reported to be expressed by terminally differentiated osteoblasts (Hall and Miyake, 2000). It is also commonly used as a marker of osteogenesis and osteoblastogenesis (Yousfi et al., 2001, Nakamura et al., 2009). It was expected however, that in the case of the coronal suture, where the *Twist1<sup>+/−</sup>* mice exhibit suture fusion unlike the WT mice there would be a greater difference in the expression of *Oc*. The fact that the expression levels in the WT and *Twist1<sup>+/−</sup>* mice at any one timepoint were comparable may be due to a few different reasons. Calvarial osteoblasts cultured from patients affected with Saethre-Chotzen syndrome have shown dysregulation of *OC* expression (Yousfi et al., 2001). In that study, a surprising result was seen where, despite having increased capacity for bone growth and osteogenesis, cells
expressed less \(OC\) than normal during this process. This was further analysed and showed to be related to Runx2 inhibition (Yousfi et al., 2002). From this, the expectation could be that during osteogenesis, a lower level of \(Oc\) should be seen in the \(Twist1^{+/−}\) sutures. A lower level of \(Oc\) was seen at day 5 in the PF and sagittal suture of \(Twist1^{+/−}\) compared to WT, however there was no difference in the other timepoints.

Previously, it was believed that \(OC\) was only expressed in osteoblasts (Mariani et al., 1989), however more recent studies have reported more widespread expression in the liver, brain and muscle of rats (Fleet and Hock, 1994, Thiede et al., 1994). It has also been detected in the osteocytes at the osteogenic fronts of sutures by immunohistochemistry (Coussens et al., 2007). During the extraction of the suture for processing for RT-qPCR it was inevitable that some of the surrounding bone and tissue was also collected. As seen from the histological pictures, particularly in day 5 and 10 coronal sutures, the bone can overlap significantly over the suture. Although great care was taken during dissection of the sutures, with bone trimmed as much as possible, it is not possible to trim this overlapping bone from the suture tissue. Thus, along with suture there may also be contaminating cells from bone. The expression pattern of \(Oc\) seen in parietal bone, with a steady significant increase to day 10 may arguably have influenced the results seen for \(Oc\) in our sutures, particularly the sagittal, which despite remaining patent, exhibited a significant upregulation of \(Oc\) at day 10.

Ideally techniques such as laser microdissection could enable more precise collection of suture tissue. This would allow for isolation of the cells of interest, in this case the mesenchymal tissue in the suture, and minimization of contaminating bone. This approach was trialled for isolation of suture tissue alone but was unsuccessful due to technical difficulties associated
with handling the small tissue pieces dissected from the animals. Another alternative, or complementary experiment that could be performed is in situ hybridisation. The use of in situ hybridisation would enable localisation of RNA expression, helping to provide an estimate of Oe expression in suture cells versus parietal bone.

In addition to osteocalcin, a marker of terminally differentiated osteoblasts, alkaline phosphatase (ALP) was also measured. ALP is commonly used as a marker of the early stages of osteoblast differentiation and osteogenesis (Braga et al., 2004, Huang et al., 2007, Stein et al., 1990, Risteli and Risteli, 1993). The presence of Alp expression in the sutures is controversial, with reports suggesting that expression is absent in the coronal suture (Markens and Oudhof, 1978) but present in the PF and sagittal sutures in rats (Winograd et al., 2001). The data from the present study showed expression of Alp in all sutures and parietal bone. A significant increase in Alp in the coronal sutures of Twist1+/- mice at day 5 and day 10 in association with the observed bony fusion was observed. This may indicate that during the fusion of these sutures the differentiation of pre-osteoblastic cells is accelerated. This also correlates with previous research which reported a significant upregulation of Alp during the fusion of rat coronal sutures (Winograd et al., 2001). In the other sutures however, there was little change in Alp expression at any point. This was not unexpected as those sutures do not fuse and hence there may not be a large amount of osteoblastogenesis occurring. At any one time in a suture there is a milieu of different cell types at different stages of differentiation. Additionally, the timepoints measured in the present study represent early stages of skull growth. Mice do not reach skeletal maturity until approximately 4 weeks of age (Squire, 2004; Haston et al. 2008). Therefore, these timepoints may be too early to see the downregulation that is expected at later stages of osteoblast differentiation, although the
results presented here suggest it is possible that these mice may reach skeletal maturity earlier than previously reported.

STRA6 is the cellular receptor for serum RBP4 (Kawaguchi et al., 2007). Little research has been done on Stra6 into its possible actions in bone and sutures. Stra6 expression has been seen in developing limb buds of mice where it was thought to be playing a role in the control of endochondral ossification (Chazaud et al., 1996). The data presented in this thesis show that Stra6 is expressed in mice PF, sagittal and coronal sutures, as well as in parietal bone. In all sutures analysed, and in bone, Stra6 expression increased over time. In the PF and sagittal sutures, Twist1+/− mice expressed a significantly higher level of Stra6 at day 10 than their WT counterparts. The expression of Stra6 in the suture and parietal bone tissue indicates that these tissues are capable of responding to serum Rbp4 and retinol. Recent research in an animal model of the fatal Matthew-Wood syndrome in which STRA6 is mutated demonstrated that Stra6 can function as a bidirectional transporter of retinol and RBP4 (Isken et al., 2008). The presence of Stra6 on suture cells and in bone is an exciting discovery, in that these cells may be both responding to Rbp4 and retinol in the serum, but may also contribute to the levels of retinol and Rbp4 in the body.

Importantly, the results in this chapter demonstrate that Rbp4 is expressed in all sutures and in parietal bone in post-natal skull development in mouse. The Rbp4 expression profile varied in the different sutures over time. Despite this the overall trend was for a decrease across the timepoints in both suture and bone. There is no reported change in the parietal bone of Twist1+/− mice so the fact that there was no difference between the Twist1+/− and WT mice was not surprising. The parietal bone samples were taken from the centre of the bone, taking care
to avoid all sutures. Between day 0 and day 10 the parietal bones increased 2 fold in thickness. The thickening corresponded to a decrease in Rbp4 expression. This correlation could indicate a role for Rbp4 in parietal bone growth whereby the decrease in Rbp4 expression is allowing increased retinol availability, as hypothesised in sutures. In the sagittal suture the histology and microCT confirmed that this suture remained patent at all times points as expected. However, a decrease in Rbp4 expression was noted in the Twist1+/− mice from day 5. This was unexpected and may be attributed to parietal bone contamination as there was significant overlap of the osteogenic fronts at these timepoints and they experienced thickening as seen in the parietal bone samples.

In the PF suture, the Rbp4 levels were maintained through days 0-5. By day 10 however, there was a significant decrease in both the WT and Twist1+/− mice. The PF suture began to fuse from day 10. This significant decrease in Rbp4 may signify the beginnings of fusion in the PF suture that was identified in these mice. The microCT and histology demonstrate the onset of coronal suture fusion in Twist1+/− mice at day 3 at which time the RT-PCR analysis showed a significant decrease in Rbp4 expression. The expression of Rbp4 in the Twist1+/− mice continued to decrease to day 10, the last point of analysis. In contrast, in the patent coronal suture of WT mice, Rbp4 expression remained constant through day 5. However, the WT mice also experienced a significant drop in Rbp4 at day 10 to a level comparable to that seen in the Twist1+/− fusing coronal suture.

Recently, Rbp4 expression has been reported in cells from the PF, coronal and sagittal sutures in CD-1 mice along with other members of the retinol-signalling pathway (James et al., 2010a). Despite reporting its presence, no mention was made of the relative levels of Rbp4,
however, this report does suggest that the expression of these members of the retinol pathway confirm that the cells of the sutures are capable of responding to retinol.

The expression of *Rbp4* and *Stra6* in sutres and parietal bone of mice seen in this chapter, and their apparent changes of expression during suture fusion and bone growth, are consistent with the hypothesis of a possible role for of RBP4 and STRA6 in bone growth and suture fusion. Additionally, these results may suggest a potential direction for research into therapeutic agents to treat Saethre-Chotzen craniosynostosis. To further study this it is important to analyse which cells are expressing RBP4, whether RBP4 mRNA is being translated to protein and whether the changes in RBP4 have a causative effect on bone growth.
Chapter 4

Cell culture models for studying the role of RBP4 in osteogenesis
4.1 INTRODUCTION

In Chapter 3 a Saethre-Chotzen mouse model was used to demonstrate the changes in \textit{Rbp4} expression during calvarial bone growth and suture formation \textit{in vivo}. In this chapter an \textit{in vitro} model of osteogenesis was investigated for further analysis of the role of RBP4 during this process.

Many studies use cell lines as an \textit{in vitro} model of an \textit{in vivo} phenomenon. The difficulty in accessing viable clinical patient samples or difficulties with animal studies often drives the need for a suitable equivalent in the study of disease. Many studies have successfully used cell culture to emulate the osteogenesis that occurs in the prematurely fusing sutures of craniosynostosis (De Pollack et al., 1996, Dry et al., 2001, Coussens et al., 2009, James et al., 2010a). Primary cells derived from murine tissue are a valuable tool for the study of osteogenesis \textit{in vitro}. With relative ease of growth and more ready availability of mouse tissue many research groups rely on these primary cells. There are multiple methods for generating cultured calvarial cells, with the majority involving outgrowth of cells from a small bone fragment (Zambonin-Zallone et al., 1982, Coussens et al., 2009). Cells isolated from the calvaria of mice have been shown to form nodules of mineralised matrix likened to bone formation (Ecarot-Charrier et al., 1988).

The other alternative to primary cell culture is use of a cell line. One of the most commonly used cell lines in the study of calvarial osteogenesis is the MC3T3-E1 cell line. MC3T3-E1 cells are a line derived from the calvaria of newborn mice and they possess a pre-osteoblastic phenotype. These cells express \textit{Alp} and are able to differentiate to an osteoblastic phenotype.
(Sudo et al., 1983). Under the induction of various cell culture additives, particularly ascorbic acid (Harada et al., 1991) and β-glycerophosphate (Sakamoto et al., 1989) these cells have displayed the ability to mineralise, forming small calcified nodules (Sudo et al., 1983, Park et al., 2011). One of the useful aspects of these cells is their relatively predictable behaviour. The cells are known to initially proliferate, producing Alp as they differentiate and then Osteocalcin as they mineralise (Quarles et al., 1992).

Since early papers outlining the osteogenic potential of these cells, they have been used to identify many possible contributors to osteogenesis and osteogenic cell differentiation. Increased levels of mineralisation have been induced by melatonin (Roth et al., 1999) and simvastatin, a cholesterol lowering medication (Maeda et al., 2001). Differentiation of these cells has also been demonstrated in response to treatment with vitamin A and carotenoids (Park et al., 1997). MC3T3-E1 cells have also been used as a model of craniosynostosis in the study of craniosynostosis causing fgfr2 mutations (Hatch et al., 2006), FGF-2 signalling (Eda et al., 2008) and bone grafts (Aghaloo et al., 2006).

Although published research has previously demonstrated the usefulness of MC3T3-E1 cells in the study of osteogenesis, it has not yet been ascertained whether these cells express RBP4 and hence whether they are a useful tool to study RBP4 function in osteogenesis.
4.2 RESULTS

4.2.1 PARENTAL MC3T3-E1 CELLS DO NOT MINERALISE

To assess the role of RBP4 during osteogenesis, 3 different populations of MC3T3-E1 cells were sourced. Initially, these cells were tested in osteogenesis assays. During an initial osteogenesis experiment using MC3T3-E1 M (gift from Dr. M. Kansara, Peter McCallum Cancer Centre, Melbourne, Australia) the cells failed to show a morphological response to induction. The morphology did not appear to change greatly from the fibroblastic appearance that was present at the beginning of the trial and no osteogenic nodules were visible after staining (Figure 4.1).

Upon repeating the experiment with MC3T3-E1 S (gift from Dr. M. Cunningham, University of Washington, Seattle, USA) cells, a different morphology was seen initially, with cells appearing less fibroblastic and more rounded (Figure 4.2). Over time, the cells became over-confluent and began to appear less rounded. There was also cell death beginning to occur at the Day 21 time point. There was again, however, no evidence of any mineralisation at any timepoint or induction state in any of these cells either pre or post staining.

Gene expression of MC3T3-E1 S cells was assessed using RT-qPCR, and although these cells had not mineralised, both Alp and Oc expression was seen in these cells. There was a trend in Alp expression in both induced and uninduced cells, increasing after day 0 and then decreasing towards day 21. The changes in Alp expression were not significant however (Figure 4.2d).
Figure 4.1: Representative images of staining of MC3T3-E1 M cells. Day 0, 3, 7, 14 and 21 cells induced with ascorbic acid and β-glycerophosphate (Induced) or grown in normal minimal media (Uninduced). Passage 28 cells were plated at a density of $2.5 \times 10^4$ per cm$^2$ and, when confluent, induction was started and this was considered day 0. A) Light microscopy images of cells pre Alizarin Red staining. B) Light images post Alizarin Red staining. Images are shown at 20x magnification.
Figure 4.2: Representative images and gene expression of MC3T3-E1 S cells. A) Day 3, 7, 14 and 21 cells induced with ascorbic acid and ß-glycerophosphate (Induced) or grown in normal minimal media (Uninduced). Passage 30 cells plated at a density of 2.5 x 10^4 per cm^2 and when confluent, induction was started and this was considered day 0. Images captured at 20x magnification. B) High magnification (100x) cells pre staining. C) High magnification (100x) cells post Alizarin Red staining. D) Alp expression in MC3T3-E1 S cells. E) Oc expression in MC3T3-E1 cells. RT-qPCR data was normalised to ß-actin and fold change calculated to day 0 uninduced using the Delta Ct method. Bars are representative of average + SEM of n=3 biological replicates. * represents significantly greater than Day 0 uninduced and # represents significantly less than Day 0 uninduced. P<0.05.

Day 0 | Day 7 | Day 14 | Day 21
---|---|---|---
Uninduced | Induced | Uninduced | Induced

B)

C)

D)

E)
In the case of \textit{Oc}, expression also increased after day 3 and then decreased towards day 21. \textit{Oc} expression was significantly greater in induced samples then in uninduced (Figure 4.2e).

### 4.2.2 MC3T3-E1 SUBCLONE 4 CELLS MINERALISE UNDER OSTEOGENIC CONDITIONS

MC3T3-E1 subclone 4 cells purchased from the ATCC responded favourably to osteogenic induction with ascorbic acid and β-glycerophosphate and after initial changes in morphology began to mineralise (Figure 4.3). Small nodules which were visible under low power microscopy began to appear at approximately day 6 of culture in the induced cells and, by day 7, the induced culture wells stained positive to Alizarin Red S. After 21 days in culture, the induced cells displayed a large amount of mineralisation, covering approximately 90% of the surviving cell areas. Additionally, by day 21 very small amounts of mineralisation were apparent in the uninduced cell culture wells.

\textit{Oc} and \textit{Alp} expression was assessed in the subclone 4 cells by RT-qPCR (Figure 4.4). \textit{Alp} expression increased after osteogenic induction and began to decrease towards the end of culture (Figure 4.4a). There was a large difference in the expression of induced and uninduced cells, with uninduced cells failing to register a significant increase until day 21, unlike their induced counterparts. \textit{Oc} levels were increased greatly after induction and continued to rise to an approximately 50 fold increase in expression at the end of the culture (Figure 4.4b). Uninduced cells did not exhibit a significant \textit{Oc} response, however a small rise in expression was seen at day 21.
Figure 4.3: Representative images of mineralisation in MC3T3-E1 subclone 4 cells. A) Microscopic images of mineralisation on cells after osteogenic induction. Mineralisation can be seen as a small dark ‘grainy’ texture over cells and is indicated by arrows. Passage 9 cells were plated at a density of $2.5 \times 10^4$ per cm$^2$ and, when confluent, osteogenic induction was started and this was considered day 0. Uninduced cells were maintained in normal minimal media. Images shown are 20x magnification. B) Cells stained with Alizarin Red. Positive staining for calcification of nodules can be seen in red.
Figure 4.4: Gene expression of MC3T3-E1 subclone 4 cells during mineralisation. A) Alp expression in MC3T3-E1 subclone 4 cells. B) Oc expression in MC3T3-E1 subclone 4 cells. RT-qPCR data was normalised to β-actin and fold change calculated to day 0 uninduced using the Delta Ct method. Bars are representative of average + SEM of n=3 biological replicates. * represents significantly greater than day 0 uninduced. P<0.05.
4.2.3 MC3T3-E1 CELLS DO NOT EXPRESS RBP4

*Rbp4* expression was not detected in either induced or uninduced MC3T3-E1 M or S cells (data not shown) or in MC3T3-E1 subclone 4 cells (Figure 4.5a,b). The legitimacy of these results was confirmed by using a positive control tissue, liver (Figure 4.5a,b). Additionally, samples were run on a gel to confirm the RT-qPCR data, showing no evidence of any *Rbp4* expression in these cells (Figure 4.5c). Subsequent analysis of all experimental timepoints from subclone 4 cells also failed to show any *Rbp4* expression (selected timepoints shown in Figure 4.5b).

4.2.4 PRIMARY OSTEOBLAST-LIKE CELLS WERE SUCCESSFULLY GROWN FROM MOUSE SUTURE AND BONE TISSUE

The lack of RBP4 expression in MC3T3-E1 cells led to a search for a more suitable cell type to test the hypothesis. Primary cells were established from *Twist1*+/− mice and WT littermates. Three different isolation methods were used to grow cells from mouse sutures and parietal bone. The three methods chosen were; bone chip outgrowth (BCO) with cells grown from a bone fragment without any digestion; partial digestion (PD) where bone fragments were partially digested using collagenase and trypsin and then plated, allowing cells to migrate from the bone chips (Helfrich and Ralston, 2003); or finally full digestions (FD) involving repeated digestion of the bone and plating of the supernatant containing released cells, not the bone fragments (Helfrich and Ralston, 2003) (Figure 4.6). While cells isolated by the FD method grew quickly, they were of finite number, unlike those seen in BCO and PD samples (Figure 4.7).
Figure 4.5: Rbp4 expression in MC3T3-E1 subclone 4 cells. A) Melt curve analysis from RT-qPCR of RNA extracted from induced and uninduced MC3T3-E1 subclone 4 cell osteogenesis assay at day 0 and day 21 and from mouse liver. B) Ct values from the same samples. C) Gel electrophoresis of PCR products demonstrating the lack of amplification from RT-qPCR reactions using Day 0 and Day 21 induced and uninduced MC3T3-E1 samples. Orange arrow indicates Rbp4 and black β-actin in MC3T3-E1 subclone 4 samples. Blue indicates Rbp4 and green β-actin in liver samples.
Figure 4.6: Growth of murine primary cells using various isolation methods. Representative images of primary cells grown by 3 different methods. BCO is bone chip outgrowth only, PD is a chip outgrowth with a collagenase digestion and FD is a full digestion method. Arrows indicated examples of plated bone chips. Images shown at 20x magnification.
The FD method also yielded cells of questionable morphology; in addition to cells of expected fibroblastic morphology there were also present small rounded cells, similar to those seen in the early MC3T3-E1 experiments where the cells failed to mineralise (Figure 4.7b). These rounded cells comprised up to 90% of cells yielded from these preparations. The PD method produced a reliable source of primary cells, however this technique also yielded some cells (less than 5%) with a more neuronal morphology (Figure 4.7c).

4.2.5 PRIMARY CELLS FROM MOUSE SUTURES FAILED TO MINERALISE

Cells obtained by these 3 isolation methods were tested for their ability to mineralise. None of the 3 isolation methods yielded cells with the ability to mineralise when exposed to OG, Dex or RA. Cells from PF, sagittal, coronal and lambdoid sutures as well as parietal bone all failed to mineralise as tested by Alizarin Red S staining (Figure 4.8). Additionally, cells grown from C57BL/6 mice by the BCO method also failed to mineralise (data not shown).

4.2.6 PRIMARY CELLS FROM HUMAN SUTURE SAMPLES MINERALISE RELIABLY

Following the failure of mouse primary cells to mineralise, cells derived from human sutures were tested as a model of osteogenesis. Cells from human sagittal, lambdoid and coronal sutures were all able to mineralise under osteogenic induction. Cells isolated from unfused coronal (Figure 4.9) and fused coronal suture (Figure 4.10) were induced with ascorbic acid
Figure 4.7: Growth rates of murine primary cells from the different isolation methods and examples of the morphology of cells grown. A) Rounded morphology typical of cells grown using the FD method. B) Cells with many long cytoplasmic protrusions grown using the PD method. C) Large flat cells with morphology similar to that seen in human cells grown using the FD method. Images A, B and C are shown at 60x magnification. D) Graphical representation of the number of cells isolated from each method before sample exhaustion, typically after 4-5 passages. E) Calculation of growth rate based on the number of cells isolated per passage and the time taken to reach confluence in 24 well plates. D and E are representative of n=3 biological replicates from 5 different cell types. # represents significantly less than BCO.
Figure 4.8: Representative images of mouse coronal suture derived cells. Day 0, 3, 7, 14 and 21 cells induced with ascorbic acid and β-glycerophosphate (Induced) or with normal minimal media (Uninduced). Passage 8 cells were plated at a density of 2.5 x 10^4 per cm² and, when confluent, induction was started and this was considered day 0. Images are shown at 20x magnification.
Figure 4.9 Representative images of mineralisation in human coronal suture derived cells from unfused suture samples of patient AC115. Microscopic images of mineralisation on cells after induction. Mineralisation can be seen as a small dark ‘grainy’ texture over cells and is indicated by arrows. Passage 11 cells were plated at a density of $2.5 \times 10^4$ per cm$^2$ and, when confluent, induction was started and this was considered day 0. Cells were induced with ascorbic acid and β-glycerophosphate (OG), dexamethasone and OG media (DEX), retinoic acid and DMEM media (RA) or with normal minimal media (DMEM). Images shown are 20x magnification. Smaller images are Alizarin Red staining at each timepoint.
Figure 4.10 Representative images of mineralisation in human coronal suture derived cells from fused suture samples of patient AC115. Microscopic images of mineralisation on cells after induction. Mineralisation can be seen as a small dark, ‘grainy’ texture over cells and is indicated by arrows. Passage 11 cells were plated at a density of $2.5 \times 10^4$ cells/cm$^2$ and, when confluent, induction was started and this was considered day 0. Cells were induced with ascorbic acid and β-glycerophosphate (OG), dexamethasone and OG media (DEX), retinoic acid and DMEM media (RA) or with normal minimal media (DMEM). Images shown are 20x magnification. Smaller images are Alizarin Red staining at each timepoint.
and β-glycerophosphate, dexamethasone and RA. Cultures with ascorbic acid and β-

glycerophosphate began to mineralise from day 14, as did cells induced with dexamethasone
(Figure 4.9 and 4.10). There was no difference in mineralisation between the cells from tissue
of fused and unfused origin, although cells of fused origin did respond more quickly to
dexamethasone treatment (Figure 4.11). Neither group of cells responded to induction with
RA.

\textit{ALP} and \textit{OC} expression in human coronal cells was measured during osteogenesis by RT-
qPCR. \textit{ALP} showed no significant change in expression during mineralisation (data not
shown) but a strong \textit{OC} response was seen. A response was seen in \textit{OC} from day 14 and this
was significant in day 21 and day 28 OG and Dex-treated cells (Figure 4.12a) and
corresponded to the Alizarin Red quantification of mineralisation (Figure 4.11). No change in
\textit{OC} expression was seen at any point in cells without induction or with RA.

\textbf{4.2.7 HUMAN SUTURE-DERIVED CELLS EXPRESS RBP4 AND IT IS
DOWNREGULATED DURING OSTEOGENESIS}

All the cultures of human cells expressed \textit{RBP4} (Figure 4.12b). The cells that mineralised
exhibited significant downregulation of \textit{RBP4} compared to day 0 and to uninduced samples at
day 14, 21 and 28 (Figure 12b). Levels of \textit{RBP4} expression in RA and uninduced samples did
not alter significantly at any time.
Figure 4.11: Graphical representation of the levels of mineralisation of cultured human cells. A) Alizarin quantification from unfused coronal cell osteogenesis assay (Figure 4.9). B) Alizarin quantification from fused coronal cell osteogenesis assay (Figure 4.10). Absorbance was normalised to number of cells. Values shown are average + SEM of replicates n=3. * indicates significantly greater than day 0 DMEM and ^ indicates significantly different to the OG counterpart of that particular timepoint. P<0.05.
Figure 4.12: Gene expression from mineralising human suture cells by RT-qPCR. A) OC measurements from osteogenesis assay of fused and unfused cells from patient AC115. B) RBP4 measurements from osteogenesis assay of fused and unfused cells from patient AC115. RT-qPCR data was normalised to β-actin and fold change calculated to day 0 uninduced using the Delta Ct method. Values shown are average ± SEM of replicates n=6. # indicates significantly less than day 0 DMEM and ^ indicates significantly different to the OG counterpart of that particular timepoint. P<0.05.
4.3 DISCUSSION

In this chapter several cell culture model systems were investigated for their suitability for studying the role of RBP4 during osteogenesis. Two of the three types of MC3T3-E1 cells sourced did not mineralise, and none of them expressed \textit{Rbp4}. Although primary mouse suture-derived cells expressed \textit{Rbp4}, they too did not mineralise. Human suture-derived cells proved to be a suitable model as they both mineralised and expressed \textit{RBP4}.

MC3T3-E1 cells have been identified previously by their ability to mineralise under various induction conditions (Sudo et al., 1983, Chung et al., 1992). Therefore, the lack of mineralisation seen in the MC3T3-E1 M and S lines was unexpected. The laboratories the cells were sourced from used them for studies of cell fingerprinting and analysis of craniosynostosis-related FGFR2 mutations, MC3T3-E1 S (Hu et al., 2004, Hatch et al., 2006), and studies related to the cell cycle and osteosarcoma; MC3T3-E1 M (Thomas et al., 2004, Galindo et al., 2007). Those publications demonstrated the ability of those clones to mineralise. The difficulty experienced in inducing mineralisation of these cells in this study may be due to a variety of reasons. The morphology of the M cells suggested that there was a lack of differentiation from the pre-osteoblastic phenotype (usually expected to have a fibroblastic appearance) to an osteoblastic phenotype (expected to have a more rounded/cuboidal appearance)(Sudo et al., 1983). A lack of differentiation to osteoblasts may explain a lack of mineralisation, as pre-osteoblastic cells do not, by themselves, mineralise. In the case of the MC3T3-E1 S cells, the rounded appearance of the cells received suggested that those cells may have already differentiated before inducement and hence exhausted their ability to mineralise.
Both MC3T3-E1 M and MC3T3-E1 S lines of cells were used at a high passage number, P28 and P30 respectively. This was unavoidable as the cells were received at passages above 20 and the cell population had to be expanded to get the number of cells required for experimentation. There has previously been evidence that MC3T3-E1 cells do not respond favourably to passaging to high numbers. A decrease in proliferation has been seen in MC3T3-E1 cells at high passage numbers (passage 40-60) which has been likened to the process of replicative senescence (Peterson et al., 2004). It has also been demonstrated that at high passage MC3T3-E1 cells (passage 65 and above) lose responsiveness to inductive factors and can change morphology, similar to the observations presented here (Chung et al., 1999).

There have also been reports of MC3T3-E1 cells losing their ability to mineralise after long periods in LN2 storage (approximately 10 years) (Baba, 2000). Although the cells were used immediately upon arrival, it is not certain how they were stored prior to arrival in the laboratory. There is evidence that loss of mineralisation arising from long term storage could be reversed by addition of dexamethasone to cell culture (Baba, 2000), but due to the lack of RBP4 expression use of these cells was abandoned.

The MC3T3-E1 subclone 4 cells that were purchased from the ATCC mineralised. In addition to the subclone 4 cells, the ATCC also provides MC3T3-E1 subclone 30 cells which do not mineralise. These were derived from the same parental MC3T3-E1 population and kept at low passage and this indicates that MC3T3-E1 clonal lines can have greatly varying gene expression and mineralisation abilities (Wang et al., 1999a). This indicates that the MC3T3-E1 phenotype can change in culture.
Like the earlier MC3T3-E1 cells, the failure of the mouse primary suture-derived cells to mineralise was unexpected. The ability of mouse primary calvarial cells to mineralise has been established previously (Mansukhani et al., 2000, Zhang et al., 2003). However, there were two main differences between those studies and that presented here. The first was the method of primary cell isolation, with those experiments using enzymatic digestion and the original ones used in the present study based on cells derived from non-digested bone chip outgrowth. This led us to trial different methods of isolation using differing levels of enzymatic digestion. The different isolations produced viable cells, however these too failed to mineralise. Some of the unusual cell morphologies seen with those isolations may indicate that not all cells were pre-osteoblastic. The cells were a heterogeneous population and there was the possibility of contaminating factors like dura mater or brain. Care was taken to remove all brain and dura from the skull, however in small animals the dura is quite sticky and has a strong association at sites of the suture. The cells that were grown using the FD method with the more rounded appearance may have differentiated down the path of adipogenesis during isolation. Alternatively, this isolation may have isolated a population of adipocytes rather than osteoblast precursor cells. There are reports of adipocyte precursor cells being isolated from both bone marrow and calvaria (Marko et al., 1995, Steenhuis et al., 2009). To test this hypothesis in future experiments these cells could be tested with a lipid stain such as oil red O staining which should indicate whether adipocyte differentiation has occurred.

The other major difference between the isolation methods used in the present study and the majority of the published literature is that unlike other groups who specifically exclude suture tissue from their calvarial cell cultures, the aim of the present study was to preferentially isolate cells from the suture. Another research group has had success in isolating cells from
the posterior frontal and sagittal sutures of mice (James, 2008; James, 2010; James, 2010). In addition to the upregulation of osteoblast markers Alp, Runx2, BMP2 and 4 and osteopontin (James, 2010; James, 2010) the authors also showed mineralisation of these suture-derived cells (James et al., 2008). As dexamethasone had been reported to rescue the mineralising phenotype in MC3T3-E1 cells (Baba, 2000), the mouse primary suture cells were tested under dexamethasone induction but still no mineralisation occurred.

Finally, the testing of human suture-derived cells proved successful. Cells isolated from fused and unfused coronal sutures mineralised under induction with OG and Dex. The only unexpected result with these cells was their lack of response to treatment with RA. The conditions used in these experiments to promote RA-induced osteogenesis were based on a protocol previously published in rats (Song et al., 2005). In that paper, the authors reported mineralisation of cells treated with DMEM and RA after only 7 days in culture. Contrary to that study, however, other studies have used RA in conjunction with other commonly used inducers of osteogenesis ascorbic acid and ß-glycerophosphate (Kitching, 2002). The quality of the RA used in these experiments was tested and confirmed via HPLC. It is possible that if this media were tested it might have induced osteogenesis in the human cells. Unfortunately there was not time to trial the experiment again under those conditions. The other consideration is that the studies that have shown mineralisation in response to RA had been in calvarial bone derived cells, not suture cells as were being used in this trial.

*Alp* and *Oc* are commonly used determinants of the stage of cell behaviour in MC3T3-E1 cells (Casser-Bette et al., 1990, Franceschi and Iyer, 1992). Normally, an initial increase in *Alp* activity occurs after induction followed by a decrease towards later stages, whereas *Oc*
increases as the cells mineralise (Franceschi et al., 1994). The initial increase in Alp expression seen in the MC3T3-E1 S cells that did not mineralise suggested that some osteogenic processes in those cells may have occurred normally. For Oc in these MC3T3-E1 S cells the expected large increase in expression did not occur. Although this was not expected it does accurately reflect the lack of mineralisation that occurred in those cells. This response was different to the MC3T3-E1 subclone 4 cells that mineralised as detected by Alizarin Red staining and where Oc increased dramatically as mineralisation occurred.

In the MC3T3 subclone 4 cells a more ‘typical’ expression of those osteogenic markers was observed. In the induced cells there was an increase in Alp as cells began to mineralise and Oc increased steadily as cells mineralised. Also, the large difference in expression between the induced and uninduced cells reflected the different behaviour of those cells. Oc expression was also substantially different between the induced and uninduced cells, with expression increasing as mineralisation occurred. The comparatively small increase in Oc expression in the uninduced cells at day 21 was consistent with the small amount of mineralisation that was apparent in those cells. Although inducing agents are generally used to promote mineralisation in MC3T3-E1 cells, the cells have been shown to have the capacity for spontaneous mineralisation without induction, so this result is not surprising (Sudo et al., 1983). The expected mineralisation and gene expression seen in the subclone 4 cells suggested that they may be more suitable for use with these experiments if they expressed Rbp4.

As microarray analysis of gene expression in fusing human sutures had recorded a decrease in RBP4 expression as bone formed, it was likely that if Rbp4 was expressed in MC3T3-E1 cells
it should occur at early timepoints. To this end, samples from all subclones of MC3T3-E1 cells were collected before treatment with osteogenic induction were tested and all proved negative for Rbp4 expression. Although according to the hypothesis it was unlikely to be the case, all timepoints from the successful subclone 4 mineralisation experiment were then analysed for Rbp4 expression in case expression could be detected in later stages. They too proved negative for Rbp4 expression at any timepoint.

The lack of Rbp4 expression in the MC3T3-E1 S and M cells was unexpected, as data presented in Chapter 3 had shown Rbp4 expression in mouse calvarial bone, the location of origin for those cells (Sudo et al., 1983). The lack of mineralisation in the MC3T3-E1 S and M cells prompted doubts that the lack of Rbp4 expression was genuine and it was thought that it may be an artefact of the problems occurring with those particular cells lines; hence the experiments were repeated in the MC3T3-E1 subclone 4 cells. The MC3T3-E1 subclone 4 cells however, confirmed the lack of Rbp4 expression seen in the earlier experiments. This may indicate, as discussed earlier, that the MC3T3-E1 cells have differentiated to stage post Rbp4 expression and are therefore no longer pre-osteoblastic cells. This does however, make the MC3T3-E1 subclone 4 cells a potentially useful model for the study of Rbp4 during osteogenesis using the exogenous RBP4 expression methods such as transfection of an RBP4 expression vector (for more information see Chapter 6).

Human cells isolated from patient samples extracted during surgery exhibited an expected pattern of expression of osteoblast markers in response to osteogenic induction. OC was significantly upregulated upon induction and correlated with mineralisation. Compared to the MC3T3-E1 cells the fold increase in expression was much greater. An increase in OC
expression has been reported in human primary bone marrow cells during mineralisation previously (Schecroun and Delloye, 2003) and in cells derived from human sutures (Coussens et al., 2009).

As expected, expression of \textit{RBP4} was seen in the human suture-derived cells. The expression of \textit{RBP4} behaved as hypothesised, decreasing during mineralisation. The decrease in \textit{RBP4} in cell culture was not as pronounced as that seen in tissue but this was not unexpected (Coussens et al., 2007). A paper recently published from our laboratory demonstrates that expression of bone growth-related genes was significantly different between tissue and cell culture (Coussens et al., 2009). That study reported data for only 2 timepoints during osteogenesis and did not achieve as thorough an analysis as reported here.

From the experiments presented here it was concluded that neither MC3T3-E1 nor primary mouse suture-derived cells were a suitable model for the study of \textit{Rbp4} in osteogenesis. Primary cells derived from human suture tissue however, reliably mineralised under induction conditions, expressed \textit{RBP4} and displayed a decrease in \textit{RBP4} expression during \textit{in vitro} osteogenesis. This made the primary human suture derived cells a suitable candidate for further research into RBP4 function in osteogenesis.
Chapter 5

Subcellular localisation of RBP4 in human suture cells
5.1 INTRODUCTION

In the previous chapters downregulation of RBP4 was identified during murine bone growth and suture fusion, as well as during mineralisation of human primary suture cells. In this chapter the aim was to identify the subcellular localisation of RBP4 in human suture cells to help gain insight into its function in these cells.

RBP4 has been well characterised as a transport protein for retinol in serum. RBP4 is fundamentally implicated in conditions linked to diabetes (Tamori et al., 2006) and metabolic disease (Qi et al., 2007, Aeberli et al., 2007). In these conditions, RBP4 is assessed as a serum transport molecule alone, without consideration of an intracellular role. The expression of RBP4 by suture tissue and cells, and the change of expression during mineralisation, as demonstrated in the previous chapters indicate that in addition to an extracellular transport molecule, there is a possibility that in these cells RBP4 is playing a site-specific intracellular role.

There is limited literature on the possible roles of RBP4 in tissues other than serum. The few reports that exist however often show distinct patterns of expression between different tissues or disease states, suggesting a role of RBP4 in multiple other processes. By microarray analysis RBP4 has been identified as a possible candidate gene in cholesterol reduction in monkeys (Nantermet et al., 2008), vasculogenesis in zebrafish (Sumanas et al., 2005), zebrafish hepatogenesis (Li et al., 2007), human kidney disease (Frey et al., 2008), human celiac disease (Diosdado et al., 2004) and in human suture tissue (Coussens et al., 2007). RBP4 has also been implicated in altering the susceptibility of humans to hepatitis B and C.
infection (Kinoshita and Miyata, 2002). These studies all highlight the extrahepatic expression of RBP4 and indicate the likelihood of a role for RBP4 in tissues other than serum.

The function of RBP4 in these tissues may be related to its cellular location. Immunocytochemistry has been used to detect RBP4 in the human digestive tract, in particular in the pancreas and intestinal mucosa where it was seen to be co-localised with TTR (Kameko et al., 1991). The use of immunocytochemistry has also been able to localise RBP4 in the liver of rats (Poole et al., 1975) in particular, accumulation of RBP4 has been seen in the endoplasmic reticulum of these animals during retinol storage (Suhara et al., 1990).

5.2 RESULTS

5.2.1 PREDICTION OF RBP4 SUBCELLULAR LOCALISATION BY SEQUENCE ANALYSIS

To identify possible cellular locations of RBP4 several protein localisation prediction programs were compared to indicate potential locations. Cell-PLoc (Hum-mPLoc) predicts localisation to one of 14 different subcellular locations (Chou and Shen, 2008) and predicted RBP4 to be secreted (Figure 5.1). BaCelLo, which predicts localisation to one of 5 different subcellular locations (Pierleoni et al., 2006) also predicted that RBP4 would be secreted. HSLPred predicts localisation to one of 4 different subcellular locations (Garg et al., 2005) and SubLoc predicts localisation to one of 4 different subcellular localisations (Hua and Sun, 2001) both predicted that RBP4 would be localised to the cytoplasm of cells. SecretomeP 2.0,
Figure 5.1: Prediction of subcellular localisation of RBP4 by computerised prediction software.  

**A)** Table of results comparing output predictions and possible subcellular localisation by program. NS indicates not supplied by prediction program.  

**B)** An example of output from prediction program HSLPred.
which predicts whether a protein will be secreted by a ‘classical’ pathway, predicted that RBP4 would be secreted. According to 3 of the 5 subcellular localisation prediction programs, RBP4 would be secreted. Two of the programs used predicted that RBP4 would remain in the cell and be cytoplasmic.

5.2.2 RBP4 HAS A DISTINCT LOCALISATION IN BOTH HUMAN SUTURE CELLS AND LIVER CARCINOMA CELLS

Human suture cells are large flat cells with many cellular protrusions. An anti-human RBP4 antibody was used to perform immunocytochemistry of sub-confluent human suture cells cultured from coronal and sagittal sutures. This demonstrated that positive staining for RBP4 surrounded the nucleus and tended to be polarised to one side of the cell (Figure 5.2). The staining did not differ when cells from different sutures were tested; cells from the coronal (Figure 5.2b), sagittal (Figure 5.2d) and lambdoid (data not shown) all had the same distinctive staining polarised to one side of the cell, surrounding the nucleus. To further examine this staining, confocal microscopy was used. A series of 0.5 µm optical slices revealed a low level of RBP4 throughout the cytoplasm closest to the base of the cell (Figure 5.3, panels 1-6). More intense RBP4 staining appeared to surround and cover the nucleus, rather than being present inside the nucleus (Figure 5.3, panels 7-28). Analysis of cells from 3 different patients all displayed the same localisation of RBP4.

For comparison, Huh7 cells derived from human liver carcinoma were also tested. Huh7 cells are small rounded cells (Figure 5.4). In Huh7 cells RBP4 appeared to be localised to a small ‘pouch’ in each cell (Figure 5.4a,b). There is also a less intense level of RBP4 staining
Figure 5.2: Immunofluorescent staining of RBP4 in human suture cells.  A) Coronal suture cells seen using light microscopy.  B) Sub-confluent cells grown from AC125 coronal suture at P10 stained for RBP4 using DAKO antibody.  C) The same cells with a white trace highlighting the outline of the cell.  D) Cell from AC125 sagittal suture at P7.  E) No primary antibody control.  Regions positive for RBP4 are seen in green.  Blue staining indicates DAPI staining of the nucleus.  Images B, C and E were taken on an epifluorescent microscope, and image D is an optical slice captured by confocal microscopy.  Scale bar represents 25 μm.
Figure 5.3: ‘Z stack’ optical sections of a subconfluent AC125 coronal human suture cell stained for RBP4. 1-28) Optical sections were taken at 0.5 μm intervals. Green staining indicates regions positive for RBP4 and blue indicates the DAPI-stained nucleus. Base of the stack is 1.
Figure 5.4: RBP4 expression in Huh7 cells.  A) Confluent Huh7 cells at P26 imaged by epifluorescent microscopy.  B) 0.5 μm optical slice of confluent Huh7 cells imaged with confocal microscopy.  C) Confluent Huh7 cells imaged by light microscopy.  D) No primary antibody control.  Regions positive for RBP4 are seen in green.  Blue staining indicates the DAPI-stained nucleus.  Scale bar represents 25 μm.
throughout the cytoplasm that is not seen in the isotype (data not shown) and no primary antibody controls (Figure 5.4d). Both confluent and non-confluent antibody controls did not stain positively for RBP4. The localisation of RBP4 in human suture cells appears to differ from its localisation in Huh7 cells, however due to the large difference in cell size and morphology is is difficult to make a direct comparison.

5.2.3 DUAL IMMUNOCYTOCHEMISTRY STAINING SUGGESTS THAT RBP4 IS LOCATED IN THE ENDOPLASMIC RETICULUM OF SUTURE CELLS

The distinctive pattern of RBP4 staining in suture cells prompted further analysis of localisation, using markers of cellular organelles and compartments. Phalloidin detects F-actin, and in the human suture cells highlights a complex organisation of actin fibres. The actin fibres are organised radially from the points of cytoplasmic extension (Figure 5.5). Rab-5 is a marker of endosomes, and in both Huh7 (data not shown) and human suture cells it stains a discrete tightly packed region near the nucleus. Calnexin (CANX) is a marker of endoplasmic reticulum and stains human suture cells around the nucleus and is generally polarised to one side of the cell. When dual immunocytochemistry was performed, both CANX and RBP4 were localised to the same region in human suture cells. CANX and RBP4 both localised to the area surrounding the nucleus, however CANX staining was more intense than RBP4 (Figure 5.6).
Figure 5.5: Immunofluorescent staining of cellular markers in AC124 human coronal suture cells at P6. Cells were stained with phalloidin, Rab-5 or calnexin (CANX). Positive staining was seen for CANX (red), phalloidin (green) and RAB-5 (green). Blue in all images represents the DAPI-stained nucleus. Scale bar represents 25 μm.
Figure 5.6: Colocalisation of CANX and RBP4 in AC124 human coronal suture cells at P6. A) RBP4 staining. B) CANX staining. C) Merge of RBP4 and CANX staining. Blue staining represents DAPI-stained nucleus. Scale bar represents 25 μm.
5.2.4 RBP4 AND CANX CELLULAR LOCATIONS ARE LARGELY RESISTANT TO CYCLOHEXIMIDE TREATMENT

Following on from dual immunocytochemistry data suggesting that RBP4 was located in the endoplasmic reticulum, cycloheximide treatment was used to block protein synthesis and analyse whether the RBP4 was resident in the endoplasmic reticulum or in transit. Initial experiments showed 10 mM to be the optimal concentration to use in experiments in human suture cells (data not shown). Use of 5 mM elicited less response, but 15 mM proved to be toxic and resulted in a high level of cell death. Staining at 12 and 24 hours after cycloheximide treatment showed little change in the localisation of CANX in suture cells, with staining persisting around the nucleus (Figure 5.7) like that shown in the untreated cells. RBP4 also persisted in the region surrounding the nucleus after cycloheximide treatment, compared to 24 hours post cycloheximide and untreated cells. However, in these suture cells 12 hours post treatment there were “stringy” and “granular” regions of RBP4 seen in the cytoplasm not detected previously. β-tubulin was detected in untreated cells and was seen in an expected morphology throughout the cell. Twelve hours post treatment, β-tubulin was not detected in the human suture cells but the expression of β-tubulin had recovered 24 hours post-cycloheximide treatment. After treatment however, β-tubulin appeared disorganised throughout the cytoplasm of the cell.

5.2.5 HUMAN SUTURE CELLS DO NOT APPEAR TO SECRETE RBP4

As liver cells can accumulate RBP4 in the endoplasmic reticulum and are known to secrete
Figure 5.7: Immunocytochemistry of AC124 coronal suture cells treated with cycloheximide. Subconfluent cells were treated with 10 mM cycloheximide for 1 hour then fixed at 12 hours and 24 hours. CANX, RBP4 and β-tubulin staining can be seen in green. Blue indicates DAPI-stained nucleus. White arrows indicate ‘stringy’ regions of staining. Scale bar represents 25 μm. Insets are no primary antibody controls for the relevant secondary antibodies.
RBP4 (Marinari et al., 1987, Mariani et al., 1989, Suhara et al., 1990) western blot was used to test if RBP4 was also being secreted from human suture cells. Higher than expected levels of RBP4 detection in negative control ‘media only’ western blots led to the suspicion that the antibody may be detecting RBP4 in the FBS used for cell culture maintenance. CLUSTALW protein analysis showed that RBP4 of bovine and human origin has 96% identity (Figure 5.8a). Western blot analysis showed that in addition to RBP4 of human and murine origin as previously reported, the antibody also detected RBP4 of bovine origin. RBP4 was readily detected in 10% FBS media (normally used for cell culture maintenance) down to 2% FBS media (Figure 5.8b).

Like the cellular localisation, western blot analysis of conditioned culture supernatants from human suture and Huh7 cells again showed differences in RBP4. Cell culture supernatant from cells grown in serum-free conditions was run neat on a western blot. RBP4 was detected in the supernatant of Huh7 cells but not in the supernatant of human suture cells (Figure 5.9). Albumin, a positive control for secretion was present in the samples from both Huh7 and human suture cells. Cell lysate from human suture cells had RBP4 present as detected by western blot, as did Huh7 cells. The levels of RBP4 protein intracellularly appeared to be greater in Huh7 cells than human suture cells, however without further experimentation a ratio of expression cannot be estimated.
Figure 5.8: Antibody detection of RBP4 in fetal bovine serum used in cell culture experiments. A) ClustalW alignment of human, mouse and bovine RBP4 protein. * indicates same amino acid, : indicates a different amino acid but of similar nature and blank indicates no similarity. B) Western blot detection of RBP4 in varying concentrations of FBS in neat media. -ve indicates no primary antibody control.
Figure 5.9: RBP4 detection using western blot from cell lysate and culture medium after overnight serum starvation. Coronal suture cells from AC124 and AC125, along with Sagittal cells from AC124 and Huh7 cells were grown to confluence. After this supernatant was removed and replaced with serum-free media overnight. Supernatant was then collected and cells lysed using RIPA buffer. Samples were then analysed by western blot. -ve indicates relevant no primary control membranes. Albumin is a positive control for secretion and β-tubulin a positive control for cell lysate.
5.3 DISCUSSION

The aim of this chapter was to investigate the subcellular localisation of RBP4 in human suture cells. The data presented here demonstrate a distinctive subcellular localisation of RBP4, similar to that of the endoplasmic reticulum marker CANX, suggesting that RBP4 was located in the endoplasmic reticulum. RBP4 did not appear to be secreted from human suture cells and when its localisation was tested using cycloheximide treatment RBP4 localisation remained largely unchanged.

Difficulty in detecting RBP4 appears to be a problem experienced by researchers and may contribute to some of the conflicting data that is seen in the literature (Graham et al., 2007). During the present research great difficulty was also encountered in finding suitable methods of RBP4 detection. Although a variety of RBP4 antibodies were commercially available, it was difficult to find an antibody that would reproducibly and reliably stain RBP4 in suture tissue and cells. To be able to detect RBP4 in both mouse and human cells, antibodies were purchased that were designed to detect RBP4 from both species. Abcam goat anti-human RBP4 (Abcam, Cambridge UK), Everest goat anti-human RBP4 (Everest Biotech, Oxfordshire, UK) and R&D sheep anti-mouse RBP4 (R&D systems, Minneapolis, USA) were all tested for use in western blot, immunohistochemistry and immunocytochemistry and failed to produce reliable detection of RBP4. The Abcam antibody had not previously been used in any published reports, but was recommended for use in western blot. The antibodies from Everest and R&D were also recommended by the manufacturer for western blot of RBP4. Recently, a publication identified an RBP4 antibody that was raised against human epitopes that could also detect mouse RBP4, albeit with lesser affinity (Yang et al., 2005b). This
antibody proved successful in the western blot and immunocytochemistry analyses in the present research and was used for all further experiments.

Recently, the development of prediction tools for subcellular localisation has been used to direct research and aid the experimental approaches. These methods use in depth sequence analysis to predict the localisation of a protein. Most of the programs are based on a few main analysis techniques. The original program developed for predictions was PSORT which used comparison with sequences of proteins with known localisation, such as GPI anchors and DNA binding motifs in addition to protein folding to predict localisation (Nakai and Horton, 1999).

For confirmation, a combination of both prediction and experimental techniques must be used. Both computer prediction techniques and experimental data has strengthened many reports in the literature and increased the likelihood of a correct analysis of localisation. Three of the 5 programs tested predicted RBP4 to be secreted and the other two predicted localisation to the cytoplasm. In the human suture cells, RBP4 did not appear to be secreted, whereas it was secreted by Huh7 cells and is known to be secreted from adipocytes (Ost et al., 2007) and hepatocytes (Fujita, 2008). This indicates an inherent variability in this predictive approach because cellular context may modify location.

Subcellular localisation as a tool to study function of protein has proven highly useful and has led to drug design targets for disease. Fluorescently tagged proteins have been used to identify the subcellular localisation of bovine viral proteins (Tsai et al., 2005) but it can be argued that addition of a GFP may significantly alter the folding, size and hence the localisation of a protein. There is also the potential to use other smaller tags which may have a diminished
influence on protein folding, however there is still no definitive tag that can be used without altering the native protein (Waugh, 2005). Indirect immunofluorescence using antibodies circumvents this problem and this method has been used to localise many proteins in cell culture, for example members of the Janus Kinase (JAK) pathway (Behrmann et al., 2004). Dual immunofluorescence with a protein of interest and a known organelle marker can show co-localisation (Allan 2000). Another method which has proven useful in the identification of subcellular localisation is to separate different cellular fractions and look at their contents by mass spectrometry. An elegant review on the pros and cons of these experimental techniques can be found in a review by Simpson (Simpson, 2006).

This is the first study to look at the subcellular localisation of RBP4 in human suture cells. The fact that the staining pattern differed between human suture cells and human liver carcinoma cells suggests that RBP4 may be playing a different role in these cells. RBP4 is known to be secreted from Huh7 cells and this was confirmed here by western blot. In Huh7 cells a distinct pocket of RBP4 was located in the cytoplasm, which may indicate it is packaged in a vesicle ready for secretion. This staining looks similar to Rab-5 which also showed a single pocket of staining in Huh7 cells. Rab-5 is a GTPase involved in endocytosis and is used as a marker of endosomes (van der Blick, 2005). Dual immunocytochemistry of RBP4 with an endosomal marker such as Rab-5 would need to be performed to confirm this. However, because the primary antibodies that detected these epitopes in the present study were both raised in rabbit this was not possible.

In the human suture cells RBP4 appeared co-localised with CANX. CANX is a chaperone protein resident in the endoplasmic reticulum and is a marker for endoplasmic reticulum (Bergeron et al., 1994). In particular, dual immunocytochemistry has been used to show co-
localisation of CANX and proteins of interest in the endoplasmic reticulum of COS-7 cells (Sato et al., 2008) and HEK293T cells (Chen et al., 2009). From the co-localisation it can be assumed that in human suture cells RBP4 is localised to the endoplasmic reticulum. Previously, RBP4 has been demonstrated to be present in the endoplasmic reticulum of the liver of rats (Suhara et al., 1990). Here it is known to play a role of storage for retinol, and this may indicate the reason for its localisation in the endoplasmic reticulum in human suture cells.

In regards to RBP4 function, localisation to the endoplasmic reticulum suggests an intracellular role. The most common function of the endoplasmic reticulum is protein synthesis. Thus, the RBP4 in the endoplasmic reticulum could be there simply as this is where it is made. To test this experimentally a protein synthesis inhibitor, cycloheximide, was used. In human suture cells, cycloheximide transiently ablated β-tubulin from the cells. The localisation of both CANX and RBP4 were resistant to cycloheximide, with the similarity in behaviour suggesting they both reside in the same subcellular location in the cell. Therefore, this indicates that RBP4 may be a resident protein in the endoplasmic reticulum similar to CANX.

The specific localisation of RBP4 to the ER may indicate a role in bone growth. The ER can be a site of calcium storage in a cell and as discussed in Chapter 1, the levels of available calcium can have an effect on bone health. The endoplasmic reticulum being a site for calcium storage (Koch, 1990) means that it is possibly a site for storage of other molecules. Calcium is stored in the endoplasmic reticulum bound to calreticulin (Hassan et al., 1995). In our hypothesis, we propose that RBP4 is acting as a storage molecule for retinol in our cells.
If RBP4 is a resident protein in the endoplasmic reticulum it may be acting as a storage molecule similar to calreticulin.

Using this antibody RBP4 was also detected in the FBS that is used in the culture media. As there is a 96% sequence identity between human and bovine RBP4 this cross-species detection was not surprising. Data presented here show it is likely that the RBP4 synthesised in human suture cells remains intracellular. This does not exclude the possibility that suture cells may also respond to RBP4 signalling from external sources. In human serum, RBP4 may be able to control intracellular levels of RBP4 through binding to the RBP4 receptor, STRA6, on those cells. Human suture cells have been shown to express STRA6 by flow cytometry (Dr. P. Dwivedi, unpublished data). It is also assumed here, that the RBP4 detected by our antibody is synthesised in these cells, not internalised from the serum. This belief is due to the levels of mRNA measured in these cells (Chapter 4). It is possible however, that the RBP4 protein detected in suture cells is a combination of both locally synthesised and internalised RBP4. To address this question experimentally a comparison could be made between cells with access to serum RBP4 and cells without. However, to serum starve cells for a long period of time for assessment may not be a viable option. Experiments with antibody blockade of RBP4 in the serum may be useful to assess the influence of this extracellular RBP4.

The western blot data shows RBP4 secretion from Huh7 cells, but also demonstrates that human suture cells did not secrete detectable levels of the RBP4 under the experimental conditions. The secretion of RBP4 by Huh7 cells has been reported previously and it is well known that the liver is a site of production and secretion of RBP4 (Tamori et al., 2006, Shea et al., 2007). Another similar liver carcinoma cell line, HepG2 has also been demonstrated to secrete RBP4 (Marinari et al., 1987). The lack of RBP4 secretion by human suture cells
strengthens an argument for a localised function. The positive control for secretion, albumin, demonstrates those cells were functionally active and able to secrete proteins. Unfortunately, time constraints restricted further experiments but, in future, immunoprecipitation of the culture supernatant may concentrate any RBP4 that is secreted but below detectable levels for our western blot analysis. This could confirm the lack of secretion of RBP4 from suture cells or if detected, may be able to quantitate the amount of secreted protein.

The conflicting results between the computerised prediction and experimental procedures also raise the question of RBP4 isoforms. Isoforms of RBP4 have been detected in patients with renal failure (Jaconi et al., 1995) but are not routinely tested for in other diseases. These isoforms lack 1 to 6 of the C-terminal residues of the protein. The C-terminal end of the RBP4 protein is located on the 3D surface of the molecule (Newcomer et al., 1984). This suggests that these isoforms with altered C-terminal ends may have differing interactions with other proteins in the cell. In serum, RBP4 binds with TTR to prevent its passage through the kidneys into the urine (Jaconi et al., 1995). Intracellularly, it is not known if RBP4 is bound to a companion molecule but the possibility of different interactions from different isoforms cannot be ignored. The antibody used in the present study was raised against human urinary RBP4 and hence there was no specific sequence to which the antibody is raised.

In this chapter, a distinctive subcellular localisation of RBP4 in human suture cells was demonstrated, which was likely to be in the endoplasmic reticulum. It was also shown that human suture cells lack detectable RBP4 secretion.
Chapter 6

Overexpression of RBP4 and its effect on osteogenesis in human suture cell culture
6.1 INTRODUCTION

In Chapter 3, a correlation was demonstrated between RBP4 expression, suture fusion and bone mineralisation. Following on from this, in Chapter 4 a downregulation of RBP4 during mineralisation in cell culture models of osteogenesis was identified. In this chapter, experiments were conducted to test whether increasing the level of RBP4 in cells would affect their ability to mineralise. According to the hypothesis, if decreased RBP4 allows for bone formation, an increase in RBP4 may halt mineralisation seen in osteogenic cell culture.

Overexpression has allowed for functional analyses of proteins of interest in in vitro and in vivo models. The most common methods of delivery of expression vectors are transfection and infection. Although chemical transfection has been shown to be a useful method for introduction of exogenous DNA into cell lines in vitro, it is known to be a particularly difficult method for use with primary cells (Hamm et al., 2002). There has, however, been a higher success in tranfecting primary cells when using methods of electroporation (Jordan et al., 2008). Transfection efficiencies of >30% have been achieved in cartilage-derived chondrocytes and >45% in mesenchymal stem cells, similar to the suture cells used in this thesis (Hamm et al., 2002). In addition, studies have shown that human bone marrow derived mesenchymal stem cells react favourably to electroporation, with cells maintaining their morphology, proliferation and multipotency that can be lost with other transfection methods (Helledie et al., 2008). Recently, electroporation has shown potential as a therapeutic strategy, with groups demonstrating the feasibility of gene transfer as a treatment for diseases such as hypercholesterolemia (Stevenson et al., 1995), cystic fibrosis (Stocker et al., 2009) and lung fibrosis (Sime et al., 1997).
In trials in our laboratory a variety of transfection reagents have been tested, revealing that the human suture cells are particularly resistant to chemical transfection methods (Dr. Prem Dwivedi and Dr. Jodie Hatfield, personal communication). Electroporation using the Amaxa Nucleofector has been shown to be highly successful in other difficult to transfect cells such as human primary neurons (Gärtner et al., 2006), human T cells (Yin et al., 2006) and osteoclasts (Taylor et al., 2007). In the laboratory, a high level of transfection (greater than 90%) has been achieved with the Amaxa Nucleofector, however, it also resulted in a high level (also greater than 90%) of cell death (Dr. Prem Dwivedi, personal communication). In addition, the human primary suture cells are slow growing and the large numbers required for these electroporations was restrictive. This led to an investigation of the use of infection using viral vectors for overexpression.

Despite the difficulty in conducting over-expression studies in primary cells there have been some successful studies using infection of cranial and suture tissue. The small number of such studies is likely a reflection on the difficulty involved with the technique. Adenoviral vectors have been used to create FGFR1 and FGF2 overexpression in both organ culture and in animal models and these studies have shown the ability of these FGFRs to alter the fate of the suture (Greenwald et al., 2001). In addition to this, studies have used adenovirus to demonstrate a role for TGF-β in suture fusion (Song et al., 2004a). However, there is no published evidence that adenovirus achieves a high rate of infection in these cranial tissues.

The three main viral infection methods used in overexpression studies are retrovirus, adenovirus and lentivirus. Each method has positive and negative factors associated with its use. Lentivirus was chosen for this study due to its ability to infect and replicate in dividing and non-dividing cells. Additionally and in contrast to adenoviral vectors, DNA encapsulated
in lentiviral vectors is able to integrate into the genome of the target cell, allowing for long
term expression of a gene of interest (Cockrell and Kafri, 2007). Stable transfection is highly
desirable as mineralisation studies in primary suture cells can take over 28 days to perform.
Furthermore, local expertise in lentiviral technology was available from A/Prof Don Anson
and the ability of lentivirus produced by this method to infect murine tissue *in situ* (Stocker et
al., 2009) made this an attractive option for these studies.

In this chapter a lentiviral delivery system was used to increase the expression of RBP4 in
human suture derived primary cells and to assess whether it had an effect on their ability to
mineralise.

### 6.2 RESULTS

6.2.1 CONSTRUCTION OF AN RBP4 EXPRESSION VECTOR

To construct an RBP4 expression vector a human RBP4 clone (Genecopeia, USA) and pHIV
lentiviral vector (Koldej et al., 2005) were used. A summary of the process used to prepare
the vectors is shown in Figure 6.1. Primers were used to amplify the target hRBP4 sequence
and add the ClaI and NdeI restriction enzyme sites. This PCR of the hRBP4 insert from the
commercially available vector was successful and yielded products of predicted size (633bp)
as seen on an agarose gel (Figure 6.2a). To prepare the ends of the PCR product for ligation
into the lentiviral vector it was digested with ClaI and NdeI. This produced no visible size
Figure 6.1: Diagram representing the process used to create RBP4 expression vector. The hRBP4 insert was prepared by PCR from a human RBP4 vector purchased from Genecopoeia. Primers included restriction enzyme sites. The PCR product was digested with ClaI and NdeI to prepare the ends for ligation into the empty eF1α vector. The empty vector and hRBP4 coding region were then ligated.
Figure 6.2: Diagnostic digests from production of RBP4 vector. The coding region of RBP4 was amplified using PCR and ligated into an empty pHIV vector. A) Purified RBP4 PCR product on an agarose gel. B) RBP4 vector digested with BamHI and NdeI. C) RBP4 vector digested with NotI. M1 indicates 100 bp ladder, M2 indicates 1kb ladder, P indicates PCR product and V indicates vector. Arrows identify the size of expected products.
change on an agarose gel as expected. Diagnostic digestion of the hRBP4 PCR product with XhoI, which has a site within the coding region of RBP4 the PCR product, yielded two bands of expected size (data not shown).

After the hRBP4 PCR product was ligated into the lentiviral vector, enzymatic digestions were used to confirm insertion by digest size. When digested with BamHI and NdeI the vector yielded the expected two bands of predicted sizes 1818bp and 5530bp (Figure 6.2b). Digestion with NotI yielded 2 bands of predicted sizes 3185bp and 4163bp (Figure 6.2c). The RBP4 expression vector map can be seen in Figure 6.3. To further confirm the correct sequence of RBP4 the vector was purified and sequenced (Figure 6.4). Sequencing results identified the RBP4 insert and confirmed it to be human RBP4 (Figure 6.5).

6.2.2 TRANSFECTION OF THE 5 VECTOR SYSTEM SUCCESSFULLY CREATED FUNCTIONING LENTIVIRUS

After successful production of the hRBP4 expression vector, a 5 vector approach was used for lentivirus production. This method involves transfection of the expression vector, along with 4 helper vectors into HEK293T cells (Koldej et al., 2005). An overview of the system can be seen in Figure 6.6. eYFP virus was produced by the same method described for RBP4 and used as a control. Production of lentivirus was shown by the ability to infect cells and express eYFP. eYFP was shown to be present after infection with lentivirus in HEK293T (Figure 6.7a), MC3T3-E1 subclone 4 (Figure 6.7b), mouse primary coronal suture cells (Figure 6.7c), NIH3T3 (data not shown) and human primary coronal suture cells (Figure 6.8). The intensity of the eYFP expression did not change over time, however the expression did vary in the
Figure 6.3: Diagrammatic representation of RBP4 overexpression vector. Vector map produced in vectorNTi showing the placement of the hRBP4 coding region under control of the EF1α promoter and the enzymatic sites used for diagnostic digests. The sequence from 3′LTR to 5′LTR is discarded during lentiviral packaging. Black line indicates base 1.
Figure 6.4: Sequencing of the RBP4 vector. Sequencing was performed in two regions, extent of sequencing in each direction is indicated by arrows. Underlining indicates the vector backbone, green indicates restriction enzyme sites, pink indicates the start codon of RBP4 and the coding region is seen in yellow. The stop codon is highlighted in red and the EF1α promoter is highlighted in blue. Protein translation is above the sequence.
Figure 6.5: Nucleotide BLAST of sequencing results. BLAST comparison of sequencing results confirming the correct ligation of RBP4 product into the vector. Query represents the sequencing results and Subject the matching found by BLAST.
Figure 6.6: Diagram representing the process used to make lentivirus particles. Vectors were transformed into DH1a E.coli to amplify. DNA was then purified. Vectors were transfected into HEK293T cells for packaging. Supernatant was collected after 48 hours and this contained the viral particles.
Figure 6.7: Images of cells infected with eYFP lentivirus. Cells were grown to 70% confluence and then infected with eYFP virus overnight. Pictures were taken 48 hours post infection. A) HEK293T cells, B) MC3T3-E1 subclone 4 cells and C) WT coronal mouse primary cells. Green arrows indicate highly expressing cells and red arrows indicate regions of low GFP expression. Scale bar represents 200μm (all panels at same magnification). Insets are relevant uninfected controls.
**Figure 6.8: Persistence of eYFP expression in suture cells after serial passage.** Cells were grown to 70% confluence then infected overnight with eYFP lentivirus. Infected cells were then passaged when confluent, approximately every 5 days. **A**) cells 48 hours post infection and **B**) after 4 passages. Green arrows indicate cells expressing eYFP and red arrows indicate cells not expressing eYFP. Scale bar represents 100μm (all panels taken at the same magnification).
different cell types, with a higher intensity of eYFP seen in the HEK293T (Figure 6.7a) and mouse primary coronal suture cells (Figure 6.7c) when compared to MC3T3-E1 subclone 4 (Figure 6.7b). The expression vectors appeared to have integrated into the genome of the target cells as shown by serial passage of the cells. Expression of eYFP was maintained after several passages and was still visible by fluorescence microscopy (Figure 6.8).

6.2.3 LENTIVIRAL INFECTION OF MOUSE PRIMARY SUTURE CELLS AND MOUSE CALVARIAL CELL LINE, MC3T3-E1 SUBCLONE 4

To validate the quality of infection with the lentivirus two approaches were taken. The first was to assess the percentage of infected cells by flow cytometry and the second was to assess the level of expression of the target by RT-qPCR. The LV numbers here are indicative of different preparations of lentivirus, with LV91 and LV106 containing the expression vector for eYFP and LV89 and LV105 containing the expression vector for RBP4. The percentage of MC3T3-E1 subclone 4 cells infected by lentivirus differed greatly with different preparations of lentivirus, from less than 10% with LV89 and up to 80% with LV105. This difference in infection efficiency was also seen in other cell types such as the mouse primary suture cells, although with the mouse primary cells the highest percentage of infection was approximately 35% with LV106 (Figure 6.9). The differences in overexpression between groups in general correlated with the percentage of cells infected. LV91 was the most reproducible, having approximately 2000 fold overexpression above background fluorescence in both MC3T3-E1 subclone 4 and mouse primary coronal suture cells (Figure 6.9a). The lowest levels of induction were seen with LV106 and LV105 in mouse primary coronal suture cells, and LV89 in MC3T3-E1 subclone 4 cells. However these lentiviral vectors still increased expression by
Figure 6.9: Infection efficiency by flow cytometry and gene expression by RT-qPCR of eYFP and RBP4 in MC3T3-E1 subclone 4 and mouse WT coronal primary cells. Cells were infected with either an RBP4 or eYFP lentivirus. After 48 hours cells were either prepared for flow cytometry or PCR as described in chapter 2. A) eYFP expression by RT-qPCR. B) RBP4 expression by RT-qPCR. RT-qPCR analysed using the Delta CT method. Samples normalised to β-actin and control was an uninfected sample. C) eYFP infection efficiency by flow cytometry and D) RBP4 infection efficiency by indirect immunofluorescence flow cytometry. LV# denotes different lentiviral preparations. LV89 and LV105 contain the expression vector encoding RBP4 and LV91 and LV106 contain the expression vector encoding eYFP. Columns are representative of n=3 technical replicates + SEM.
approximately 100 fold over background. In the eYFP lentiviral infections of mouse primary coronal suture cells a small amount of RBP4 expression was detected and this was due to the fact that the primers used for analysis did not distinguish between endogenous and exogenous RBP4 expression (Figure 6.9b). Statistical analysis was not performed on these data as there were only technical replicates rather than biological replicates.

As shown in Chapter 4, MC3T3-E1 subclone 4 cells did not endogenously express Rbp4 (Figure 4.5). The RBP4 lentivirus infected MC3T3-E1 subclone 4 cells and resulted in the expression of RBP4 protein as determined by immunocytochemistry (Figure 6.10a). In MC3T3-E1 subclone 4 cells infected with eYFP lentivirus no RBP4 was detectable (Figure 6.10b). However, when infected with lentivirus containing the RBP4 overexpression vector, positive regions of RBP4 staining were seen. In those cells the RBP4 appeared diffuse throughout the whole cell with some brighter regions of staining at the cell-cell junctions. In the mouse primary suture cells, RBP4 was detected in both cells infected with eYFP and RBP4 vectors but a higher intensity of RBP4 staining was seen in the cells infected with the RBP4 lentivirus (Figure 6.10c).

6.2.4 HUMAN PRIMARY SUTURE CELLS INFECTED WITH EYFP LENTIVIRUS HAVE THE ABILITY TO MINERALISE

To analyse the effect of eYFP and hRBP4 overexpression on the ability of cells to mineralise, human suture cells were infected with lentivirus and then an osteogenesis assay was performed. Human primary coronal suture cells still have the ability to mineralise after lentiviral infection (Figure 6.11). Cells infected with eYFP virus mineralised under the
Figure 6.10: Immunocytochemistry of RBP4 in MC3T3-E1 cells and mouse suture primary cells infected with LV105 lentivirus. Cells were infected with an RBP4 lentivirus. After 48 hours cells were fixed and stained using an anti-human RBP4 antibody. A) represents MC3T3-E1 cells infected with RBP4 virus, B) represents MC3T3-E1 cells infected with eYFP virus, C) represents mouse suture primary cells infected with RBP4 virus and D) represents mouse suture primary cells infected with eYFP virus. E) No primary control for the secondary antibody on mouse primary cells. F) Isotype control for the primary antibody on mouse primary cells. Green regions are positive staining for RBP4. Scale bar represents 200μm (all panels captured at the same magnification).
Figure 6.11: Representative timepoints of Alizarin Red staining of osteogeness assay in lentivirally infected human primary coronal suture cells. Cells were infected with either RBP4 (LV105) or eYFP (LV91) lentivirus overnight and passaged until sufficient numbers were available for osteogenesis assay (approximately 10 passages). Cells were plated at $2.5 \times 10^4$. Day 0 was considered when wells were confluent. Red staining indicates regions positive for mineralisation. DMEM indicates cells grown in DMEM alone, OG indicates osteogenic media and Dex indicates osteogenic media with dexamethasone. Days indicate days grown in the relevant media. Day 3, 7 and 21 images were omitted as they were very similar in appearance to those timepoints presented here.
induction of both osteogenic media and OG media plus dexamethasone. Cells began to mineralise under the additional influence of dexamethasone by day 14 and continued to mineralise through to day 28. When induced with osteogenic media alone cells began to mineralise at day 21. Although cells under the influence of OG media and dexamethasone began mineralisation early, by day 28 the levels of mineralisation in the osteogenic media and dexamethasone wells appeared similar by Alizarin Red staining after quantification (Figure 6.12).

6.2.5 OVEREXPRESSION OF RBP4 IN HUMAN SUTURE CELLS DID NOT PREVENT THEIR MINERALISATION

When tested with osteogenic induction, cells infected with RBP4 virus were still able to mineralise (Figure 6.11). Although there appeared slight differences in mineralisation, quantification of Alizarin Red staining demonstrated that there was no significant difference between the levels of mineralisation seen in the cells infected with RBP4 virus and eYFP virus (Figure 6.12).

OC expression was measured by RT-qPCR during the mineralisation experiments. OC expression increased significantly at day 14, corresponding with the beginning of mineralisation (Figure 6.13a). Expression of OC continued to increase at day 21 and day 28. Both eYFP and RBP4 lentivirally infected cells exhibited a significant increase in OC expression and there was no difference in expression levels between the groups (Figure 6.13a). In cells infected with eYFP lentivirus, RBP4 expression was significantly decreased during mineralisation (Figure 6.13b). In the cells infected with RBP4 lentivirus the levels of RBP4
Figure 6.12: Quantification of Alizarin Red S staining in human coronal primary cells infected with lentivirus. Alizarin Red quantification was performed on the osteogenesis assay represented in Figure 6.11 in which cells were infected with either RBP4 (LV105) or eYFP (LV91). DMEM indicates cells grown in DMEM alone, OG indicates osteogenic media and Dex indicates osteogenic media with dexamethasone. Days indicate days grown in the relevant media. Columns indicate average + SEM for n=3 biological replicates. # indicates significantly greater than day 0 DMEM and ^ indicates significantly greater than OG. P<0.05.
Figure 6.13: Gene expression from mineralising human suture cells infected with eYFP (LV91) or RBP4 (LV105) lentivirus by RT-qPCR. A) OC expression from osteogenesis assay in Figure 6.11. B) RBP4 expression from osteogenesis assay in Figure 6.11. Values shown are average + SEM of replicates n=3. Data analysed using the Delta CT method. Samples normalised to β-actin and control was day 0 DMEM sample. # indicates significantly different to day 0 DMEM. P<0.05.
expression were high and although there was a small trend towards a decrease in expression during day 21 and 28, large error bars meant that this decrease was not significant (Figure 6.13b).

6.3 DISCUSSION

In this chapter, experiments were performed to assess whether an increase in RBP4 expression would alter the ability of human suture cells to mineralise. This was performed by creating a human RBP4 overexpression vector and lentiviral infection of human suture cells. An hRBP4 expression vector was successfully produced and, in turn, infectious lentivirus was able to infect human primary suture cells, resulting in the overexpression of RBP4.

An RBP4 lentiviral expression vector had not been produced previously. Here an overexpression vector was constructed and demonstrated its ability to induce RBP4 expression in multiple cell types including human primary coronal suture cells and mouse primary coronal suture cells. The lentivirus induced a significant increase in expression of RBP4 and eYFP in both of these cell types. The difficulty with over expression studies in primary cells has driven the need for alternatives to transfections. Along with evidence from other cell types (Blomer et al., 1997, Rubinson et al., 2003, Stewart et al., 2003), the data presented here demonstrate the usefulness of lentiviral infection in overexpression studies in primary cells derived from the suture mesenchyme. There are also reports of successful infection of tissue in vivo. Lentiviral infection has been used to elicit biologically significant responses in mouse models of neuropathology (Consiglio et al., 2001), bone repair (Sugiyama...
et al., 2005) and sickle cell disease (Pawliuk et al., 2001). The levels of gene expression achieved with the lentivirus produced for these experiments suggest that these would be viable for use in vivo experiments in mice.

A good control of the ability of these lentiviral particles to induce overexpression was MC3T3-E1 subclone 4 cells which have been shown previously to not possess detectable endogenous RBP4 expression when measured by both RT-qPCR and immunocytochemistry. As seen here, there was a high level of expression when infected with the hRBP4 lentiviral vector. In cells with endogenous hRBP4 expression, the experiments presented here did not distinguish between endogenous and exogenous RBP4. The vector was designed incorporating human RBP4, which meant that the antibody and primers used for validation and testing in this study were able to detect both the exogenous and endogenous expression in cells of human origin (human primary coronal suture cells and HEK293T). Due to high levels of homology between human and mouse RBP4 this antibody and primers can also be used to detect RBP4 of mouse origin. In the MC3T3-E1 subclone 4 cells however, the lack of endogenous RBP4 indicated that in the infected cells RBP4 was expressed from the vector alone. In future experiments there are ways of distinguishing between exogenous and endogenous expression. A protein tag engineered into an RBP4 expression vector could allow for detection of exogenous RBP4 alone, as could primers designed to the untranslated regions of RBP4. Time did not permit the use of these methods in the current study.

Despite achieving infection by these lentivirus, the levels of infection were highly variable. In the mouse primary cells and cell lines, infection rates varied from 10% to 80% infection, and overexpression varied from 500 fold to 2000 fold. It is likely that, with optimisation, the infection efficiency of these lentivirus could be improved achieving a higher rate of infection.
and more reproducible results. The lentivirus was used as neat supernatant, however it is possible to concentrate the lentivirus by centrifugation to achieve a high multiplicity of infection (Marino et al., 2003). There is also the potential of altering the concentration of the packaging vectors transfected into the HEK293T cells to make lentivirus and the amount of polybrene used to aid lentiviral infection. The ratio of vectors transfected into HEK293T cells during lentiviral production was based on a previously published method (Anson et al., 2007). Relative ease of use of this system, the gift of helper vectors from A/Prof Don Anson and the ability of lentivirus produced by this method to infect murine tissue *in situ* (Stocker et al., 2009) made this an attractive option for the present studies.

Although no difference was seen in the mineralisation ability between cells infected with RBP4 and eYFP virus, from this data it cannot necessarily be concluded that RBP4 does not play a role. The slow growth of the human suture cells meant that it was not feasible to grow enough cells to work out the infection efficiency of the cells used in these osteogenesis assays. It was however, assumed, that we had achieved the same levels of infection as seen in primary trial experiments. As seen here and in other studies a high level of infection is very hard to achieve and the average percentage of cells infected here was 40%. It is possible that if a high rate of infection was achieved a difference in mineralisation may have been seen. Additionally, knowledge of the titre of each virus may have allowed for a higher and more consistent level of infection, unfortunately the small amount of lentivirus produced did not allow for this calculation for each lentivirus. An RBP4 expression lentivirus containing an IRES-GFP cassette to enable selection of cells by FACS (Levenson et al., 1998) is currently being produced in the laboratory but due to time constraints it could not be used for this study. It is hypothesised that if a high percentage of infection was achieved, a difference in the
mineralisation ability of these cells would be seen, based on the changes of RBP4 expression during mineralisation seen here and in Chapter 4.

An important factor to consider in the analysis of these osteogenesis assay results is the origin of RBP4. Although in other cell types RBP4 is secreted into the media, it was recently demonstrated that human primary suture cells lack detectable RBP4 secretion (Chapter 5). In a cell type which is able to secrete RBP4, a low infection rate may be overcome by the large amount of RBP4 being secreted into the media from the cells that are infected. This may mean that a response could be seen from both cells that are infected and those that are not, as RBP4 in the media may be able to elicit a response from uninfected cells through its receptor Stra6. These recent results demonstrating that in normal, uninfected suture cells, RBP4 remains intracellular and is not secreted however suggests that RBP4 would not be readily released into the local endocrine environment.

Another factor to consider is the role of retinoic acid in mineralisation. As discussed previously, RA is a potent inducer of osteogenesis in cell culture models (Song et al., 2004b, Wan et al., 2007). If RBP4 is functioning as a storage molecule for retinol, its effect may not be seen with the levels of retinol in our culture medium. It is possible that if these experiments were repeated with the high and low levels of RA seen to promote mineralisation in other studies, that the role of RBP4 may become more apparent. There is evidence from in vivo models that alteration of the level of retinol is required to fully identify the function of RBP4. Thus, mice lacking functional RBP4 are reportedly phenotypically normal unless maintained on a low retinol diet (Quadro et al., 1999). This may also be the case with cells. To identify the role of RBP4 in future experiments, altered levels of RA, both high and low, may be able to elicit a response.
The expression of cell culture marker OC during mineralisation mimics that which was seen in Chapter 4. Despite the infection with lentivirus there was no difference between the OC levels in eYFP and RBP4 infected cells. This lack of difference in OC expression was not unexpected due to the fact that there was no difference in the mineralisation. RBP4 expression was expected to decrease during mineralisation, and this was seen during mineralisation in the eYFP infected samples. The high level of RBP4 expression measured in the RBP4 infected cells may have obscured any decrease in expression during mineralisation. Additionally, for future experiments, analysis of cellular localisation of RBP4 in these lentivirally infected cells may also indicate why there was no difference in mineralisation. Should this RBP4 overexpression be located outside the ER, it may lose the capacity to influence mineralisation.

From the experiments presented in this chapter it has been demonstrated that lentiviral infection is a useful method for inducing expression in primary human suture cells. An RBP4 vector was created that induced overexpression. Pilot experiments were performed to look at the role of overexpression of RBP4 during mineralisation and despite the lack of changes in mineralisation seen, further optimisation of this method may assist in discovering the role of RBP4 during mineralisation.
Chapter 7

Conclusions and Future Work
7.1 SUMMARY

Craniosynostosis is the developmental condition, involving the premature fusion of the fibrous regions between cranial bones, sutures. The cause of craniosynostosis remains unknown however current research has a strong focus on genetic mutations. The more common craniosynostosis mutations affect FGFR1, 2 and 3, as well as TWIST1 and MSX2 (reviewed in Passos-Bueno 2008). How these mutations result in the premature bone growth seen in sutures is generally unknown, however some molecular mechanisms have been suggested. The gain of function mutations seen in FGFR2 have been shown to increase alkaline phosphatase activity, and proliferation and differentiation of osteoblasts (Ang et al., 2010) as well as activating the MAPK signalling pathway (Wang et al., 2010). Mutations in FGFR1 can alter mesenchymal stem cell fate to that of chondrocytes instead of osteoblasts (Maruyama et al., 2010) and Twist1 mutations have been associated with altering the response of suture cells to FGFs (Connerney et al., 2008) and interacting with ephrins (Plotkin et al., 2005). Recently, during a microarray study of gene expression in patients with craniosynostosis, a 37x downregulation of the retinol binding protein, RBP4, was identified during suture fusion (Coussens et al., 2007).

In this PhD thesis, research was conducted to address the hypothesis that localised downregulation of RBP4 in the suture tissue of patients with craniosynostosis (compared to unaffected controls) caused premature fusion by dysregulation of RA availability at sutures. The following specific aims were proposed:
1. To investigate the expression of RBP4 in suture fusion in a natural mouse model of suture fusion and in a genetic mouse model of Saethre-Chotzen craniosynostosis

2. To investigate the subcellular localisation of RBP4 in cell culture models

3. To generate a method of overexpression of RBP4 in human and mouse cells and tissue

Research presented in this thesis demonstrated a link between Rbp4 downregulation and coronal suture fusion in Twist1+/− mice, a model of human Saethre-Chotzen syndrome, with Rbp4 downregulated 5 fold during suture fusion. However, Rbp4 was also downregulated 4.5-5 fold during growth of the parietal bone in both Twist1+/− and WT mice. Histological analysis suggested that the downregulation may be not only linked to suture fusion, but to the normal process of thickening and growth of bone. In addition to Rbp4, a link between bone growth and suture fusion and Stra6 was identified, with Stra6 upregulated 4 fold during coronal suture fusion and 8 fold during parietal bone growth. Mineralisation of human suture cells in vitro also exhibited a significant downregulation of RBP4 during the process of calcified nodule formation.

To build on these observations, functional studies were performed to address a possible cause and effect relationship of the downregulation of RBP4 during bone growth and suture fusion. This was performed by creation of an RBP4 expression lentivirus. The RBP4 lentivirus enabled exogenous expression of RBP4 in human suture cells significantly greater than endogenous levels, with a significant induction of expression seen in human suture cells. Overexpression of RBP4, did not alter the ability of these cells to mineralise in vitro, however the infection efficiency was not determined. Analysis of the subcellular distribution of RBP4 in human suture cells identified a distinctive localisation in the endoplasmic reticulum. Additionally, it was shown that unlike Huh7 liver cells, human suture cells do not secrete
detectable levels of RBP4 into the media \textit{in vitro}.

7.2 PROPOSED MECHANISM OF RBP4 ACTION IN HUMAN SUTURES CELLS AND CALVARIAL BONE

The original hypothesis stated that RBP4 controls suture fusion by acting as a storage molecule in cells and regulating the availability of retinol in the local microenvironment and that by this, RBP4 can control osteogenesis and bone growth in the suture. From the work presented in this thesis, demonstrating downregulation of RBP4 during parietal bone formation and osteogenesis in cell culture, it is now believed that RBP4 may also have a role in normal bone maintenance in the calvaria.

From the data presented here, a modified hypothesis is proposed for the action of RBP4 in suture cells. Namely, it is hypothesized that the RBP4 that is produced locally in sutures and bone cells acts locally to store retinol in the endoplasmic reticulum of the suture and parietal bone cells. A possible mechanism for this storage is that retinol is initially delivered to suture and bone cells by serum RBP4 via STRA6. The hydrophobic retinol may be delivered to RBP4 resident in the endoplasmic reticulum via cRBP1 or cRBP2 which are responsible for trafficking of retinol to sites of retinol metabolism in the cell (Rong, 1997). When bound to RBP4, retinol is located inside a $\beta$-barrel structure and its release prevented by TTR (Goodman, 1980).

A mechanism for the control of bone growth may also involve interactions with STRA6.
Namely, upon induction to undergo osteogenesis, RBP4 is downregulated in the cell. The RBP4 present in the endoplasmic reticulum is signaled to release retinol, possibly by disassociation from TTR. The result is an increase in available retinol in the cell. This high concentration of available retinol may drive the metabolism towards a critical level of retinoic acid production, which in turn can promote osteogenesis. An increase in RA should also promote production of Stra6, which is upregulated in response to RA (Kawaguchi et al., 2007). In context of bone growth disorders such as craniosynostosis, this potential role for RBP4 in regulating bone growth may be reliant on other environmental factors such as Vitamin A intake and other genetic mutations. Additionally, the inverse relationship between STRA6 and RBP4 seen here during bone growth may allow for early detection and correction of bone growth defects.

In addition to detection, another important factor to consider is prevention. Our data, in conjunction with previous studies have suggested that vitamin A, its metabolites, and now binding and transport molecules required in the retinol signaling pathway may all have an influence on the aberrant bone growth that is experienced during craniosynostosis. As vitamin A is a dietary vitamin, there is the potential for implementation of dietary recommendations during pregnancy.

7.3 FUTURE WORK

The localisation of RBP4 to the endoplasmic reticulum by immunocytochemistry is a novel finding in human suture cells. This localisation could further be confirmed by analysing
individual cellular fractions. Techniques for separation of cells into their individual compartments is well characterised and should allow for western blot to detect RBP4 in specific cellular locations.

Pivotal to the hypothesis are changes in the availability of RA. Despite attempts to test this by both high-pressure liquid chromatography (HPLC) and a RA responsive cell line F9-RARE-LacZ, those studies were unsuccessful. HPLC is commonly used to detect and quantitate retinoids (Frolik et al., 1978) and has proven to be successful in detection of retinoids in human and mouse serum, cosmetics and tissue (reviewed in McCaffery 2002). Despite the lack of reproducibility in the attempts to measure RA by HPLC in this laboratory, the technique has been used for small samples in other models (Arafa 1996, (Kochhar and Penner, 1992). It is likely that with further time, this technique could be optimised for use with the small mouse amounts of suture tissue available.

The F9-RARE-LacZ cell line is an embryonic carcinoma cell line, stably transfected with a retinoic acid inducible response element driving a LacZ reporter gene (Wagner et al., 1992). Time constraints restricted optimisation of the F9-RARE-lacZ cell line, however other groups have used these cells for analysis of RA levels in small samples such as mouse testis (Deltour et al., 1996), mouse adrenal gland (Haselbeck et al., 1997) and E7.5 mouse embryos (Deltour et al., 1996). The development of a viable RARE-lacZ transgenic mouse also opens other avenues for RA testing in this model (Rossant et al., 1991). RARE-lacZ mice have offered insights into the role of RA in the development of lungs (Malpel et al., 2000), heart (Mossa et al., 1998) and the forebrain (Silvestri et al., 2008). Cross breeding of RARE-LacZ mice with Twist1+/- mice could allow for analysis of RA responses in situ during suture fusion, with changes in RA able to be visualised over the timcourse of suture fusion by lacZ staining. This
technique would allow for the use of computerised histological analysis programs to quantify the levels of RA by the amount of positive staining present.

The apparent link between RA and craniofacial defects, in particular craniosynostosis (Gardner et al., 1998, Song et al., 2005, James et al., 2010a) do make this direction of study especially interesting. Should these experimental analyses of RA been optimised in this thesis, it could have given a greater level of understanding into the role of RBP4 during bone growth. Additionally, a correlation could have been made between dietary levels of RA and cranial suture expression of RBP4.

Functional analysis of RBP4 was started in this thesis using an RBP4 expression vector contained within a lentiviral delivery system. Despite successful infection of human primary suture cells, and induction of high levels of RBP4 expression, no difference in mineralisation was seen. Optimisation of the lentiviral technique, however, may provide a different result. The rate of infection achieved with the lentivirus produced in this study varied, though the most successful infections produced infection rates below 100%. The design and production of an RBP4 lentivirus incorporating an IRES-GFP would allow for selection of infected cells based on GFP fluorescence. Sorting cells by FACS allows for the purification of a population of cells which have been infected, such that all cells express exogenous RBP4 (Cormack et al., 1996). A population of suture cells where most are infected may show a difference in mineralisation.

Organ culture is a model system that could be used as the next step in testing RBP4 during suture growth and fusion. Unfortunately, during this thesis there was insufficient time to explore organ culture models, however sutures and surrounding calvaria have been shown to
be suitable for this type of culture (Wenig et al., 1988, Shaughnessy et al., 1995). Following on from this, lentivirus has been used to infect mice tissue in vivo (Stocker et al., 2009). If our hypothesis is correct, introduction of RBP4 lentivirus to the PF suture of a mouse may abolish, or delay, suture fusion. Proof of this model has been demonstrated using adenovirus with infection of a truncated FGFR1 expressing adenovirus showing biologically significant results in the sutures of rats in vivo (Greenwald et al., 2001). Additionally, lentiviral expression in vivo would also allow for the introduction of short interfering/short hairpin RNA, to further decrease the levels of RBP4. This process has been used to successfully decrease expression of target genes in mice (Tiscornia et al., 2003). As we have categorised the timing of suture fusion and Rbp4 expression in our Twist1+/− mice, a knockdown of Rbp4 may also be able to give a novel indication of the role of Rbp4 in suture fusion and craniosynostosis as seen in these mice.

The data presented in this thesis demonstrates that both human suture cells and mouse suture tissue express RBP4 mRNA. Hence, it is expected that the RBP4 detected by immunocytochemistry in the human suture cells is endogenous to these cells. However, it is also possible that extracellular RBP4 from the FBS may contribute to the pool of protein being detected. Blockade of extracellular RBP4 in culture by an antibody may allow for testing of this as it would eliminate the possibility of extracellular RBP4 becoming internalised. This process has proven successful in prevention of the internalization of virus particles in cell culture (de Parseval et al., 1997, Gerna et al., 2008). Although RBP4 was undetectable in the supernatant of the cells when grown in serum-free media, it does not eliminate the possibility of RBP4 secretion. More sensitive methods to detect low protein levels could be employed to test this. There are well characterised methods for concentration of cell culture supernatants and although most commonly used for purification of antibodies (Veeraragavan et al., 1991)
and virus particles (Adams, 1973), the process has also been used to analyse proteins in media (Cao et al., 1999) and there are now commercially available kits for this purpose. Another approach is immunoprecipitation, which would allow the concentration of the RBP4 in supernatant for analysis by western blot.

The Rbp4−/− mouse model would also be a useful tool in analysis of the role of RBP4 in sutures. The original report of the development of these mice did not analyse any skeletal or craniofacial defects (Quadro et al., 1999). Unfortunately, after importing the mice a breeding colony of these mice was not established in time for this study. Their sensitivity to vitamin A levels in the diet proved to be a major factor in their ability to produce viable offspring. Change of diet to a high vitamin A diet did rescue this phenomenon, but unfortunately too late for use in this thesis.

Finally, it should also be assessed whether changes in RBP4 presented in this study are reflected in changes in expression of other retinol binding proteins in suture cells. In addition to RBP4, there are other retinol binding proteins that act intracellularly. Cellular retinol binding protein 1 (cRBP1) and cellular retinol binding protein 2 (cRBP2) are important members of the retinol-signaling pathway intracellularly. They act to both store retinol and deliver it to enzymes to prepare for storage or conversion to RA (Molotkov et al., 2004). Testing of cRBP1 and 2 by PCR, immunocytochemistry and western blot would allow for analysis of levels of expression of these proteins during suture fusion and bone growth. The large cranial suture tissue bank present in our laboratory would allow for this analysis in the tissue that displayed the downregulation of RBP4. Although changes in expression have not yet been tested, data from our laboratory demonstrates that cRBP1 and 2 are expressed in human suture tissue (Miss I. Dewiputri Wan Burhanuddin, unpublished data).
7.4 CONCLUDING REMARKS

The data presented in this thesis indicates that RBP4 may play an important role in the growth of parietal bone and sutures. Its subcellular localisation in the endoplasmic reticulum in human suture cells, and the inverse relationship with Stra6 in mouse suture tissue suggest a storage role in calvarial tissue. Finally, an RBP4 lentiviral expression vector was developed that will enable investigation of the function of RBP4 in osteogenesis. These finding present a direction for future investigation into therapeutic interventions in the treatment of craniosynostosis.
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APPENDIX

Primer locations and PCR product sequencing of primers designed for this study.

Other primers used in this study can be found in Coussens (Coussens, 2007).
MOUSE β-ACTIN

PRIMER LOCATION

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1  ctgtcgagtc gcgtccaccg gcgagcacag cttctttgca gctccttcgt tgccggtcca
61  caccggttcc ctacgccagc cgtgactgat tcctgtttgg tggacgccga
gtgagcatgc tgcgtttgtt tttctttgca gctccttcgt
gggagtgtat acactctatgt
121  cggcatgtgc aaagccggct tcgcgggcga cgatgctccc cgggctgtat
tcccctccat cgggccgc cctaggcacc agggtgtgat
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SEQUENCING AND BLAST RESULTS MOUSE β-ACTIN

CGTGGGCCGCCCTAGGCAACAGGGTGTTGATGTTGGGAAATGGGTCAAGGACTCTATGTGGTGA
GAGGCCAGAGCAAGAGAGGTATCTGACCCTGAGCTGAAGAACATGGGAATCCATGGCATTTGAC

ref|NM_007393.2| GM Mus musculus actin, beta, cytoplasmic (Actb), mRNA

Length=1891

Score = 233 bits (128), Expect = 4.6e-61
Identities = 129/130 (99%), Gaps = 1/130 (0%)
Strand=Plus/Plus

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Query 120
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Sbjct 301
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HUMAN β-ACTIN

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>refNM_001101.3 Homo sapiens actin, beta (ACTB), mRNA
Length=1852

GENE ID: 60 ACTB | actin, beta [Homo sapiens] (Over 100 PubMed links)

Score = 476 bits (240), Expect = 6e-132
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Strand=Plus/Plus

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MOUSE RBP4

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SEQUENCING AND BLAST RESULTS MOUSE RBP4

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>ref|NM_011255.21 G MUs musculus retinol binding protein 4, plasma (Rbp4), mRNA
Length=1176

Score = 324 bits (175), Expect = 9e-87
Identities = 175/175 (100%), Gaps = 0/175 (0%)
Strand=Plus/Plus

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Strand=Plus/Plus
MOUSE ALKALINE PHOSPHATASE

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232
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Length=2459

Score = 167 bits (90),  Expect = 3e-41
Identities = 93/94 (99%), Gaps = 1/94 (1%)
Strand=Plus/Plus

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SEQUENCING AND BLAST RESULTS MOUSE OSTEOCALCIN

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(Over 100 PubMed links)

Score = 265 bits (143), Expect = 2e-70
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235
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121  ccccaagctg gctcaaggaag ggggagaggt cccgaaaggg aaccaagga agctctcaag
181  gacccgaccc ccttgccgctgc ttagctctct ctcggtcggt
241  catcagaggaa cctctaggag ctgaggaggt gcagccagag ggggtgattc ccctttgcca
301  gctcaccgca cccccctggcc ttggcatcgt cgagaggtgt ttacccggca gccctgggtt
361  gctctacgcc acatcctcagc cggggtgata ggcctggagt tcggggtggt ggtgctggct
421  ctcctctggcc gggggtaggc cccgataact ccagaggtct

actg
184  gactg cccagccctg tggatttctt ggctggggac ctatcctgga cagtgcctgc
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tgcttcggca gggcaccaag ctgcattcct attgggtact gtgttatctt gggcccactt
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184  gactg cccagccctg tggatttctt ggctggggac ctatcctgga cagtgcctgc
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acttg
184  gactg cccagccctg tggatttctt ggctggggac ctatcctgga cagtgcctgc
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tgcttcggca gggcaccaag ctgcattcct attgggtact gtgttatctt gggcccactt
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acttg
184  gactg cccagccctg tggatttctt ggctggggac ctatcctgga cagtgcctgc
tgctgtcttt gtggtcctct tcagcaacct atgcctgttg
tgcttcggca gggcaccaag ctgcattcct attgggtact gtgttatctt gggcccactt
tggtgttcag gtctggcaga aagctgagtg tcctcaggat cctaagatct acaagcacta
tgcttgctgttt gcctggctgg tgagacggcg caggctttgg ccacgctgtg gtcatcgcgg
SEQUENCING AND BLAST RESULTS FOR MOUSE STRA6

GTCATCGCGGACTTGGACTGCCAGGCTGGATTTCTTTGCTGATGACAGTGCTGGCTGTGCTGTCTTTGCTGCTCTTCAGCAACCTATGCTGT

>ref|NM_009291.1| GM Mus musculus stimulated by retinoic acid gene 6 (Stra6), mRNA
Length=2993

Score = 214 bits (108), Expect = 1e-53
Identities = 108/108 (100%), Gaps = 0/108 (0%)
Strand=Plus/Plus

Query 1   GTCATCGCGGACTTGGACTGCCAGGCTGGATTTCTTTGCTGATGACAGTGCTGGCTGTGCTGTCTTTGCTGCTCTTCAGCAACCTATGCTGT  60
          1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60
Sbjct  591 GTCATCGCGGACTTGGACTGCCAGGCTGGATTTCTTTGCTGATGACAGTGCTGGCTGTGCTGTCTTTGCTGCTCTTCAGCAACCTATGCTGT  650
          1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60
Query 61  CAGTGGCGCGGCTGCTCCTTTCTCTCTGCAGGGCCACCTATGCTGT  108
          1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60
Sbjct  651 CAGTGGCGCGGCTGCTCCTTTCTCTCTGCAGGGCCACCTATGCTGT  698
MOUSE HPRT

PRIMER LOCATION
1
61
121
181
241
301
361
421
481
541
601
661
721
781
841
901
961
1021
1081
1141
1201
1261
1321

ggagcctggc
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tttgccgcga
atgatgaacc
tggaaaaagt
atgtcatgaa
ataagttctt
ccattcctat
gggacataaa
ttgttgaaga
agtacagccc
ttggatacag
cccttgacta
gaaaagccaa
tgttgatgtt
gctttttgca
cgtccccaga
gtcgcttatc
agattgtatc
aatttttata
agctctcgat
tttcagcagt
aaatgcaaat

cggcagcgtt
ctttccggag
gccgaccggt
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gtttattcct
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gactgtagat
agttattggt
tataattgac
caaaatggtt
gccagacttt
taatgagtac
atacaaagcc
gccagtaaaa
tgaaccttct
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ttgtaagaaa
tgtaagaagg
tattcaggag
ttcctatcag
gttggctgta
aaataaattc

tctgagccat
cggtagcacc
cccgtcatgc
ctagatttgt
catggactga
ggccatcaca
ctggattaca
tttatcagac
ggagatgatc
actggtaaaa
aaggttgcaa
gttggatttg
ttcagggatt
taagatgagc
ttagcaggtg
atgaatgtta
gcactatgag
aacaaatctc
atttaaagag
agaaagatgt
taacagcatc
ttttcccact
taaaaattc

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tcctccgccg
cgacccgcag
tttgtatacc
ttatggacag
ttgtggccct
ttaaagcact
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tctcaacttt
caatgcaaac
gcttgctggt
aaattccaga
tgaatcacgt
gcaagttgaa
ttctagtcct
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cctataggcc
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aagctatatt
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taagaggttt
ttcagtaaat

gcgagggaga
gcttcctcct
tcccagcgtc
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caagtttgtt
ttgtgtcatt
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gtggccatct
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cgaggagtcc
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tcagttgctg
ctggtagatt
aataatactg
ttggtatttt
acgagtctga
aataaacatg
agttcctttt

SEQUENCING AND BLAST RESULTS FOR MOUSE HPRT
GGGACATAAAAGTTATTGGTGGAGATGATCTCTCAACTTTAACTGGAAAGATGTCTTGATTGTTGAA
GATATAATTGAC ACTGGTAAAA CAATGCAAAC TTTGCTTTCC CTGGTTAAGC AGTACAGCCC

239

