IMMUNOLOCALIZATION OF FIBROBLAST GROWTH FACTOR-2 (FGF-2) IN THE DEVELOPING ROOT OF THE MURINE TOOTH

Anil Kumar Madan

A dissertation submitted to the Faculty of Health Sciences, University of the Witwatersrand, in fulfilment of the requirements for the Degree of Master of Science (Medicine)

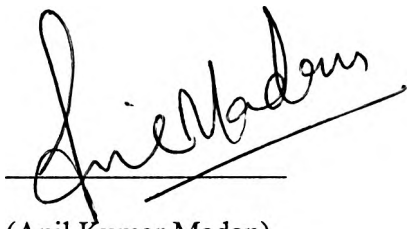
Johannesburg, 2004

"Aim for success, not perfection. Never give up your right to be wrong, because then you will lose the ability to learn new things and move forward with your life."

Dr. David M. Burns

DECLARATION

I, Anil Kumar Madan, declare that this dissertation is my own, unaided work. It is being submitted for the degree of Master of Science in Medicine in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.



(Anil Kumar Madan)

17th day of FEB 2004

In memory of my father
Tharia Lal Madaan
1941-2002

Publications and presentations arising from this study

Madan, A. K. & Kramer B. Immunocytochemical localization of the Fibroblast growth factor-2 (FGF-2) in the developing root of the murine tooth. Presented in: *XIIth International symposium on morphological sciences,* Timisoara (Romania) 11-14 September 2002

ABSTRACT

Classical epithelio-mesenchymal interactions are said to result in root development. These interactions may be regulated by a number of growth factors. Fibroblast growth factors (FGF's), members of a highly conserved family of polypeptides, the heparin binding growth factors (HBGF's) are known to play a crucial role during the development of certain vertebrate organs, including the tooth. Previously, FGF-2, 3, 4, and 8 have been shown to play a role in crown development. The aim of this study was therefore to elucidate the spatial and temporal expression of FGF-2 in the developing root. Parasagittal sections of the maxillary and mandibular arches of six age groups of post-natal mice (days 9, 10, 12, 16, 20 and 24) were cut and the developing roots of the incisor and molar teeth identified. Immunocytochemistry utilizing anti-FGF-2 was performed on sections of teeth from all stages using the strept-avidin biotin technique. Appropriate positive, negative and absorption controls were performed to ensure the specificity of the antibody. FGF-2 was immunolocalized in the cytoplasm and nuclei of the odontoblasts, fibroblasts of the periodontal ligament and pulp chamber, as well as in the osteoblasts surrounding developing bone at all the stages examined. Intense staining for FGF-2 was observed in differentiating odontoblasts at the apical end and the furcation zone of the developing root. FGF-2 localization was also observed in the cytoplasm of the ameloblasts on days 9, 10 and 12 and in cementoblasts on day 16, 20 and 24. The spatio-temporal expression pattern of FGF-2 in the developing mouse tooth root suggests that FGF-2 with other signaling molecules previously reported such as bone morphogenetic proteins-2, 3 and 7 (BMP-2, 3 and 7) participate in the signaling network during the tooth root development.

ACKNOWLEDGEMENTS

First of all, I would like to thank God for giving me the strength, knowledge and courage to start this endeavor and complete it.

I would like to express my sincere gratitude and appreciation to my supervisor, Professor Beverley Kramer for her invaluable advice, support, guidance, and dedication to Science, persistence and consistent supervision, even on weekends and after hours. I probably would not have completed this without her help.

I would like to express my heartfelt appreciation to Dr. Volkert Clausen for all his invaluable suggestions.

Special thanks to Mrs. Lesley York for her excellent technical advice.

I am grateful to my parent's -in-law and brother in-law for their continuous support throughout this project.

Finally, I would like to thank my wife, Nisha Madan for all her love, support and "patience" throughout my life and especially throughout this project.

TABLE OF CONTENTS **Page**

LIST OF FIGURES

LIST OF TABLES

Table **Page**

LIST OF APPENDICES

APPENDIX **Page**

CHAPTER ONE

1.0 INTRODUCTION

1.1 Preamble

Signals that orchestrate the expression of genes in time and space are a central theme in developmental biology. This quest to understand these mechanisms has lead scientists to explore the process of organogenesis in mammals. Organogenesis, which is a complex paradigm, is the progression of a cell or a tissue through a series of events that finally lead to the formation of a well-defined structure, both in shape and form. To achieve this final outcome, every cell passes through three fundamental stages: firstly, availability of positional information and interpretation of this information to initiate organ formation at the correct place (induction); secondly formation of a rudiment organ by the cells (morphogenesis) and finally formation of an organ-specific structure (differentiation). Morphogenesis, which is a critical stage of this process, is governed by co-coordinated, sequential and often reciprocal cascades of events between the epithelium and mesenchyme (Grobstein, 1953; Saxen *et al.,* 1976). Reciprocal inductions underlie the development of many organs such as the limbs, hair follicles, lungs, kidneys and teeth (Mina and Kollar, 1987; Neubüser *et al.,* 1997; Cancilla *et al.,* 1999; Thesleff, 2000; Paria *et al.,* 2001). Events which govern the process of morphogenesis may include cell proliferation, cell differentiation, cell migration, apoptosis [programmed cell death], positional signaling and matrix synthesis (Wolpert, 1989; Neubüser *et al.,* 1997; Hogan, 1999). This cascade of events, which are key features in the process of morphogenesis, may either be facilitated by spatial and temporal expression of conserved families of signaling molecules (Vainio *et al.,*

1993; Peters and Balling, 1999; Thesleff, 2000), or as a result of permissive and instructive inductive interactions (Thesleff, 2000).

Tooth development also follows this pattern of morphogenesis and is believed to involve signals such as fibroblast growth factor (Wilkinson *et al.,* 1989; Kettunen *et al.,* 2000), bone morphogenetic proteins (Vaino *et al.,* 1993; Thomadakis *et al.,* 1999), hedgehog genes (Dassule *et al.,* 2000 Gritli-Linde, 2002), Wnt genes (Sarkar and Sharpe, 1999; Jernvall and Thesleff, 2000; Sarkar *et al.,* 2000), expression of transcription factors [e.g. Msx-1 and Msx-2] (Chen *et al.,* 1996), the synthesis of membrane protein syndecan and extracellular protein tenascin (Tucker *et al.,* 1993).

1.2 Morphogenesis and tooth development

Tooth development is a complex morphogenetic process that is guided both by intrinsic genetic information and epigenetic environmental signals. A characteristic feature of odontogenesis and dental cytodifferentiation in mammals is the interaction between the epithelium and mesenchyme both sequentially and reciprocally, at successive key morphogenetic stages (Chen *et al.,* 1996; Thesleff and Sharpe, 1997; Jernvall and Thesleff, 2000). In mammals, classical tissue recombination experiments have demonstrated that tooth morphogenesis involves an interaction between the oral epithelium of the first pharyngeal arch (E-9 to E-11 in the mouse) covering the maxillary, median-nasal and mandibular processes and the underlying neural-crest derived ectomesenchymal cells (Kollar and Baird, 1970; Slavkin and Bringas, 1976; Mina and Kollar, 1987; Karanova *et al.,* 1992; Vaino *et al.,* 1993; Thomas *et al.,* 1997; Choi *et al.,* 2000). The neural crest cells subsequently signal reciprocally to the epithelium and regulate its morphogenesis. It is believed that the same conserved multigene signaling families mediate epithelio-

mesenchymal interactions in tooth development as in other vertebrate organs (Jernvall and Thesleff, 2000).

In the past two decades, a number of genes and gene products have been discovered that are believed to be the chemical signals for cell-to-cell communications between the oral epithelium (dental lamina, enamel organ epithelia and inner enamel epithelium) and the ectomesenchymal cells of the dental papilla that are required for tooth formation (MacKenzie *et al.,* 1992; Vainio *et al.,* 1993; Chen *et al.,* 1996; Neubüser *et al.,* 1997; Bei and Maas, 1998; Kettunen and Thesleff, 1998). These chemical signals or morphoregulatory proteins are believed to play their role by accomplishing the following tasks. They:

(i) act directly on the deoxyribonucleic acid (DNA), present in the chromosomes in the nucleus of the somatic cells [e.g. transcription control molecules such as Msx-1, Msx-2, Lef-1 and Hox genes] (MacKenzie *et al.,* 1992; Chen *et al.,* 1996; Thesleff and Sharpe, 1997; Bei and Maas, 1998).

(ii) act as extracellular chemical signals (such as bone morphogenetic proteins [BMP's], fibroblast growth factors [FGF's] and epidermal growth factors [EGF's] etc.) or their cognate receptors (Neubüser *et al.,* 1997; Kettunen and Thesleff, 1998).

To date, more than 200 genes expressed in developing teeth have been studied (http://honeybee.helsinki.fi/toothexp: Nieminen *et al.,* 1998). It is suggested that the spatial and temporal expression of these genes determines the position and the state of differentiation of individual cells in tooth morphogenesis (Thesleff, 2000).

1.3 Fibroblast growth factors (FGF's): members of the heparin binding growth factor family (HBGF)

Fibroblast growth factors (FGF's) form one of the larger groups of signaling molecules expressed in a variety of tissues and are believed to play a crucial role in successive stages of organogenesis. FGF's were initially isolated as proteins capable of stimulating growth of fibroblasts and demonstrated the ability to bind with heparin/heparan sulfate. Originally, FGF's were classified as members of the "heparin-binding growth factor" (HBGF) superfamily (Masahiro *et al.,* 2001). The member proteins of this family are structurally related, but genetically distinct (Kuzis *et al.,* 1996; Stern *et al.,* 1997).

The polypeptides of this family bind to the sulfated glycosaminoglycan heparin by their overlapping mitogenic and neurotropic actions on a variety of mesodermal, ectodermal and neuroectodermal cell types (Burgess and Maciag, 1989; Kuzis *et al.,* 1996; Courlier *et al.,* 1997; Guillonneau *et al.,* 1998). To date, 22 members of this family of mitogens have been identified (Ornitz and Itoh, 2001; Karabagli *et al.,* 2002) in organisms ranging from nematodes to humans. In vertebrates, the 22 members of the FGF family range in molecular mass from 16 to 34 KDa. They share 13-71% of the amino acid sequence in a core region of 120 amino acids and demonstrate similar activities in many biological assays (Kuzis *et al.,* 1996; Courlier *et al.,* 1997; Ford *et al.,* 1997; Stern *et al.,* 1997; Guillonneau *et al.,* 1998; Ohbayashi *et al.,* 1998; Barasch, 1999; Ornitz and Itoh, 2001). Member proteins of

this family are highly conserved across mammalian species (Ornitz and Itoh, 2001). Although considerable homology exists in the structure of FGF's, their functions differ significantly.

FGF-1 and FGF-2 were the first two members to be isolated, and are the most studied members of this multipotent family of polypeptides (Burgess and Maciag, 1989). FGF-1 and FGF-2 are monomers of approximately 155 amino acids in chain length, with molecular weight of about 18 KDa (Okada-Ban *et al.,* 2000). These two members share about 55% amino acid identity as well as both lack the classical peptide signalling sequence (Rifkin and Moscatelli, 1989). Although there is almost universal tissue distribution and action of FGF-2 and the wide distribution of FGF-1, many other members of this family are also expressed during the development of many organs. Some other important members of this family are FGF-3 to -9. FGF-3 to -6 were isolated as proto-oncogenes from tumor cells (Dickson and Peter, 1987; Miki *et al.,* 1991) and were designated as FGF-3 (int-2), FGF-4 (Kaposi sarcoma FGF/K-FGF or hst/ a product of hst-1 oncogenes), FGF-5 and FGF-6 (KGF). FGF-7 also called keratinocyte growth factor (KGF) was identified based on its selective mitogenicity for epithelial cells but not for fibroblasts (Finch *et al.,* 1989). FGF-8 (androgen-induced growth factor/AIGF) was isolated from a murine androgen-dependent carcinoma (Tanaka *et al.,* 1992). FGF-9 initially described as glial- activating factor (GAF), was isolated from a human glioma cell line (Miyamoto *et al.,* 1993). In addition, a group of four fibroblast homologous growth factors (FHF's) have been identified and which play an important role in nervous system development. They are designated as FGF-11 to -14 (Smallwood *et al.,* 1996). The discovery of new members of this family has been frequent and in future, more functions related to this group of polypeptides may be defined.

1.4 Historical perspective of fibroblast growth factor-2 (FGF-2)

FGF's in general were first isolated in the 1970's from bovine brain based on their mitogenic and angiogenic activities (Armelin, 1973). In 1975, Gospodarowicz specifically identified FGF-2 from bovine pituitary gland extracts based on its mitogenic activity in the Balb/c3T3 cell line. This growth factor was characterized as a single peptide of the molecular weight of 18 KDa. FGF-2 is produced as a 155 amino acid precursor out of which the mature 146 amino acid residue recombinant form has been synthesized (Moy *et al.,* 1996; Okada-Ban *et al.,* 2000). Its structure has been determined using nuclear magnetic resonance [NMR] (Moy *et al.,* 1996). This growth factor is also called basic fibroblast growth factor (b-FGF) because of its basic isoelectric point pI= 9.6 (Okada-Ban *et al.,* 2000). The primary structure of FGF-2 is highly conserved among the mammals including humans, bovines and rats (Rifkin and Moscatelli, 1989).

1.5 FGF-2: a pluripotent growth factor

FGF-2 is a pluripotent growth factor. Several biochemical and immunocytochemical studies have demonstrated the presence of this mitogen in a variety of rat, human and mouse tissues. These include brain (Bean *et al.,* 1991; Kuhn *et al.,* 1997), heart (Pasumarthi *et al.,* 1996), pituitary and adrenal glands (Grothe and Unsicker, 1990), thyroid gland (Grothe and Unsicker, 1990), embryonic tissues (Karabagli *et al.,* 2002), in the developing retina of different species (Mc Farlane *et al.,* 1998; Patel and McFarlane, 2000), in ovine skin during follicle morphogenesis (Du-cros *et al.,* 1993) and in blood vessels (Poole *et al.,* 2001). In addition, this growth factor has also been immunolocalized in structures such as the adult rat submandibular salivary gland (Amano *et al.,* 1993) and human and rat (adult

and embryonic) kidney (Cancilla *et al.,* 1999; Floege *et al.,* 1999) which undergo branching morphogenesis.

This multifunctional growth factor demonstrates a broad spectrum of biological activities and mediates its effects through both paracrine and autocrine mechanisms (Sato and Rifkin, 1988; Bashkin *et al.,* 1989; Jackson *et al.,* 1992). These include cell proliferation (Rifkin and Moscatelli, 1989), differentiation and cell migration (Cauchi *et al.,* 1996; Mansukhani *et al.,* 2000) and skeletogenesis (Mansukhani *et al.,* 2000). FGF-2 is also believed to be a mitogen for the process of angiogenesis of endothelial and smooth muscle cells (Reiland and Repraeger, 1993; Floege *et al.,* 1999; Kan *et al.,* 1999). In *Xenopus levis*, amphibians and mammals, FGF-2 induces induction of embryonic mesoderm (Kimelman *et al.,* 1988) and pattern formation (Amaya *et al.,* 1993; Labonne and Whiteman, 1994) as well as control, expression and deposition of the extracellular matrix components (Aktas and Kayton, 2000). This cytokine has also been implicated in the early morphogenesis in mammals (Feldman *et al.,* 1995). In addition, FGF-2 plays a critical role in bone growth and development and participates in the process of chondrogenesis and osteogenesis (Cohn *et al.,* 1995; Sutherland *et al.,* 1996; Mansukhani *et al.,* 2000).

1.6 Mechanism of action of FGF-2

FGF-2, as a member of the FGF family of ligands exerts it biological effects through a dual receptor system (Cancilla *et al.,* 1999). These receptors are believed to participate in epithelio-mesenchymal interactions and thus act as the main vehicles of organogenesis (Ornitz *et al.,* 1996). The first component of this system consists of specific transmembrane tyrosine kinase receptors that are similar in structure to other transmembrane protein kinase receptors (Lee *et al.,* 1989; Stern *et al.,* 1997; Cancilla *et al.,* 1999). To date, four membrane bound FGF-receptors (FGFR-1 to FGFR-4) have been identified (Reiland and Repraeger, 1993; Kan *et al,* 1999) and their existence is documented in all contemporary vertebrates (Courlier *et al.,* 1997). They bind to FGF's with high affinity (Basilico and Moscatelli, 1992). These receptors are monomeric in their native state and dimerize after binding with an FGF ligand. This dimerization activates tyrosine kinase and thus induces the effect of this growth factor through multiple signaling pathways.

The second component of the dual receptor system is the polysaccharide component (heparin like molecules) of the heparan sulfate proteoglycans (HSPG's) present on the cell surfaces and in the extracellular matrix (ECM) [Ruoslahti and Yamaguci, 1991; Wiedlocha *et al.,* 1994]. They bind FGF's with low affinity and do not participate in the signalling cascade. However, HSPG's provide an extracellular storage compartment and thus protect FGF's from degradation. In addition, in response to the internal or external stimuli, e.g. proteolytic processing of the extracellular matrix or phophorylation, these thermostable "FGF-heparin like polysaccharide" complexes regulate the bioavaibality of FGF's by increasing the affinity of FGF's for FGFR's (Yayon *et al.,* 1991; Givol and Yoyon, 1993; Aviezer *et al.,* 1994; Roghani *et al.,* 1994 ; Fannon and Nugent, 1996; Repraeger, 2000).

1.7 FGF's and tooth development

Recent work has shown that a variety of fibroblast growth factors play a critical role in tooth morphogenesis. For example, FGF-3 (int-2), FGF-4 (hst, kFGF), FGF-7 (KGF), and FGF-8 (AIGF) mRNAs have been detected in the developing tooth (Wilkinson *et al.,* 1989; Niswander and Martin, 1992; Finch *et al.,* 1995; Neubüser *et al.,* 1997). Most of these studies, however, primarily focused on the expression of mRNA transcripts of FGF's during the early stages of tooth development. In addition, Cam *et al.* (1992) and Russo *et al.* (1997) have reported the immunolocalization of FGF-1 and FGF-2 in early stages of tooth development. All these studies however, did not extend their work to unfold the role of FGF-2 in the development of the root of the tooth. Root morphogenesis, which follows the formation of the crown of the tooth, also exhibits a classical example of epithelio-mesenchymal interaction. The root of the tooth consists of dentin and cementum (Ten Cate, 1998). The process of root development begins with the proliferation of epithelial cells of the external and internal dental epithelium from the cervical loop of the dental organ to form a double layer of cells known as *Hertwig's epithelial root sheath* (epithelial derivative). Hertwig's epithelial root sheath grows around the dental papilla between the papilla and the dental follicle (oral ectomesenchymal derivatives) and encloses the dental papilla completely, except for the apical portion of the papilla. The rim of this root sheath (the epithelial diaphragm) encloses the primary apical foramen. The inner epithelial cells of the root sheath progressively enclose the gradually expanding dental papilla, and initiate the differentiation of odontoblasts from the cells at the periphery of the dental papilla (ectomesenchymal derivative). These cells eventually form the dentin of the root (Ten Cate, 1998). The stretching of the epithelial root sheath of Hertwig eventually results in its fragmentation. This is followed by the migration of the innermost cells of the dental follicle

(ectomesenchymal derivative) towards the root dentin surface where they, in turn, differentiate into cementoblasts. The cementoblasts secrete cementoid, which later on in development will mineralize to form cementum and form part of the root (Avery, 2001). The outermost cells of the dental follicle differentiate into osteoblasts and form the alveolar bone. The more centrally located cells of the dental follicle differentiate into fibroblasts, which take over the role of collagen fibre production. These collagen fibres become embedded in both cementum and bone as Sharpey's fibres (Avery, 2001).

Previous studies by Cam *et al.* (1992) and Russo *et al.* (1997) elucidated the expression of FGF-2 in the cells differentiating from the dental papilla and dental follicle in the region of the crown during early tooth development. These investigators suggested that FGF-2 expression is specific in the developing crown with progression in time. It is likely that later events associated with root development such as root dentin formation, alveolar bone formation, cementogenesis and the development of the periodontal ligament may be regulated by the expression of fibroblast growth factor-2. Thus, using immunocytochemical techniques, the aim of this study is to examine the pattern of localization of FGF-2 in various cell types and tissues during the development of the root of maxillary and mandibular molars and incisors.

CHAPTER TWO

2.0 MATERIALS AND METHODS

2.1 Ethical Clearance

Ethical clearance for this study was obtained from the Animal Ethics Screening Committee of the University of the Witwatersrand (AESC NO: 2001/ 012/ A).

2.2 Tissues

Animals were obtained from the Central Animal Unit of the University of the Witwatersrand. Thirty-six (36) mouse pups (strain MF-1) of different stages were used for this project. Tissue from six age groups of post-natal mice was studied (i.e. post-natal days 9, 10, 12, 16, 20 and 24). Six mice from each of the age groups were studied. The chronological ages of the mice for each stage of the project were based on mouse molar tooth development as described by Cohn (1956). The submandibular gland of 10-week old rat (strain Sprague-Dawley) was used as a positive control for immunocytochemistry (Amano *et al.*, 1993).

2.3 Animal sacrifice

The mouse pups were killed with an overdose (150mg/ kg body weight) of sodium pentobarbitone (Eutha-naze, Centaur labs), administered intraperitoneally. The animals were decapitated and the region of the face anterior to the external acoustic meatus was isolated. The calotte and brain tissue of each specimen was then also removed. The tissue was further bisected in the mid-sagittal plane to divide the maxilla and mandible into two symmetrical halves.

2.4 Tissue fixation

The tissues were fixed by immersion in a 3.7% formaldehyde/ zinc fixative [also called zinc formalin] (Electron Microscopy Sciences, Cat #15675-01), for 36 hours. The rationale for using zinc in 3.7% formaldehyde as a fixative is that, it prevents or at least inhibits cross linking by formaldehyde; it holds macromolecules in their native 3-dimensional confirmation and it creates superior morphological details at the level of the light microscope (Dapson, 1993).

2.5 Tissue decalcification

Zinc formalin fixed tissues were decalcified using 5% formic acid as an acid-decalcifying agent (Stevens *et al.,* 1996). Tissues were immersed in 5% formic acid (from 72 to 120 hrs) at room temperature depending on the stage of development of the mice. A chemical test called the "Calcium oxalate test" adapted from Arnim (1935) and Clayden (1952) and as described by Stevens *et al.* (1996) was performed to test for the completion of decalcification [Appendix I].

2.6 Tissue processing and sectioning

Following decalcification, the tissues were routinely processed in an enclosed automated tissue processor (SHANDON, citadel 1000) through a graded series of alcohols followed by two changes of chloroform and cleared in xylene. Processed mice and rat tissues were embedded in paraffin wax. Sagittal sections (5μm thick) from each mouse and rat tissue were cut on a sledge microtome (Leica 1400, Leica Instruments GmbH, Germany). The sections were mounted on aminoalkylsilane-treated slides [Appendix II] for immunocytochemistry (Rentrop *et al.,* 1986). Every tenth section was

stained with haematoxylin and eosin [Appendix III] for routine histological examination of both the mandibular and maxillary molars and their supporting structures (Steven and Wilson; 1996).

2.7 Primary Antibody

Polyclonal rabbit anti-FGF-2 antibody [FGF-2 (147) K, Cat # sc-79K, Lot #F020] was used (Santa Cruz Biotechnology, Inc. USA). This affinity purified, polyclonal rabbit anti-FGF-2 antibody is raised against a peptide mapping within the amino terminal domain of FGF-2 of human origin. It differs from corresponding mouse and chicken sequences by a single amino acid (Dionne *et al.,* 1990). As specified by the manufacturer, this polyclonal antibody is mouse, rat and human reactive. Concentration runs were performed at 1:200, 1:100 and 1:80 to determine the optimal concentration of the primary antibody required with the least amount of background staining. The dilution was performed with 10 mM phosphate buffered saline (PBS), pH 7.4 and 0.1% Tween-20 [Appendix IV] as per manufacturer's instructions and finally the primary antibody was used at a concentration of 1:80.

2.8 Secondary Antibody

Biotinylated goat anti-rabbit pre-diluted secondary antibody (Santa Cruz Biotechnology, Inc. USA) was used. The secondary antibody was supplied with the ImmunoCruz™ staining system, which was used as a detection system.

2.9 Blocking Antigen (Peptide)

Blocking antigen (Peptide) (FGF-2 [147] P, Cat # sc-79 P, Lot # J021) was used for absorption control (Santa Cruz Biotechnology, Inc. USA). This consisted of 100 µg peptide in 0.5 ml phosphate buffered saline (PBS) with 0.1% sodium azide and 100 µg bovine serum albumin (BSA).

2.10 Immunohistochemical staining system

Rabbit ImmunoCruz™ staining system (Cat #sc-2051, Santa Cruz Biotechnology, Inc. USA) [Appendix V] was used as the immunohistochemical staining system. This staining system differs from other ABC systems in that it utilizes an HRP-Strept-avidin complex rather than the avidin/biotynalated HRP complex. The advantage of this staining system is that all the reagents are supplied in pre-diluted form to the optimal concentrations, needed for tissue staining.

2.11 Chromogen

The chromogen used was 3,3' diaminobenzidine tetrahydrochloride (DAB). It produces a gold-brown stain and was supplied as part of the Rabbit ImmunoCruz™ staining system (Cat #sc-2051, Santa Cruz Biotechnology, Inc. USA)

2.12 Immunocytochemistry

Fibroblast growth factor-2 (FGF-2) localization on the tissue sections was performed using the avidin-biotin immunoperoxidase technique adapted from Hsu *et al.* (1981) and Bratthauer (1994). All steps were performed according to the research applications

procedure supplied by Santa Cruz Biotechnology, Inc. USA, the manufacturer of the primary antibody, secondary antibody and detection system.

All incubations were performed at room temperature in a humidified chamber unless otherwise stated. Briefly the sections were deparaffinized in three changes of xylene and rehydrated through a graded series of alcohols. The sections were then washed in de-ionized water for 5 minutes in the presence of a magnetic stirrer. Excess liquid from the slides was aspirated.

Antigen unmasking was performed at this stage by microwave heat treatment. For this technique, slides mounted with sections were placed in a coplin jar filled with 10 mM sodium citrate buffer, pH 6.0 [Appendix VI]. The sections were heated at 95° C for 5 minutes in a standard microwave oven. The coplin jar was then topped up with fresh citrate buffer and sections were again heated at 95° C for a further 5 minutes. The slides were allowed to cool in the buffer for approximately 20 minutes. The sections were then rinsed and washed twice in de-ionized water. Excess liquid from the sections was drained. Appropriate care was taken to keep the sections wet and moist. The sections were incubated with 1% hydrogen peroxide in de-ionized water for 10 minutes to quench intrinsic endogenous peroxidase activity, followed by two 5-minutes washes in 10 mM phosphate buffered saline (PBS), pH 7.4 and 0.1% Tween-20. The sections were then incubated for 20 minutes in 1-3 drops of 5% normal goat serum. The sections were drained and wiped carefully around the tissue to remove excess fluid and were immediately incubated for two hours at room temperature, in a moist chamber with polyclonal rabbit anti-FGF-2 antibody (1:80 diluted with PBS). The sections were then rinsed and washed twice with PBS and 0.1% Tween-20 in the presence of a magnetic stirrer. The sections were incubated with 1-3 drops of biotinylated goat anti-rabbit secondary antibody (pre-diluted) for 30 minutes, followed by two brief washes in PBS

and 0.1% Tween-20. Excess liquid was drained from the sections. The site of the immunoreaction was made visible by incubating the sections with 1-3 drops of HRP-Streptavidin complex for 30 minutes, followed by a brief wash with PBS and 0.1% Tween-20. The sections were once again wiped around the tissue to remove excess fluid, followed by incubation with 1-3 drops 3', 3'-diaminobenzidine tetrahydrochloride (DAB). This reagent was supplied as an HRP substrate mixture [Appendix VII] with the Immuno-cruz staining system.

The sections were developed for 20 minutes until the desired stain intensity developed. The sections were then washed twice for 5-minutes each in de-ionized water in the presence of a magnetic stirrer, dehydrated through ascending grades of alcohol and cleared in two changes of xylene. Finally, the sections were mounted in a permanent mounting medium, Entellan (Merck, Germany).

2.13 Negative Controls

Negative controls for immunocytochemistry were performed. The rationale for the use of the negative controls was to ensure that immunolocalization in the test sections was not due to binding of either the primary antibody or the secondary antibody alone and/or of a non-specific immunoglobulin. This confirms that the primary antibody is indeed the critical link for any "staining" to occur. Negative controls were carried out on a section adjacent to the section on which reaction had been localized.

The following negative controls were performed:

(a) Exclusion of the primary antibody and replacement with normal rabbit serum (supplied with ImmunoCruz™ staining system)

(b) Exclusion of the primary antibody and replacement with phosphate buffered saline (PBS)

(c) Exclusion of the secondary antibody and replacement with normal rabbit serum (supplied with ImmunoCruz™ staining system)

(d) Exclusion of the secondary antibody and replacement with phosphate buffered saline (PBS)

2.14 Positive Controls

A positive control section was used with every immunocytochemical run. For this purpose, tissues in which FGF-2 had previously been localized such as the submandibular gland of an adult rat were used as a positive control. Epithelial cells lining the striated and excretory ducts and the granular convoluted tubules of the submandibular gland of a 10-week old adult rat were used (Amano *et al.,* 1993).

2.15 Absorption (Peptide neutralization) Control

An absorption (peptide neutralization) control was performed to determine the specificity of the primary antibody. Polyclonal rabbit anti-FGF2 antibody (FGF-2 [147] K, at a concentration of 1:80) was combined with a five-fold excess of blocking antigen peptide (FGF-2 [147] P) in 500 µl of PBS and incubated overnight at 4°C. Sections of the tooth adjacent to a section depicting the localization of the antibody were incubated with preabsorbed antibody, substituted for the primary antibody.

2.16 Counterstaining

All the sections were counterstained briefly for 10 seconds with haematoxylin.

CHAPTER THREE

3.0 RESULTS

In the present study, the pattern of expression of fibroblast growth factor-2 in the developing root of teeth was examined. Six stages of the developing roots of teeth from mice were selected (post-natal days 9,10,12,16, 20 and 24).

This study did not attempt to quantify the expression of FGF-2, but rather, the immunocytochemical *localization* of FGF-2 was observed both spatially and temporally.

Photographic representations of teeth are oriented according to the position of the tooth either in the developing maxillary or mandibular arch. Different developing cell types at various stages of development from undifferentiated mesenchymal cells of the dental papilla were identified by their position e.g. odontoblasts in relation to the pulp cavity. Similarly, the differentiating ectomesenchymal cells in the central part of the developing periodontal ligament were identified as probably being the presumptive fibroblasts of the periodontal ligament.

For antigen retrieval, sections were boiled in 10 mM citric acid buffer twice for five minutes in a microwave oven. This technique was adopted as per manufacturer's recommendation, to be used with the specified polyclonal antibody. This technique allows for the unmasking of further/additional antigen sites over the entire section.

Although the microwave technique proved useful in unmasking antigen sites, the sections tended to lift with some loss of morphological details. This may be due to the fact that the thickness of the sections on the slide varied between areas of soft tissues to some "harder" tissue e.g. minor areas of calcification still present in the teeth.

Variation in *localization* of FGF-2 with respect to specific cellular regions occurred i.e. localization was most frequently found in the cytoplasm and the extracellular matrix and occasionally in the nuclei at different stages of morphogenesis and differentiation.

3.0.1 Positive and Negative Controls for Immunocytochemistry

For the positive control, sections of the submandibular gland of a male adult rat were used. Intense FGF-2 immunoreactivity was observed in the granular convoluted tubules, predominantly in the agranular pillar cells of the submandibular gland (Fig. 1) as described by Amano *et al.* (1993).

For the negative control, sections of tooth, adjacent to a section showing immunolocalization of FGF-2, from all the animals at each stage examined, were incubated with phosphate buffered saline (PBS) or normal rabbit serum (NRS) in place of either the primary or the secondary antibodies respectively. In these sections, no evidence of FGF-2 protein expression was detected (see e.g. Fig. 2a) compared to the adjacent FGF-2 labeled section (Fig. 2b). This confirmed that the primary antibody indeed was responsible for the immunolocalization of FGF-2 in the sections.

3.0.2 Absorption (Peptide neutralization) control

For the absorption control, sections of the tooth, adjacent to a section depicting the localization of the antibody were used. These sections were incubated with pre-absorbed antibody (antigen-antibody incubated overnight at 4°C) substituted for the primary antibody. In these sections, no evidence of immunoreactivity of FGF-2, with minimal background was observed (Fig. 3) compared to the adjacent section in which the FGF-2 antibody had been applied and in which localization was evident (Fig. 2b). This proved that the staining was not due to a non-specific immunoglobulin and/or secondary antibody binding to the sections, but due to the primary antibody itself.

Figure 1 **Positive control.** Representative section of the submandibular gland of an adult male rat. Immunolocalization of FGF-2 occurs in the granular convoluted ducts and predominantly in the agranular pillar cells (→). Counterstained with Meyer's haematoxylin. Bar: 20 μm.

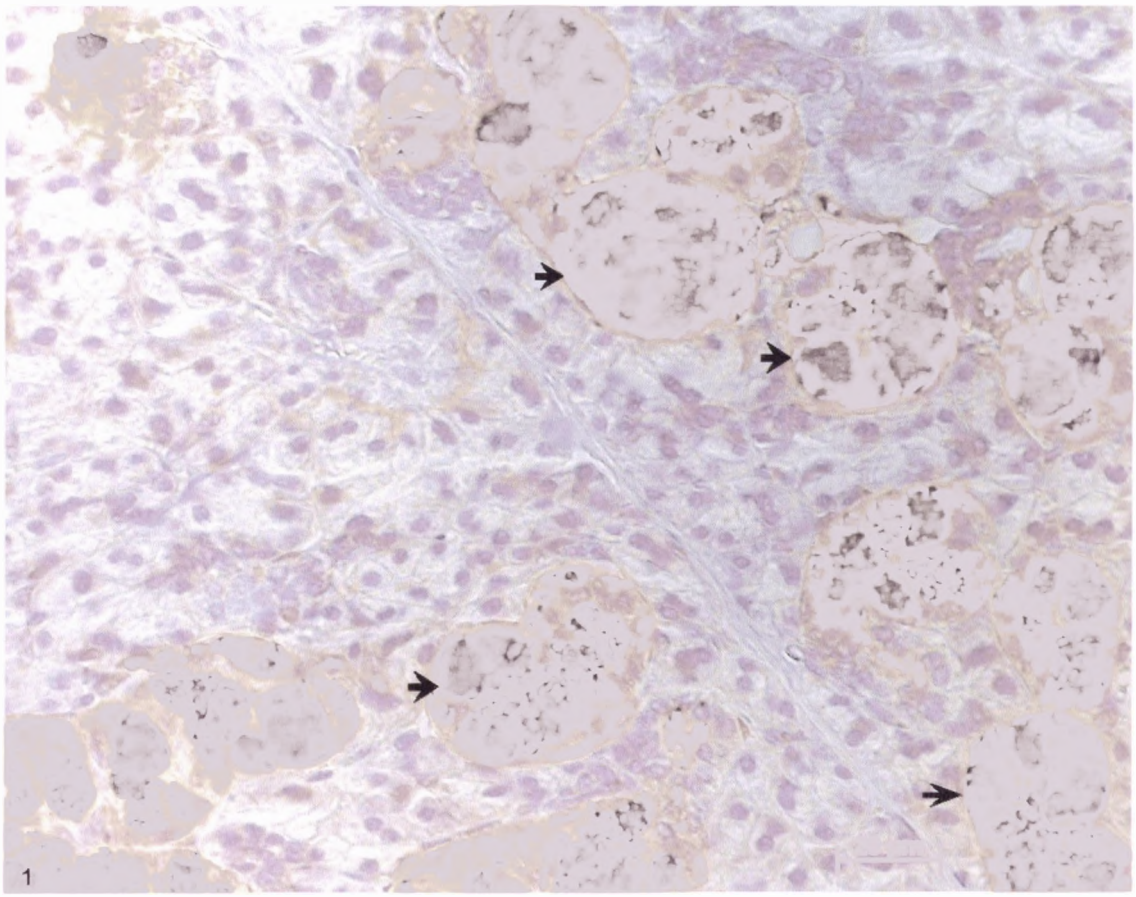
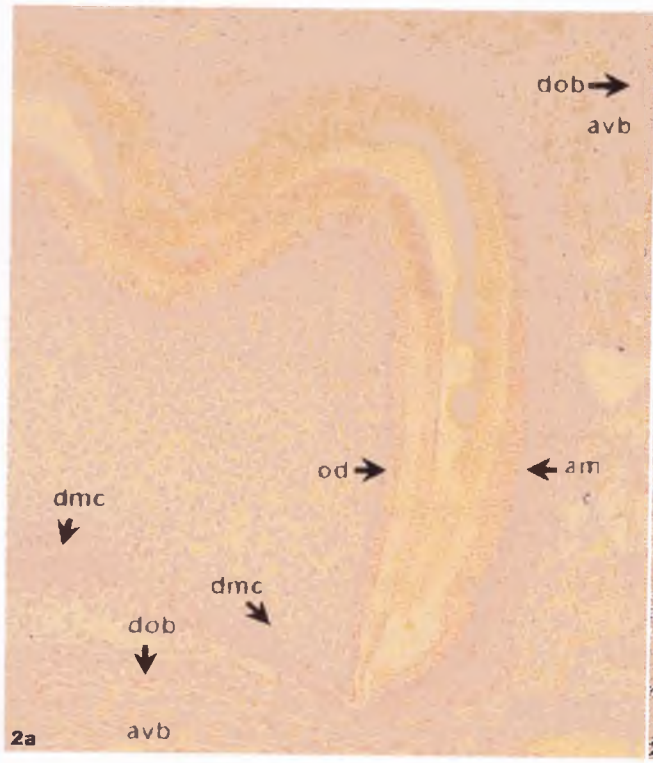
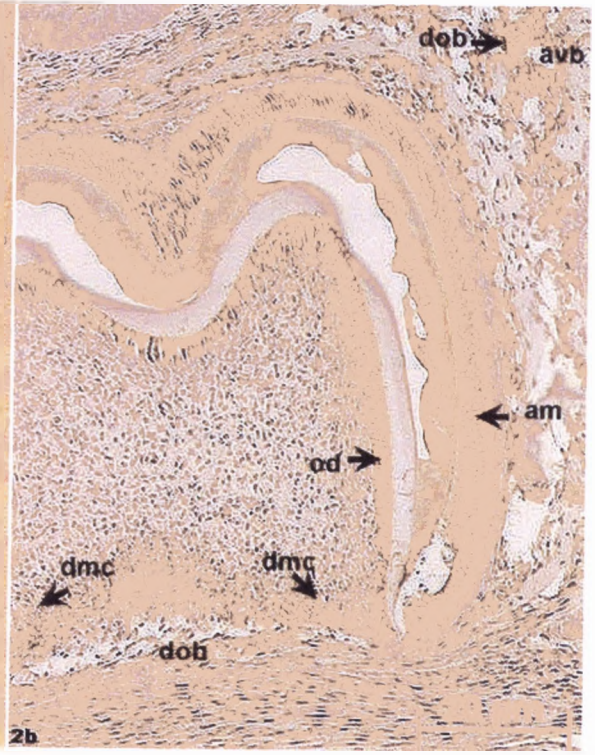

1

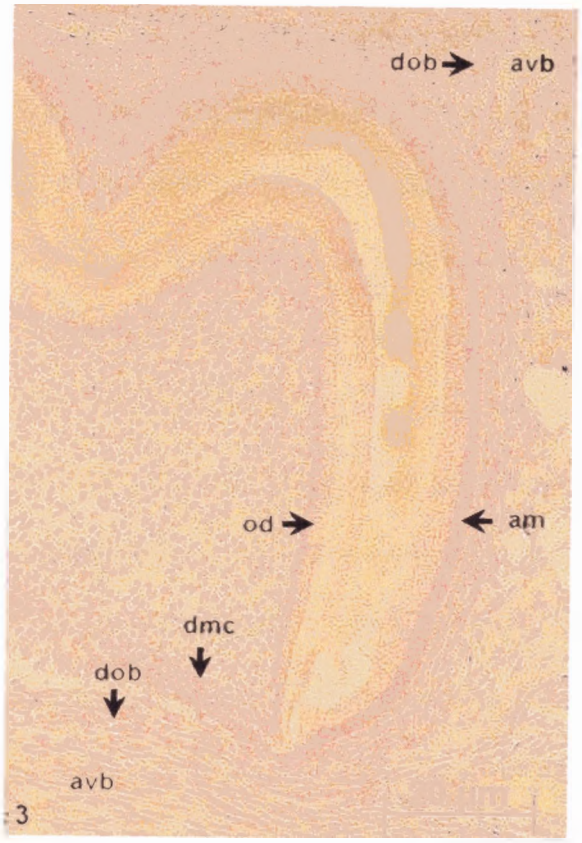
Figure 2(a) **Negative control.** Representative section of the developing root of a maxillary first molar of a 10-day post-natal mouse pup. Substitution of the primary antibody with normal rabbit serum (NRS). Note that no immunolocalization (➞) is observed in the odontoblasts (od), ameloblasts (am), presumptive odontoblasts (dmc) and in presumptive osteoblasts (dob). Counterstained with Meyer's haematoxylin. Bar: 20 μm.

Figure 2 (b) Representative section, adjacent to that in figure 2(a). Immunolocalization of FGF-2 occurred in the odontoblasts (od) lining the pulp chamber and the pulp canal, in ameloblasts (am), in individual differentiating ectomesenchymal cells (dmc) [presumptive odontoblasts] and in a few differentiating ectomesenchymal cells (dob) [presumptive osteoblasts] lining the developing alveolar bone (avb). Counterstained with Meyer's haematoxylin. Bar: 20 μm.

Figure 3 **Absorption control.** Representative section, adjacent to that in figure 2 (b). Substitution of the primary antibody with preabsorbed antibody. Note no immunoreactivity, with minimal background is observed in the cells, namely odontoblasts (od), ameloblasts (am), presumptive odontoblasts (dmc) and osteoblasts (dob), as compared to the tissue section in figure 2 (b). Counterstained with Meyer's haematoxylin. Bar: 20 μm.


dob
avb
od
am
dmc
dmc
dob
avb
2a


dob
avb
am
od
dmc
dmc
dob
2b


dob
avb
od
am
dmc
dob
avb
3

3.1 Immunolocalization of FGF-2 in the root of the murine tooth on post-natal day 9

The teeth of post-natal day 9 mice pups were the earliest stage examined for the expression of FGF-2 (Fig. 4). Every tenth (10^{th}) section from each animal was stained routinely with haematoxylin and eosin stain to examine the tooth morphology and the development. In the developing maxillary and mandibular arches, all three molar teeth (i.e. first, second and third), as well as the central incisors were present.

The development of the maxillary molars lagged slightly behind that of the mandibular molars. However, by post-natal day 9, the shape of the future crown of the first and second molars in the maxillary and mandibular arches had been established. In both arches, the first and second molars were in the late bell stage, while the third molar was still in the cap stage.

In both sets of teeth, differentiating ectomesenchymal cells were present in the pulp chamber, in the developing periodontal ligament and around the developing spicules of the alveolar bone. By this stage, cells of the inner dental epithelium had differentiated into ameloblasts and the process of amelogenesis was in progress (Fig. 4). The presence of enamel was noted in the sections (Fig. 4). The cells of the outer dental epithelium and stellate reticulum had lost their individual identity. The stratum intermedium was observed as a stratified layer of epithelial cells adjacent to the ameloblast layer (Fig. 4). The formation of the diaphragm of the epithelial root sheath had begun (Fig. 4).

In the maxillary first molar, root development had just started (Fig. 4), while in the second and third molar, the diaphragm of the epithelial root sheath was still in the initial stage of formation.

In the mandibular first molar, evidence of formation of the diaphragm of the epithelial root sheath was observed and a condensation of the differentiating ectomesenchymal cells was noted in the apical part of the teeth. The second mandibular molar did not show the formation of a diaphragm of the epithelial root sheath at this stage. The dental follicle was evident.

In both the maxillary and mandibular central incisors, differentiating odontoblasts and ameloblasts were seen (Figs. 5 and 6). The periodontal ligament was still undifferentiated (Figs. 5 and 6). Bony spicules forming the developing alveolar bone were observed (Figs. 5 and 6). The formation of the diaphragm of the epithelial root sheath was complete and had progressed along the apical axis of the root formation.

Thus, on post-natal day 9, as seen in Fig. 4, the presence of ameloblasts, odontoblasts, enamel, dentin, differentiating ectomesenchymal cells (presumptive fibroblasts) in the pulp chamber, region of the developing root (presumptive odontoblasts) and lining the developing alveolar bone (presumptive osteoblasts) was noted both in mandibular and maxillary teeth. Cementum and cementoblasts were absent at this stage.

In general, immunolocalization of FGF-2 both in the maxillary and mandibular molars and incisors was evident (Refer to Table 3.1). In the ameloblasts, FGF-2 distribution was primarily in the cytoplasm (Figs. 6 and 7). In the differentiated odontoblasts, distribution of FGF-2 was homogeneous in the cytoplasm (Figs. 5, 6 and 7). However, in a few differentiating ectomesenchymal cells of the pulp chamber and in individual cells of the developing periodontal ligament (presumptive fibroblasts), localization of

FGF-2 was noted in the nuclei and extracellular matrix (Figs. 5 and 6). FGF-2 was also localized to the nuclei and the extracellular matrix of the differentiating ectomesenchymal cells lining the developing alveolar bone (presumptive osteoblasts) and in the presumptive odontoblasts in the region of root development (Figs. 5 and 6). The stratum intermedium did not show immunolocalization of the antibody (Fig. 7). In the ameloblast cell layer, most of the cells were immunoreactive to the FGF-2 antibody and a uniform pattern of immunolocalization was observed (Figs. 6 and 7).

The odontoblasts located more coronally in the developing root exhibited more intense localization of FGF-2 as compared to those located apically, although this was not quantifiable (Figs. 5, 6 and 7). A few of the presumptive odontoblasts in the region of the root demonstrated intense localization of FGF-2, primarily in the nucleus (Fig. 5).

A few presumptive fibroblasts in the pulp chamber and in the developing periodontal ligament as well as a few presumptive osteoblasts in the region of developing alveolar bone also exhibited evidence for immunoreactivity for FGF-2 in the nuclei (Figs. 5 and 6).

As referred in Table 3.1, although faint "localization" of FGF-2 was also observed in the dentin (Figs. 6 and 7), it was not as definitive as when localized in specific cells.

The stratum intermedium and the predentin exhibited no evidence of immunolocalization of FGF-2 (Refer to Table 3.1 and Fig. 7).

Table 3.1 **Immunolocalization of FGF-2 in the root of the murine tooth on post-natal day 9**

	Maxillary				Mandibular		
	Central Incisor	First Molar	Second Molar	Third Molar	First Molar	Second Molar	Third Molar
Stratum intermedium (si)	-	-	-	-	-	-	-
Ameloblasts (am)	+	+	+	+	+	+	+
Odontoblasts (od)	+	+	+	+	+	+	+
Differentiating ectomesenchymal cells (dmc)	+	+	+	+	+	+	+
Predentin (pd)	-	-	-	-	-	-	-
Dentin (d)	+	+	+	+	+	+	+
Zone of Cementum (c)	A	A	A	A	A	A	A
Differentiating ectomesenchymal cells in the cementum zone (cb)	A	A	A	A	A	A	A
Differentiating ectomesenchymal cells lining the developing alveolar bone (dob)	+	+	+	+	+	+	+
Differentiating ectomesenchymal cells in the pulp chamber (dfb)	+	+	+	+	+	+	+
Differentiating ectomesenchymal cells of the periodontal ligament (pdlfb)	+	+	+	+	+	+	+

Legends:

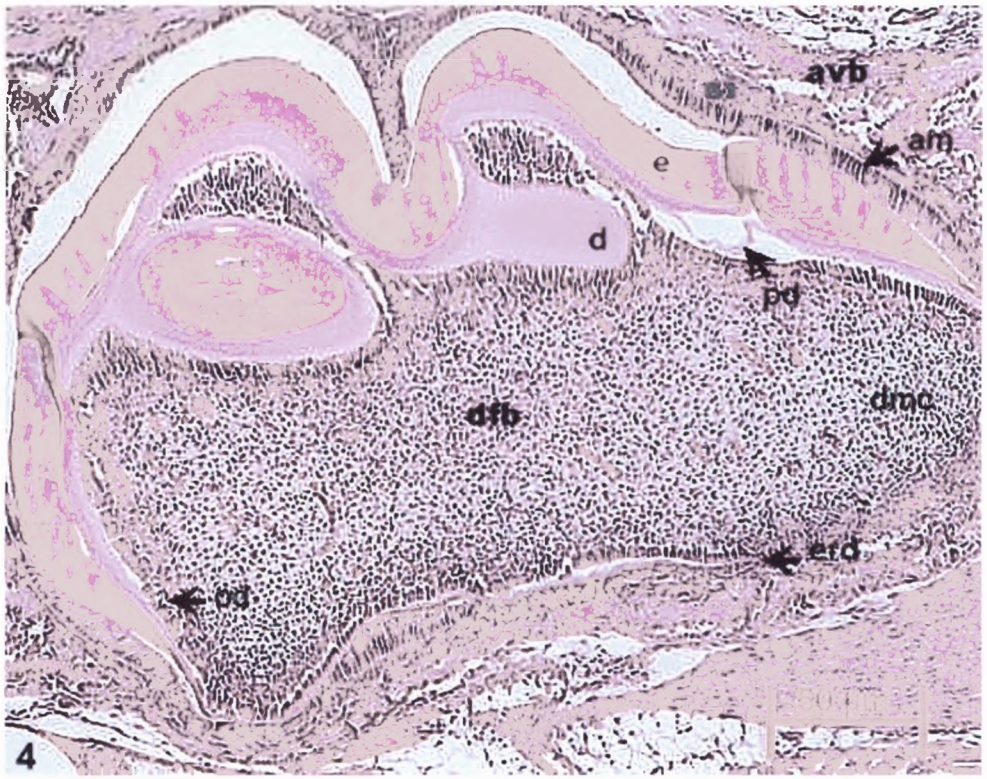
(+): Positive for immunoreactivity for FGF-2

(-): Negative for immunoreactivity for FGF-2

(A): Absent at this stage

Figure 4 Representative section of a maxillary first molar of a 9-day post-natal mouse pup. Note the presence of the ameloblasts (am), enamel (e), dentin (d), predentin (pd), odontoblasts (od), stratum intermedium (si), diaphragm of the epithelial root sheath (erd) and differentiating ectomesenchymal cells (dmc) in the pulp chamber and in the region of the future root development. Haematoxylin and eosin stain. Bar: 50 μm

Figure 5 Representative section of the developing root of a maxillary central incisor of a 9-day post-natal mouse pup. Immunolocalization of FGF-2 in the cytoplasm of the odontoblasts (od), in a few differentiating ectomesenchymal cells (dfb) of the pulp chamber [presumptive fibroblasts], in individual differentiating ectomesenchymal cells (pdlfb) of the periodontal ligament [presumptive fibroblasts] and in the differentiating ectomesenchymal cells (dob) [presumptive osteoblasts] lining the developing alveolar bone (avb). Note intense localization of FGF-2 antibody in the nuclei of differentiating ectomesenchymal cells (dmc) [presumptive odontoblasts] at the apical end of the root region. Counterstained with Meyer's haematoxylin. Bar: 10 μm.


avb
afn
e
d
pd
dmc
dfb
erd
od
4

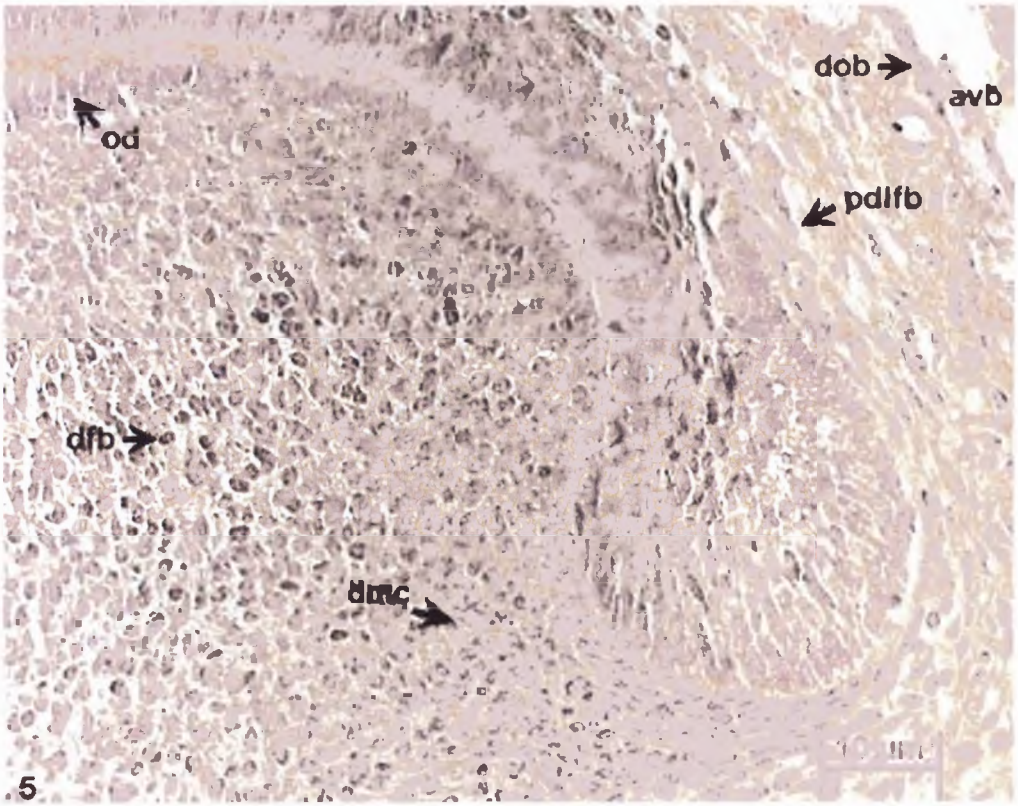
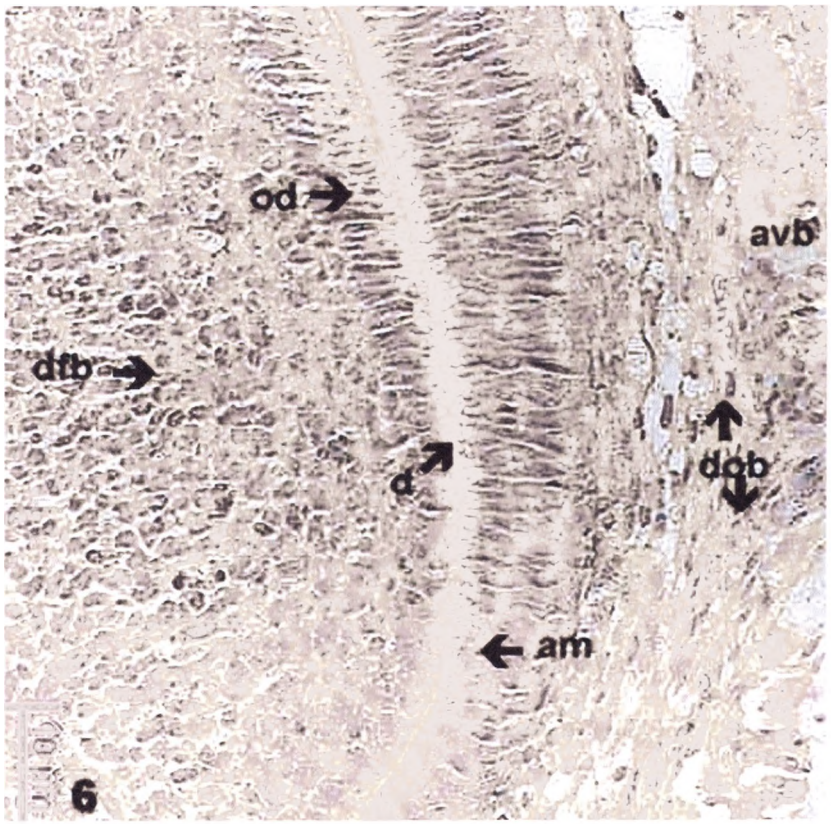
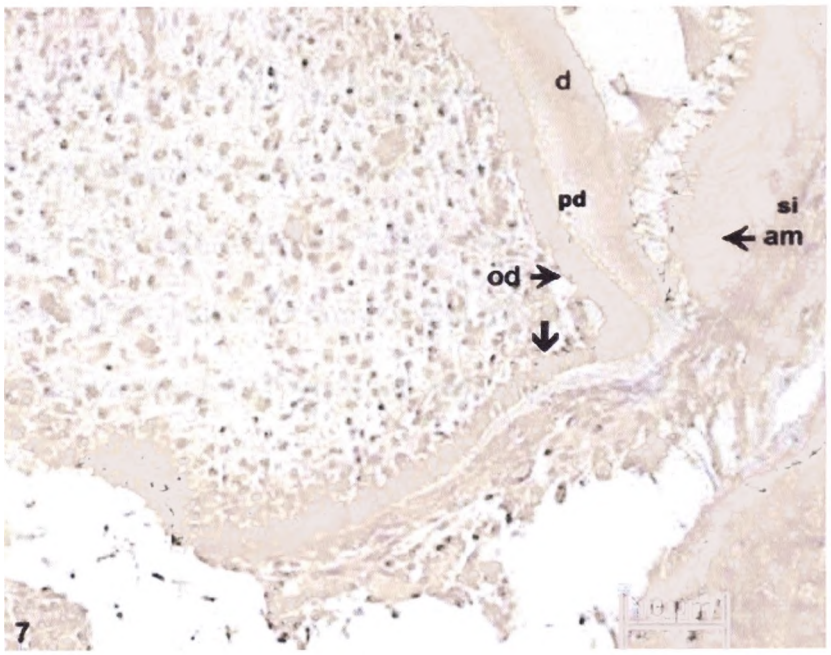

dob
avb
od
pdlfb
dfb
dmc
5

Figure 6 Representative section of the developing root of a maxillary central incisor of a 9-day post-natal mouse pup. Note FGF-2 immunoreactivity in a few differentiating ectomesenchymal cells (dob) [presumptive osteoblasts] lining the developing alveolar bone (avb), in the ameloblasts (am), the odontoblasts (od) and in a few individual differentiating ectomesenchymal cells (dfb) of the pulp chamber [presumptive fibroblasts]. Also note a very faint signal in the early deposition of dentin (d). Counterstained with Meyer's haematoxylin. Bar: 10 μm.

Figure 7 Representative section of a maxillary first molar of a 9-day post-natal mouse pup. Note FGF-2 immunolocalization in the cytoplasm of the odontoblasts (od) and ameloblasts (am). Also note a very faint signal in the early deposition of dentin (d). No immunolocalization of FGF-2 occurred in the stratum intermedium (si) and predentin (pd). Counterstained with Meyer's haematoxylin. Bar: 10 μm.


od
avb
dtb
d
dob
am
6


d
pd
si
am
od
7

3.2 Immunolocalization of FGF-2 in the root of the murine tooth on postnatal day 10

On post-natal day 10, sections stained with haematoxylin and eosin showed the presence of all three developing molars and the central incisors as in the previous stage i.e. 9-days postnatal. The first and second molars were in the late bell stage while the third molars were now in the early bell stage of tooth development (Fig. 8). In the first molar, maturation of enamel at the tips of the cusp and the beginning of root formation was observed. In the second molar, dentin and enamel were observed but root formation had not yet begun. The presumptive furcation zone of the first and second molars was noted and a condensation of the differentiating ectomesenchymal cells (presumptive odontoblasts) was observed. Formation of the diaphragm of the epithelial root sheath was evident in the third molars as well as the presence of the stratum intermedium (Fig. 8).

Once again, as seen in the Fig. 8, the presence of various structures and cells types namely the ameloblasts, odontoblasts, enamel, dentin, predentin, presumptive odontoblasts in the region of the developing root, presumptive fibroblasts of the pulp chamber, developing periodontal ligament and the developing alveolar bone was noted at this stage. Cementoblasts and cementum were absent.

As seen in Table 3.2 and Figs. 9, 10 and 11, cytoplasmic as well as nuclear localization of FGF-2 was present both in the maxillary and the mandibular molars and incisors of 10-day postnatal mice pups. Ameloblasts and odontoblasts exhibited evidence of cytoplasmic immunoreactivity with a few cells having nuclear reactivity (Figs. 9 and 10). Presumptive fibroblasts, in the pulp chamber and in the developing periodontal ligament, and presumptive osteoblasts lining the developing alveolar bone exhibited nuclear immunoreactivity. There was also some localization for FGF-2 in the extracellular matrix (Figs. 9, 10 and 11). The stratum intermedium, however, did not show immunoreactivity (Table 3.2 and Figs. 9 and 10).

In the ameloblast layer, a homogeneous distribution of FGF-2 was noted in the cytoplasm of most of the cells (Figs. 9 and 10). A uniform cytoplasmic and nuclear localization of FGF-2 in the odontoblasts was present apically, as root formation extended (Fig. 10). Once again, a few presumptive odontoblasts in the region of root development showed specific nuclear immunoreactivity for FGF-2 (Table 3.2 and Fig. 10).

Nuclear and extracellular matrix localization of the antibody was also observed in presumptive fibroblasts of the pulp chamber (Fig. 11). Expression appeared to be evident in more cells as compared to the previous stage i.e. post-natal day 9.

Ectomesenchymal cells of the dental follicle and cells lining the developing alveolar bone were also immunoreactive for FGF-2 (Table 3.2 and Fig. 9). At this stage many cells in the dental follicle expressed FGF-2. The dentin, once again showed faint immunolocalization, which may or may not be true immunoreactivity (Table 3.2 and Fig. 10). Once again, as seen in Table 3.2, the predentin (Fig. 10) and the stratum intermedium (Figs. 9 and 10) did not exhibit any evidence of immunolocalization.

Table 3.2 **Immunolocalization of FGF-2 in the root of the murine tooth on post-natal day 10**

	Maxillary				Mandibular		
	Central Incisor	First Molar	Second Molar	Third Molar	First Molar	Second Molar	Third Molar
Stratum intermedium (si)	-	-	-	-	-	-	-
Ameloblasts (am)	+	+	+	+	+	+	+
Odontoblasts (od)	+	+	+	+	+	+	+
Differentiating ectomesenchymal cells (dmc)	+	+	+	+	+	+	+
Predentin (pd)	-	-	-	-	-	-	-
Dentin (d)	+	+	+	+	+	+	+
Zone of Cementum (c)	A	A	A	A	A	A	A
Differentiating ectomesenchymal cells in the cementum zone (cb)	A	A	A	A	A	A	A
Differentiating ectomesenchymal cells lining the developing alveolar bone (dob)	+	+	+	+	+	+	+
Differentiating ectomesenchymal cells in the pulp chamber (dfb)	+	+	+	+	+	+	+
Differentiating ectomesenchymal cells of the periodontal ligament (pdlfb)	+	+	+	+	+	+	+

Legends:

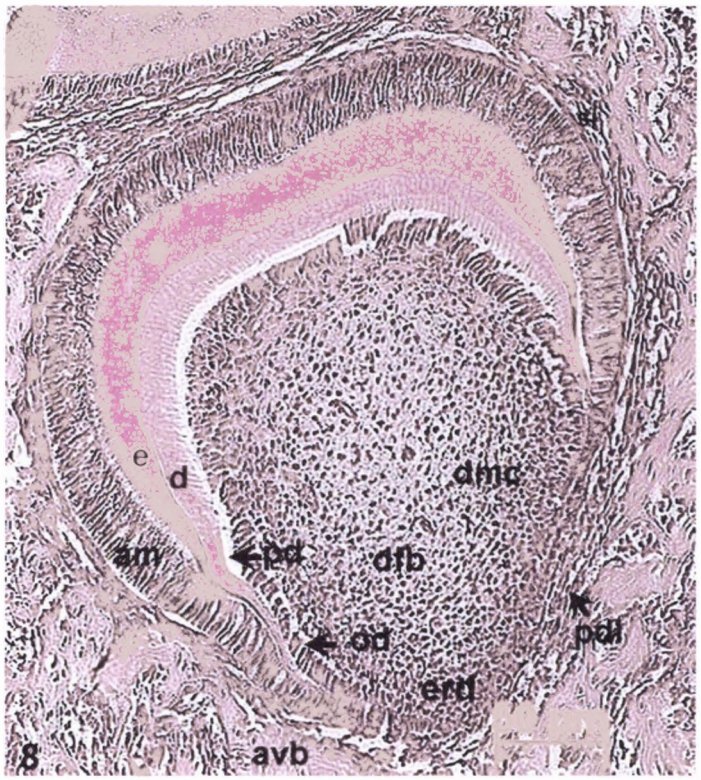
(+): Positive for immunoreactivity for FGF-2

(-): Negative for immunoreactivity for FGF-2

(A): Absent at this stage

Figure 8 Representative section of the developing mandibular third molar tooth of a 10-day post-natal mouse pup. Note the presence of ameloblasts (am), enamel (e), dentin (d), predentin (pd), odontoblasts (od), stratum intermedium (si), epithelial root diaphragm (erd), differentiating ectomesenchymal cells (dmc) [presumptive odontoblasts] in the root area, developing periodontal ligament (pdl), developing alveolar bone (avb) and the differentiating ectomesenchymal cells (dfb) [presumptive fibroblasts] in the pulp chamber. Haematoxylin and eosin stain. Bar: 50 μm.

Figure 9 Representative section of the developing crown and early root formation of a maxillary first molar of a 10-day post-natal mouse pup. Immunolocalization of FGF-2 in the ameloblasts (am), in the odontoblasts (od) and in the differentiating ectomesenchymal cells (dob) [presumptive osteoblasts] (ob) lining the developing alveolar bone (avb). No localization of the antibody is observed in the stratum intermedium (si). Counterstained with Meyer's haematoxylin. Bar: 10 μm.


e
d
dmc
am
pd
dfb
od
pdl
erd
8
avb

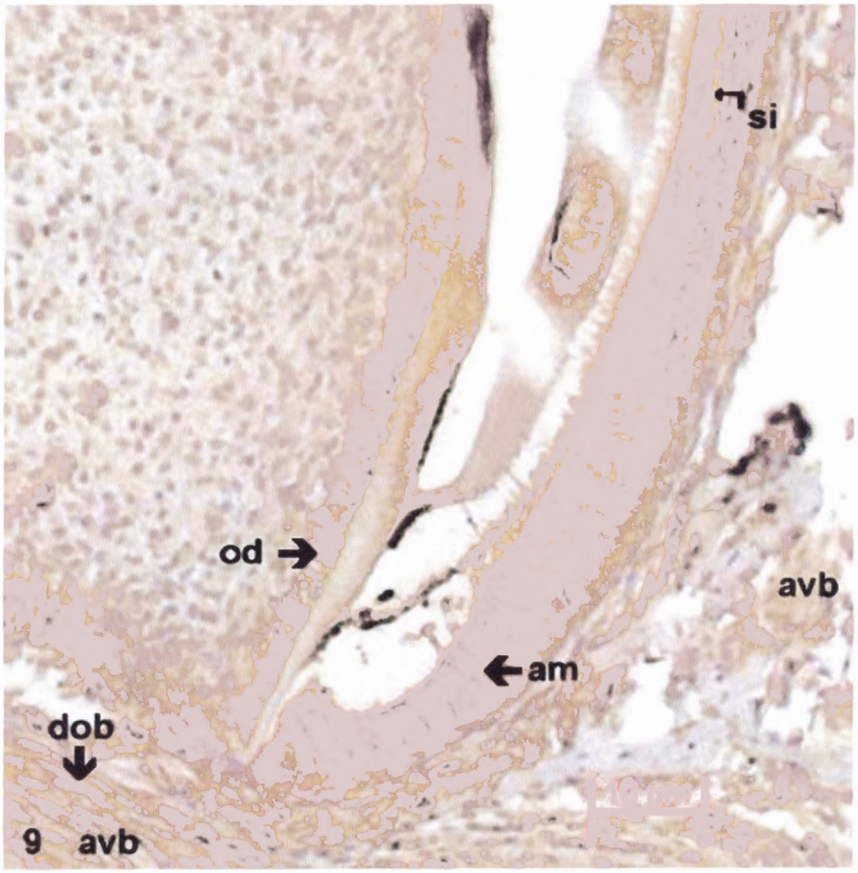
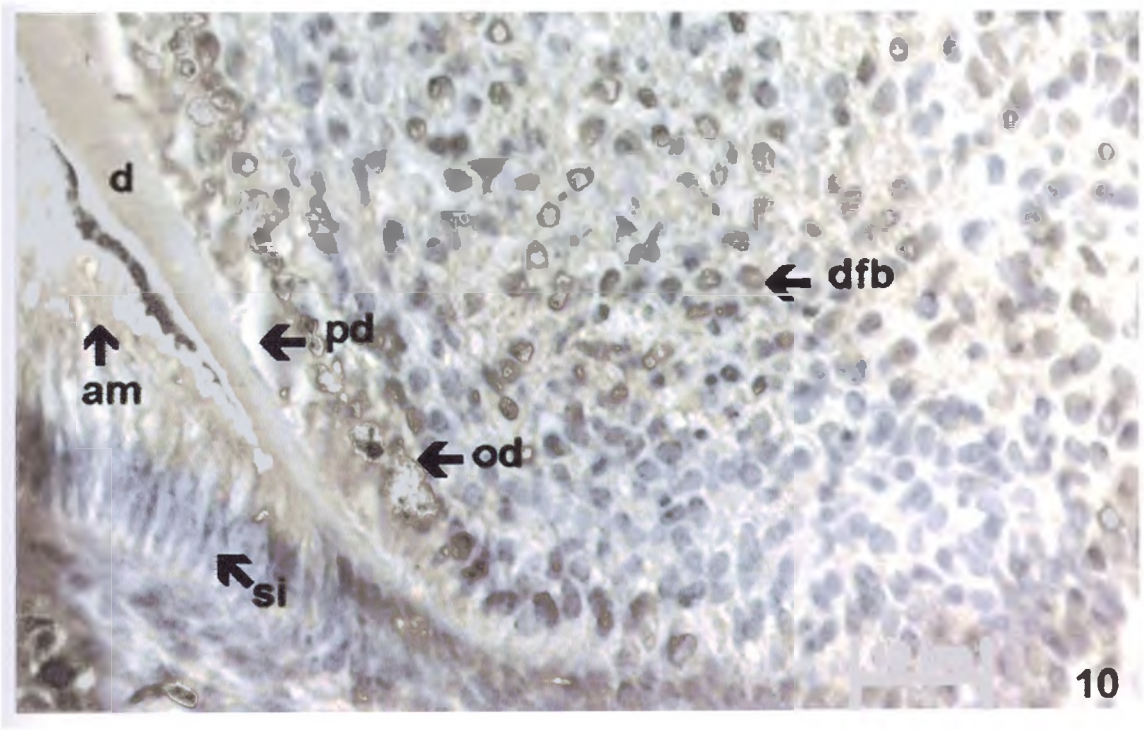
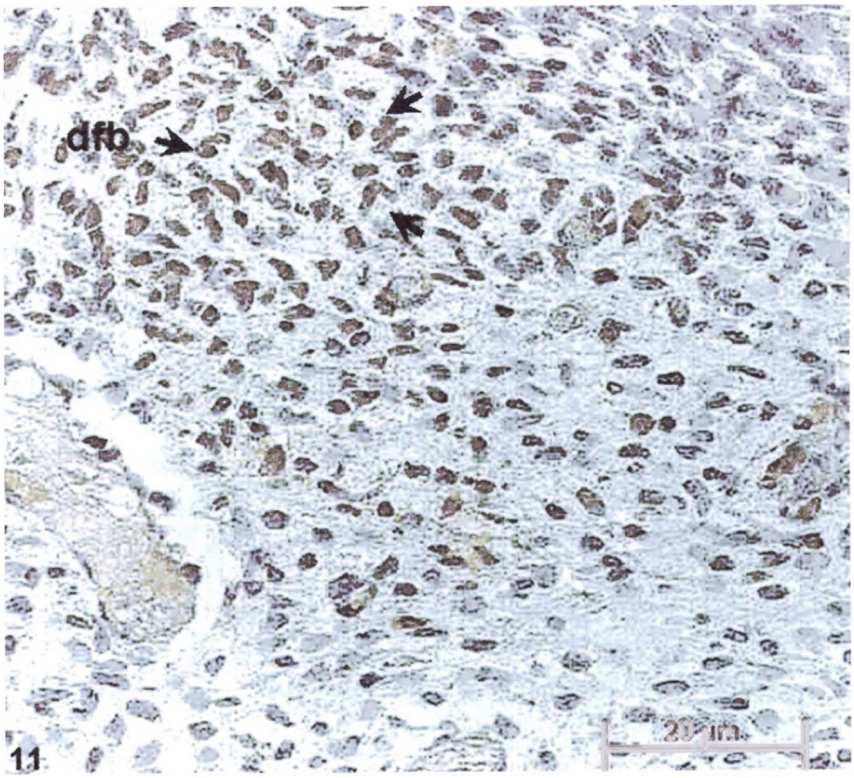

si
od
avb
am
dob
9
avb

Figure 10 Representative section of the developing root of a mandibular third molar of a 10-day post-natal mouse pup. Note immunolocalization of FGF-2 in the ameloblasts (am), odontoblasts (od) and in individual differentiating ectomesenchymal cells (dfb) [presumptive fibroblasts] of the pulp chamber. Also note the presence of dentin (d). The stratum intermedium (si) and predentin (pd) are negative for FGF-2. Counterstained with Meyer's haematoxylin. Bar: 10 μm.

Figure 11 Representative section of the pulp chamber of a maxillary first molar of a 10-day post-natal mouse pup. Note localization of FGF-2 in the nuclei of individual differentiating ectomesenchymal cells (dfb) [presumptive fibroblasts]. Counterstained with Meyer's haematoxylin. Bar: 20 μm.


d
dfb
pd
am
od
si
10


dfb
11

3.3 Immunolocalization of FGF-2 in the root of the murine tooth on post-natal day 12

Sections of the mice jaws stained with haematoxylin and eosin on post-natal day 12 showed that enamel maturation was almost complete in the first mandibular molar and root formation had progressed. Evidence of enamel maturation and the beginning of root formation was also noted in the second and third molars (Fig. 12).

Sections from all the molars demonstrated the presence of enamel and the adjacent layer of ameloblasts, the predentin, dentin and the odontoblasts layer, differentiating ectomesenchymal cells (presumptive odontoblasts) in the region of the developing root and in the pulp chamber and the developing periodontal ligament and the developing spicules of the alveolar bone (Fig. 12). Evidence of formation of connective tissue fibres in the dental follicle was also noted. Cementoblasts and cementum, once again were absent at this stage (Fig. 12).

Once again as seen in Table 3.3 and Figs. 13, 14 and 15, immunolocalization of FGF-2 in the maxillary and mandibular molars and the incisors was evident in a number of structures namely, the ameloblasts, odontoblasts and the differentiating ectomesenchymal cell types in different regions.

In the ameloblasts the signal was decreased and a faint cytoplasmic immunolocalization of FGF-2 was located (Fig. 15). In the odontoblast cell layer, homogeneous cytoplasmic immunolocalization was observed in almost all the cells. As extension of the root occurred, more odontoblasts differentiated apically and exhibited immunolocalization of FGF-2 (Fig. 13).

The differentiating ectomesenchymal cells (presumptive odontoblasts) in the apical region of the root exhibited intense nuclear and cytoplasmic immunolocalization for the antibody with some evidence in the extracellular matrix (Figs. 14 and 15). The same cell type also demonstrated the evidence of intense localization of FGF-2 in the presumptive furcation zone of the developing root of all molars and the incisors (Fig. 14).

Once again, by comparison of sections, it appeared that the differentiating ectomesenchymal cells (presumptive fibroblasts) of the pulp chamber and of the developing periodontal ligament showed immunolocalization of FGF-2 in more cells as compared to the previous two stages (Fig. 14). This however was not quantified.

Once again, no immunolocalization for FGF-2 was demonstrated in the predentin (Fig. 13) and the stratum intermedium (Fig 15) at this stage.

Table 3.3 **Immunolocalization of FGF-2 in the root of the murine tooth on post-natal day 12**

	Maxillary				Mandibular		
	Central Incisor	First Molar	Second Molar	Third Molar	First Molar	Second Molar	Third Molar
Stratum intermedium (si)	-	-	-	-	-	-	-
Ameloblasts (am)	+	+	+	+	+	+	+
Odontoblasts (od)	+	+	+	+	+	+	+
Differentiating ectomesenchymal cells (dmc)	+	+	+	+	+	+	+
Predentin (pd)	-	-	-	-	-	-	-
Dentin (d)	-	-	-	-	-	-	-
Zone of Cementum (c)	A	A	A	A	A	A	A
Differentiating ectomesenchymal cells in the cementum zone (cb)	A	A	A	A	A	A	A
Differentiating ectomesenchymal cells lining the developing alveolar bone (dob)	+	+	+	+	+	+	+
Differentiating ectomesenchymal cells in the pulp chamber (dfb)	+	+	+	+	+	+	+
Differentiating ectomesenchymal cells of the periodontal ligament (pdlfb)	+	+	+	+	+	+	+

Legends:

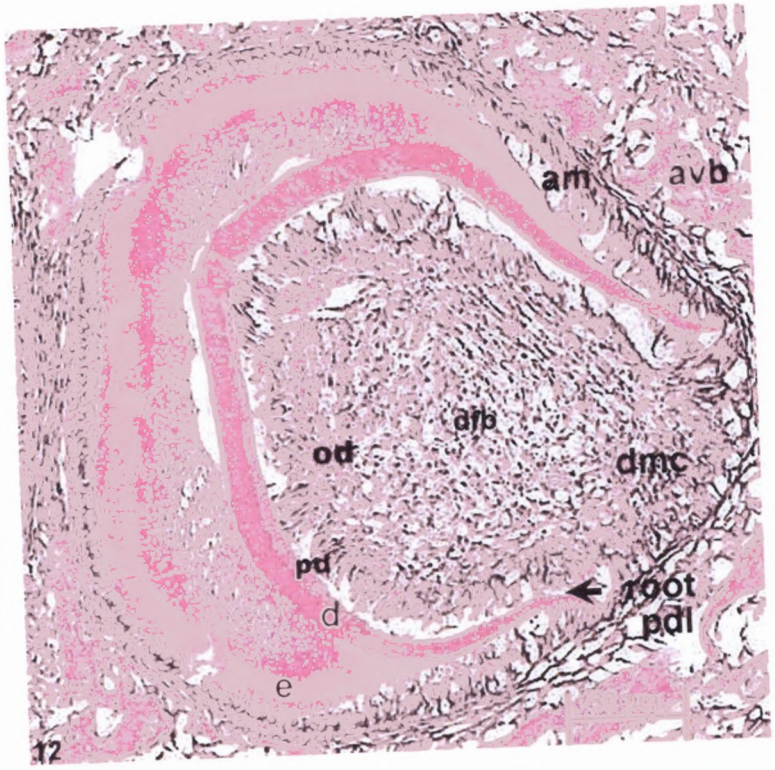
(+): Positive for immunoreactivity for FGF-2

(-): Negative for immunoreactivity for FGF-2

(A): Absent at this stage

Figure 12 Representative section of the developing mandibular third molar of a 12-day post-natal mouse pup. Note the presence of ameloblasts (am), enamel (e), dentin (d), predentin (pd), odontoblasts (od), differentiating ectomesenchymal cells (dmc) [presumptive odontoblasts], developing periodontal ligament (pdl), developing alveolar bone (avb) and the differentiating ectomesenchymal cells (dfb) [presumptive fibroblasts] in the pulp chamber. Also note the formation of the root in the apical area. Haematoxylin and eosin stain. Bar: 50 μm.

Figure 13 Representative section of the pulp chamber of a mandibular third molar of a 12-day post-natal mouse pup. Note the immunolocalization of FGF-2 in individual differentiating ectomesenchymal cells (dfb) of the pulp chamber [presumptive fibroblasts] and in the odontoblasts (od). Also note that dentin (d) and predentin (pd) do not show immunolocalization of the antibody. Counterstained with Meyer's haematoxylin. Bar: 10 μm.


am
avb
dfb
od
dmc
pd
root
pdl
d
e
12

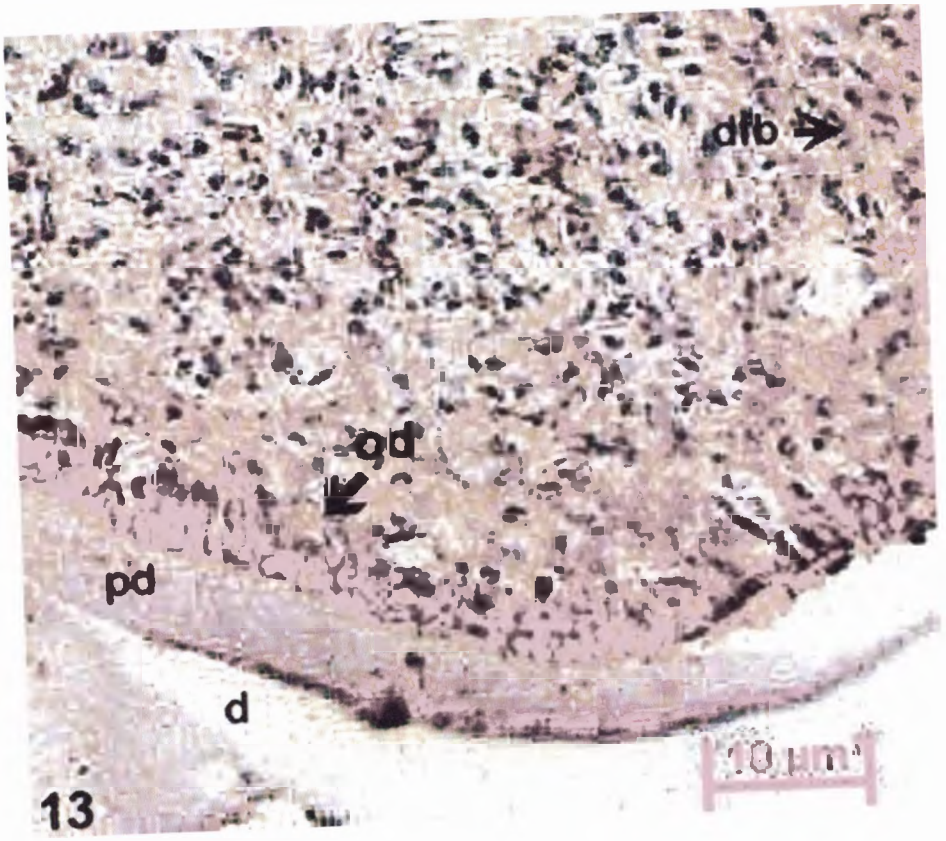
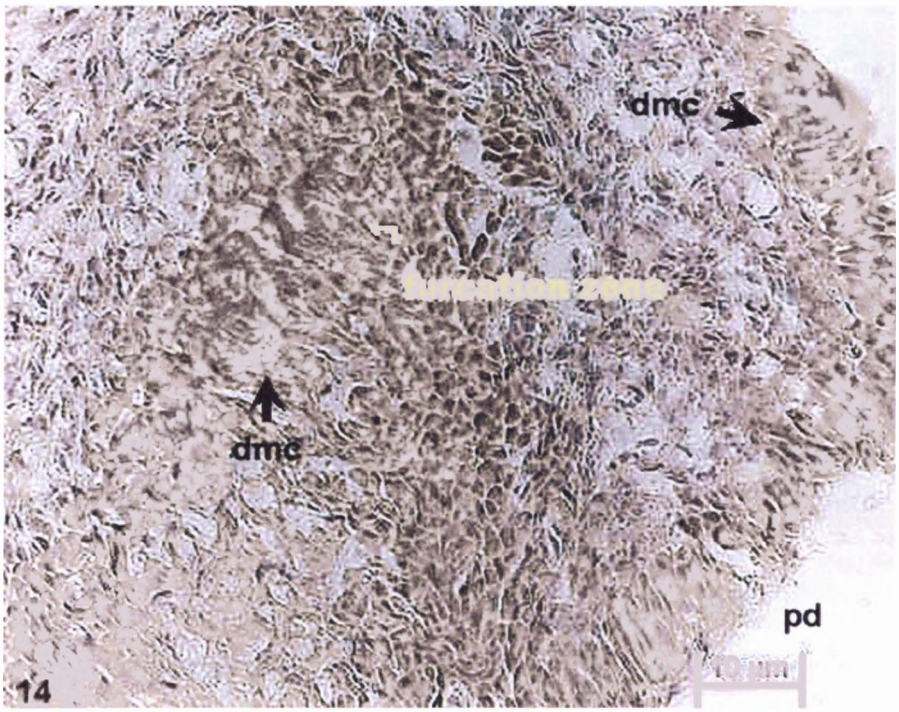
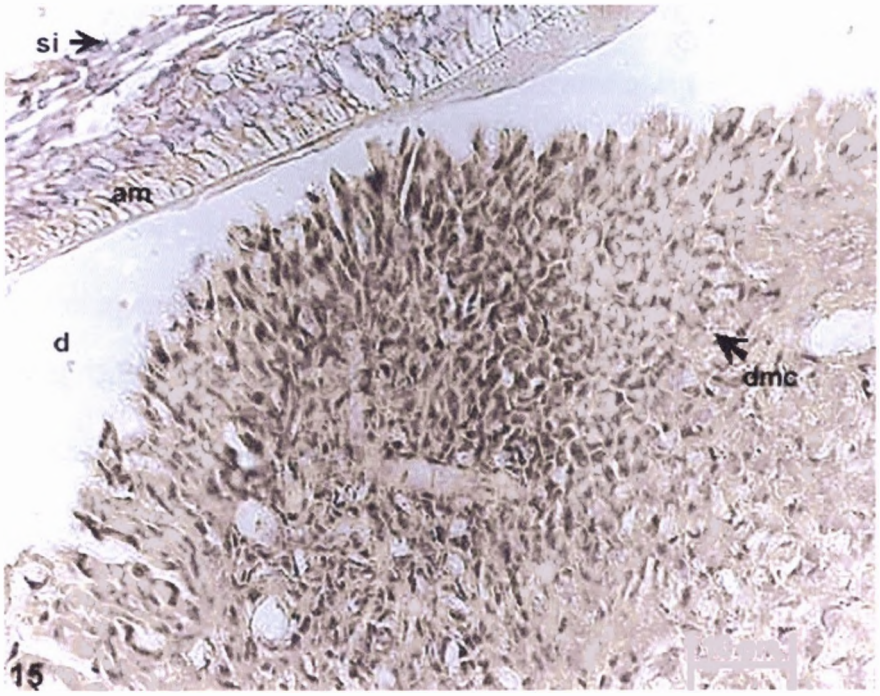

dfb
od
pd
d
10 µm
13

Figure 14 Representative sagittal section of the furcation zone of a maxillary second molar of a 12-day post-natal mouse pup. Note the intense immunolocalization of FGF-2 in the differentiating ectomesenchymal cells (dmc) [presumptive odontoblasts] while pre-dentin (pd) shows no localization of the antibody. Counterstained with Meyer's haematoxylin. Bar: 10 μm.

Figure 15 Representative section of the pulp chamber of a mandibular second molar of a 12-day post-natal mouse pup. Note the intense immunolocalization of FGF-2 in the differentiating ectomesenchymal cells (dmc) [presumptive odontoblasts] and faint immunoreactivity in the ameloblasts (am). Also note that the dentin (d) and stratum intermedium (si) do not show immunolocalization of FGF-2. Counterstained with Meyer's haematoxylin. Bar: 10 μm.


dmc
dmc
pd
14


si
am
d
dmc
15

3.4 Immunolocalization of FGF-2 in the root of the murine tooth on post-natal day 16

Progression in the development of all the types of teeth was evident on post-natal day 16. As seen in Table 3.4 and Fig. 16, no ameloblasts were present. This may be due to the fact that by this stage, the ameloblasts had degenerated and disappeared concurrent with the eruption of teeth. Also, no evidence of a stratum intermedium was found from this stage onwards (see Table 3.4 and Fig. 16). Evidence of eruption of the first molar was noted in both the arches.

By this stage, the process of cementogenesis had started and the presence of acellular cementum adjacent to the dentin layer was noted in the developing root. Differentiating ectomesenchymal cells (presumptive cementoblasts) of the dental follicle were opposed to the dentin layer (Fig. 16). Fibres of the developing periodontal ligament and the formation of the alveolar bone around the root were also noted.

As seen in Table 3.4 and Fig. 16, various cell types with the exception of ameloblasts and the stratum intermedium were present at this stage. FGF-2 was localized both in the maxillary and mandibular molars and the incisors. As referred to in Table 3.4 and Figs. 17 and 18, expression of this protein was evident in the cytoplasm of the odontoblasts, in the nuclei of the differentiating ectomesenchymal cells (presumptive fibroblasts) of the pulp chamber and the pulp canal.

Specific mention should be made of the differentiating ectomesenchymal cells (presumptive odontoblasts) in the apical part of developing root, which at this stage showed very intense immunolocalization, concentrated both in the cytoplasm and in the nucleus with some in the extracellular matrix (Figs. 17 and 18).

Immunolocalization of FGF-2 was still evident in the ectomesenchymal cells (presumptive osteoblasts) lining the developing alveolar bone as seen in the previous three stages i.e. post-natal days 9, 10 and 12 (Fig. 18).

Differentiating ectomesenchymal cells (presumptive fibroblasts) of the pulp chamber and the developing periodontal ligament also demonstrated immunoreactivity for this protein and there was faint localization in the extracellular matrix (Figs. 17 and 18).

Once again, as evident in the previous stages, the dentin, the predentin (Figs. 17 and 18) and cementum (Fig. 17) did not show any evidence of immunolocalization of FGF-2, at all.

Table 3.4 **Immunolocalization of FGF-2 in the root of the murine tooth on post-natal day 16**

	Maxillary				Mandibular		
	Central Incisor	First Molar	Second Molar	Third Molar	First Molar	Second Molar	Third Molar
Stratum intermedium (si)	A	A	A	A	A	A	A
Ameloblasts (am)	A	A	A	A	A	A	A
Odontoblasts (od)	+	+	+	+	+	+	+
Differentiating ectomesenchymal cells (dmc)	+	+	+	+	+	+	+
Predentin (pd)	-	-	-	-	-	-	-
Dentin (d)	-	-	-	-	-	-	-
Zone of Cementum (c)	-	-	-	-	-	-	-
Differentiating ectomesenchymal cells in the cementum zone (cb)	+	+	+	+	+	+	+
Differentiating ectomesenchymal cells lining the developing alveolar bone (dob)	+	+	+	+	+	+	+
Differentiating ectomesenchymal cells in the pulp chamber (dfb)	+	+	+	+	+	+	+
Differentiating ectomesenchymal cells of the periodontal ligament (pdlfb)	+	+	+	+	+	+	+

Legends:

(+): Positive for immunoreactivity for FGF-2

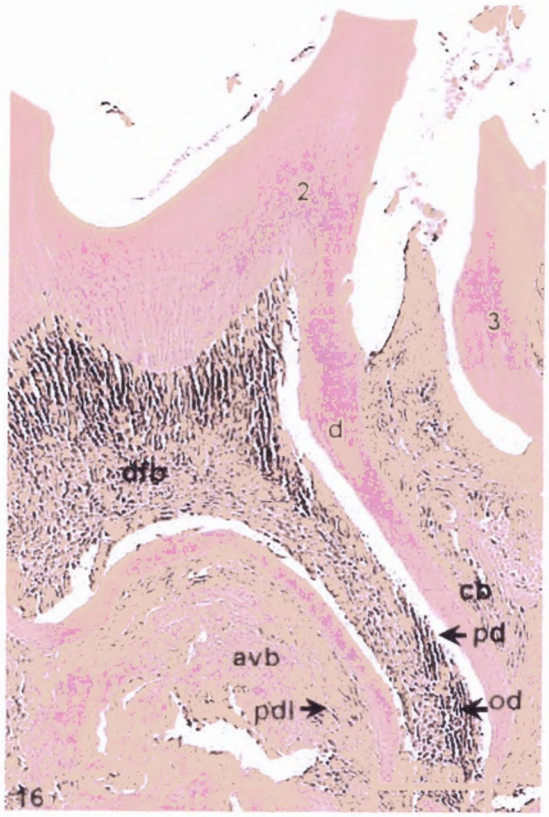
(-): Negative for immunoreactivity for FGF-2

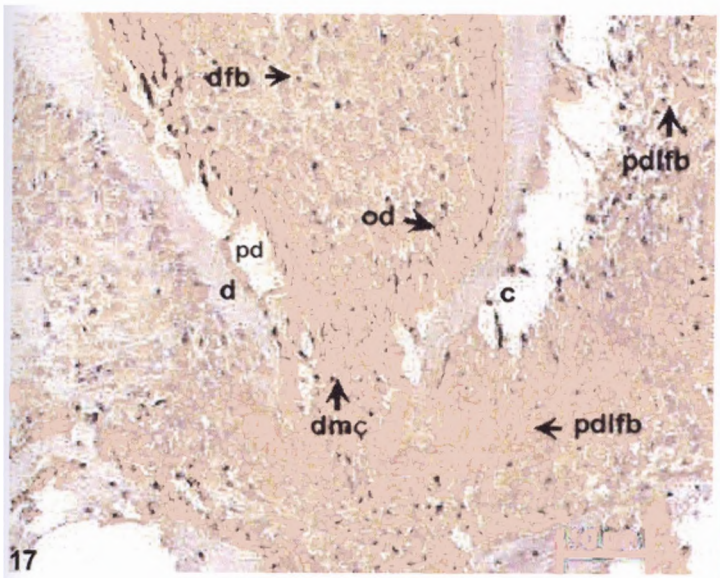
(A): Absent at this stage

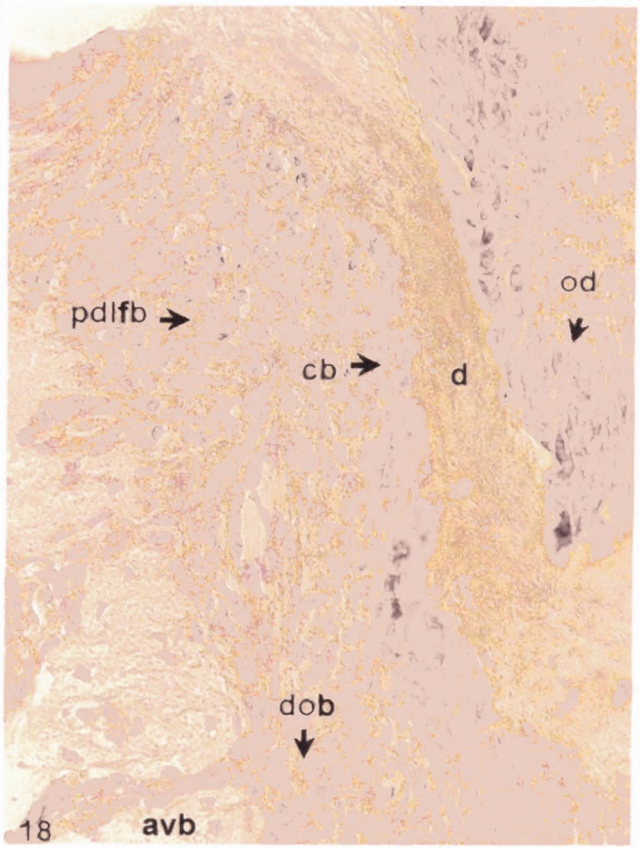
Figure 16 Representative section of the developing maxillary second and third molar of a 16-day post-natal mouse pup. Note the presence of dentin (d), predentin (pd), odontoblasts (od), developing periodontal ligament (pdl), developing alveolar bone (avb), cementoblasts (cb) and the differentiating ectomesenchymal cells (dfb) of the pulp chamber [presumptive fibroblasts]. Haematoxylin and eosin stain. Bar: 50 μm.

Figure 17 Representative section of the developing root of a maxillary third molar of a 16-day post-natal mouse pup. Note immunolocalization of FGF-2 in the odontoblasts (od), in the differentiating ectomesenchymal cells (dmc) [presumptive odontoblasts] in the apical zone of the developing root, in a few differentiating ectomesenchymal cells (dfb) of the pulp chamber [presumptive fibroblasts] and in individual differentiating ectomesenchymal cells (pdlfb) of the periodontal ligament. Also note that the dentin (d), predentin (pd) and the region of future cementum (c) formation next to the dentin layer do not show immunolocalization of FGF-2. Counterstained with Meyer's haematoxylin. Bar: 10 μm.

Figure 18 Representative section of the developing root of a maxillary first molar of a 16-day post-natal mouse pup. Note immunolocalization of FGF-2 in the odontoblasts (od), in individual cementoblasts (cb), in a few differentiating ectomesenchymal cells (pdlfb) of the periodontal ligament and in the presumptive osteoblasts (dob). Counterstained with Meyer's haematoxylin. Bar: 20 μm.


2
3
d
dfb
cb
pd
avb
pdl
od
16


dfb
pdlfb
od
pd
d
c
dmç
pdlfb
17


pdlfb
od
cb
d
dob
18
avb

3.5 Immunolocalization of FGF-2 in the root of the murine tooth on post-natal day 20

On post-natal day 20, routinely stained haematoxylin and eosin sections showed the eruption of the second molar in both the arches. Enamel maturation and progress in root formation was evident in the third molar. The process of cementogenesis was noted with the formation of the zone of cementum next to the dentin layer (Fig. 19). Neither ameloblasts nor the stratum intermedium was present.

The presence of various tissues and cell types as noted in Table 3.5 were obvious in the molars and incisors.

In general, at this stage, as seen in Table 3.5 and Figs. 20, 21 and 22, immunolocalization of FGF-2, both in the maxillary and mandibular molars and incisors was evident in the nucleus and cytoplasm of the odontoblasts, differentiating ectomesenchymal cells (presumptive fibroblasts) in the pulp chamber and in the developing periodontal ligament, presumptive osteoblasts lining the developing alveolar bone and in the presumptive cementoblasts in the zone of cementum formation next to the dentin layer.

Intense localization of FGF-2 was observed in the cytoplasm and the nucleus of the odontoblasts in the apical part of the root as compared to those placed more coronally (Figs. 20 and 21).

Expression of FGF-2 was again evident in the cytoplasm of many differentiating ectomesenchymal cells [presumptive osteoblasts] (Fig. 22).

Not all, but a few differentiating ectomesenchymal cells (i.e. presumptive cementoblasts) around the zone of cementum formation also expressed FGF-2 (Figs. 20 and 21).

The dentin (Figs. 20 and 21), the predentin (Figs 20 and 21) and the zone of cementum formation (Fig. 20) showed no localization of the antibody.

Expression of FGF-2 was again evident in the cytoplasm of many differentiating ectomesenchymal cells [presumptive osteoblasts] (Fig. 22).

Not all, but a few differentiating ectomesenchymal cells (i.e. presumptive cementoblasts) around the zone of cementum formation also expressed FGF-2 (Figs. 20 and 21).

The dentin (Figs. 20 and 21), the predentin (Figs 20 and 21) and the zone of cementum formation (Fig. 20) showed no localization of the antibody.

Table 3.5 **Immunolocalization of FGF-2 in the root of the murine tooth on post-natal day 20**

	Maxillary				Mandibular		
	Central Incisor	First Molar	Second Molar	Third Molar	First Molar	Second Molar	Third Molar
Stratum intermedium (si)	A	A	A	A	A	A	A
Ameloblasts (am)	A	A	A	A	A	A	A
Odontoblasts (od)	+	+	+	+	+	+	+
Differentiating ectomesenchymal cells (dmc)	+	+	+	+	+	+	+
Predentin (pd)	-	-	-	-	-	-	-
Dentin (d)	-	-	-	-	-	-	-
Zone of Cementum (c)	-	-	-	-	-	-	-
Differentiating ectomesenchymal cells in the cementum zone (cb)	+	+	+	+	+	+	+
Differentiating ectomesenchymal cells lining the developing alveolar bone (dob)	+	+	+	+	+	+	+
Differentiating ectomesenchymal cells in the pulp chamber (dfb)	+	+	+	+	+	+	+
Differentiating ectomesenchymal cells of the periodontal ligament (pdlfb)	+	+	+	+	+	+	+

Legends:

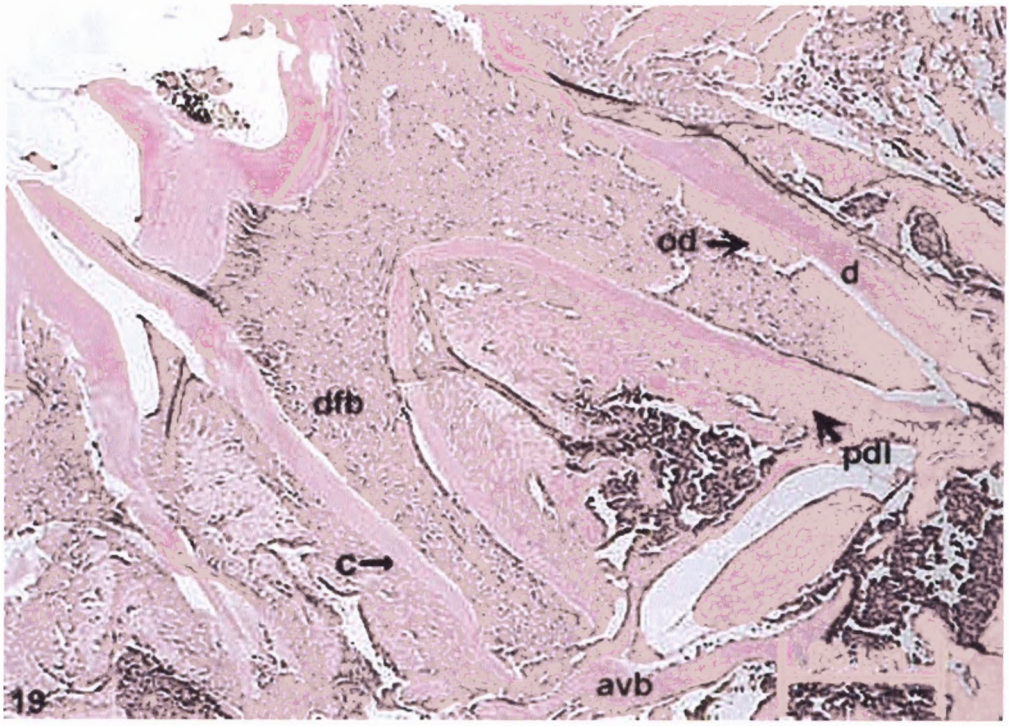
(+): Positive for immunoreactivity for FGF-2

(-): Negative for immunoreactivity for FGF-2

(A): Absent at this stage

Figure 19 Representative section of the developing mandibular first molar of a 20-day post-natal mouse pup. Note the presence of dentin (d), odontoblasts (od), developing periodontal ligament (pdl), developing alveolar bone (avb), the zone of cementum (c) formation and the differentiating ectomesenchymal cells (dfb) [presumptive fibroblasts] of the pulp chamber and canal. Haematoxylin and eosin stain. Bar: 50 μm.

Figure 20 Representative section of the developing root of a mandibular second molar of a 20-day post-natal mouse pup. Note immunolocalization of FGF-2 in the odontoblasts (od), in a few differentiating ectomesenchymal cells (dfb) [presumptive fibroblasts] and in the presumptive cementoblasts (cb). Note that the dentin (d), predentin (pd) and the zone of cementum (c) formation next to the dentin layer do not show immunolocalization of FGF-2. Counterstained with Meyer's haematoxylin. Bar: 10 μm.


od
d
dfb
pdl
c
avb
19

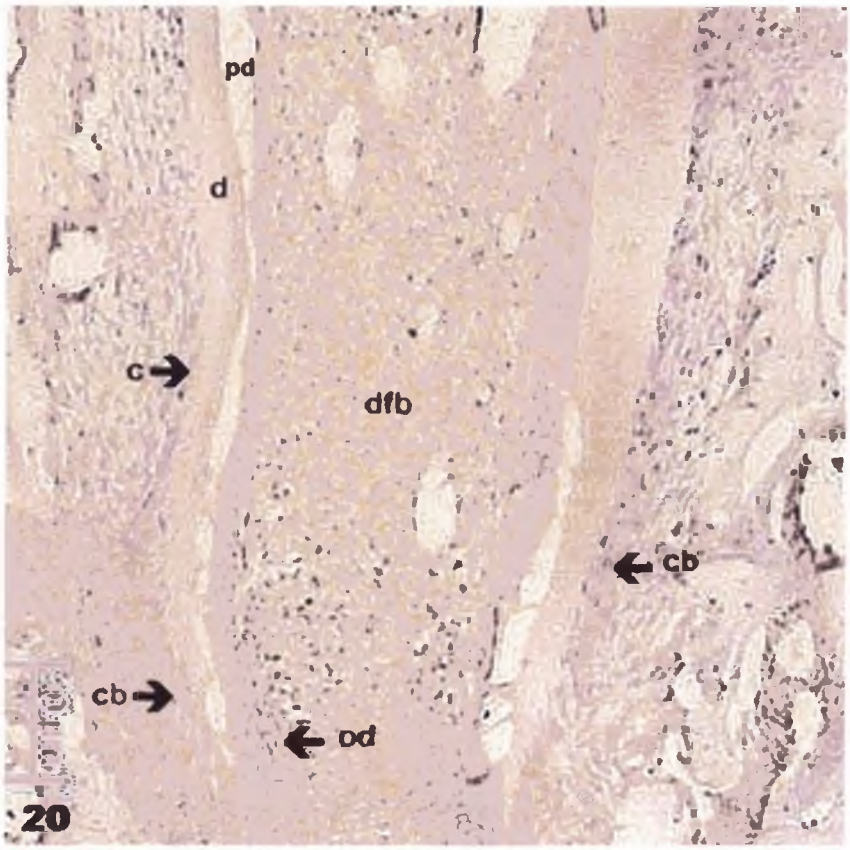
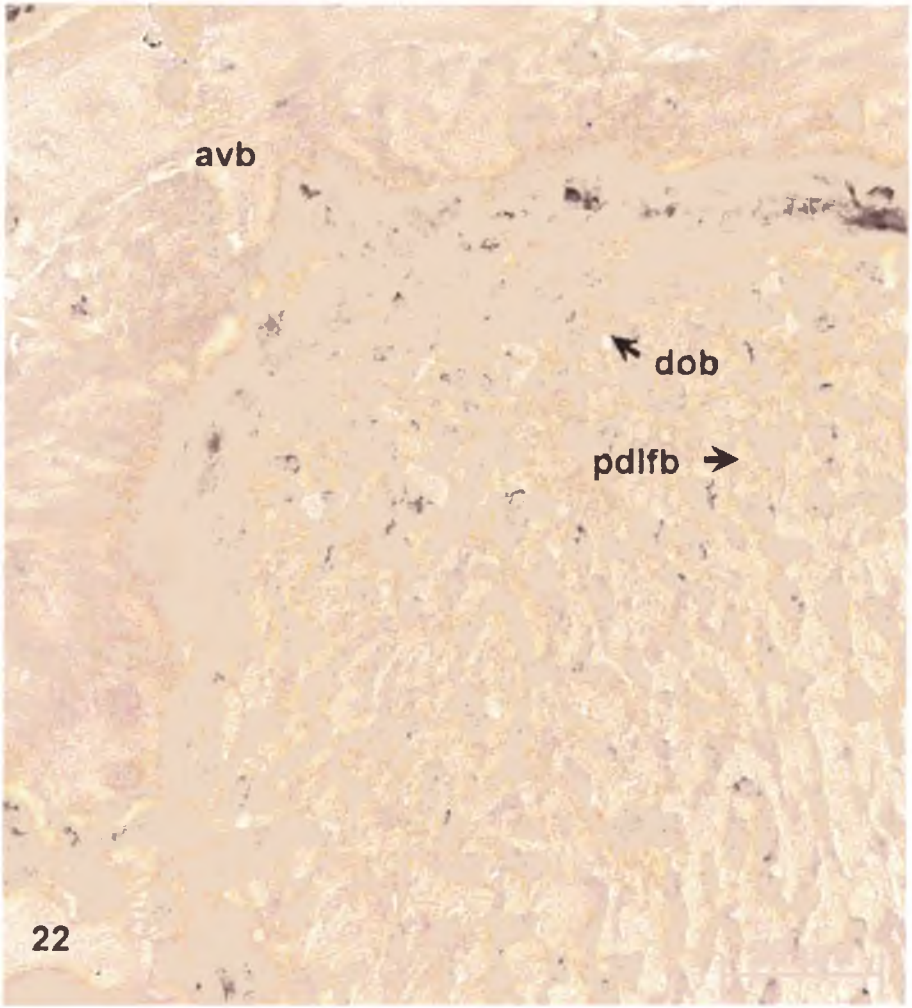

pd
d
c
dfb
cb
cb
od
20

Figure 21 Representative section of the developing root of a mandibular first molar of a 20-day post-natal mouse pup. Note the immunolocalization of FGF-2 in the odontoblasts (od), in the differentiating ectomesenchymal cells (cb) in the zone of cementum formation [presumptive cementoblasts] and in differentiating ectomesenchymal cells (dfb) of the pulp canal [presumptive fibroblasts]. Note that the dentin (d) and predentin (pd) show no localization of the antibody. Counterstained with Meyer's haematoxylin. Bar: 10 μm.

Figure 22 Representative section of the developing alveolar bone and the periodontal ligament of a mandibular first molar of a 20-day post-natal mouse pup. Note the immunolocalization of FGF-2 in the differentiating ectomesenchymal cells (dob) [presumptive osteoblasts] lining the developing bone (avb) and in few differentiating ectomesenchymal cells (pdlfb) [presumptive fibroblasts] of the periodontal ligament. Counterstained with Meyer's haematoxylin. Bar: 20 μm.


cb
dfb
pd
d
od
10 µm
21


avb
dob
pdlfb
22

3.6 Immunolocalization of FGF-2 in the root of the murine tooth on post-natal day 24

Root development in the first and second molar was almost complete by post-natal day 24. Odontoblasts, dentin, predentin, differentiating ectomesenchymal cells in the different parts of the developing root (presumptive odontoblasts and fibroblasts) and osteoblasts as well as the zone of cementum formation were obvious as seen in the previous two stages i.e. post-natal days 16 and 20 (Fig. 23).

Sections treated with the immunocytochemical procedure for FGF-2 demonstrated the same pattern of immunoreactivity in the molars and incisors, as observed in the previous stage (see Table 3.6 and Figs. 24 and 25). Intense homogeneous localization of FGF-2 was seen in the cytoplasm and nucleus of the odontoblasts (Figs. 24 and 25) and in differentiating ectomesenchymal cells (presumptive odontoblasts) in the developing root (Figs. 24 and 25).

Presumptive cementoblasts, in the developing periodontal ligament and presumptive osteoblasts lining the developing bone also exhibited localization of the antibody in the nuclei and extracellular matrix (Figs. 24 and 25).

The differentiating ectomesenchymal cells of the pulp chamber (dfb) and of the periodontal ligament (pdlfb) were also immunoreactive to this protein (Fig. 25)

Once again, no immunolocalization occurred in the dentin, the predentin and in the zone of cementum formation (Fig. 25).

Table 3.6 **Immunolocalization of FGF-2 in the root of the murine tooth on post-natal day 24**

	Maxillary				Mandibular		
	Central Incisor	First Molar	Second Molar	Third Molar	First Molar	Second Molar	Third Molar
Stratum intermedium (si)	A	A	A	A	A	A	A
Ameloblasts (am)	A	A	A	A	A	A	A
Odontoblasts (od)	+	+	+	+	+	+	+
Differentiating ectomesenchymal cells (dmc)	+	+	+	+	+	+	+
Predentin (pd)	-	-	-	-	-	-	-
Dentin (d)	-	-	-	-	-	-	-
Zone of Cementum (c)	-	-	-	-	-	-	-
Differentiating ectomesenchymal cells in the cementum zone (cb)	+	+	+	+	+	+	+
Differentiating ectomesenchymal cells lining the developing alveolar bone (dob)	+	+	+	+	+	+	+
Differentiating ectomesenchymal cells in the pulp chamber (dfb)	+	+	+	+	+	+	+
Differentiating ectomesenchymal cells of the periodontal ligament (pdlfb)	+	+	+	+	+	+	+

Legends:

(+): Positive for immunoreactivity for FGF-2

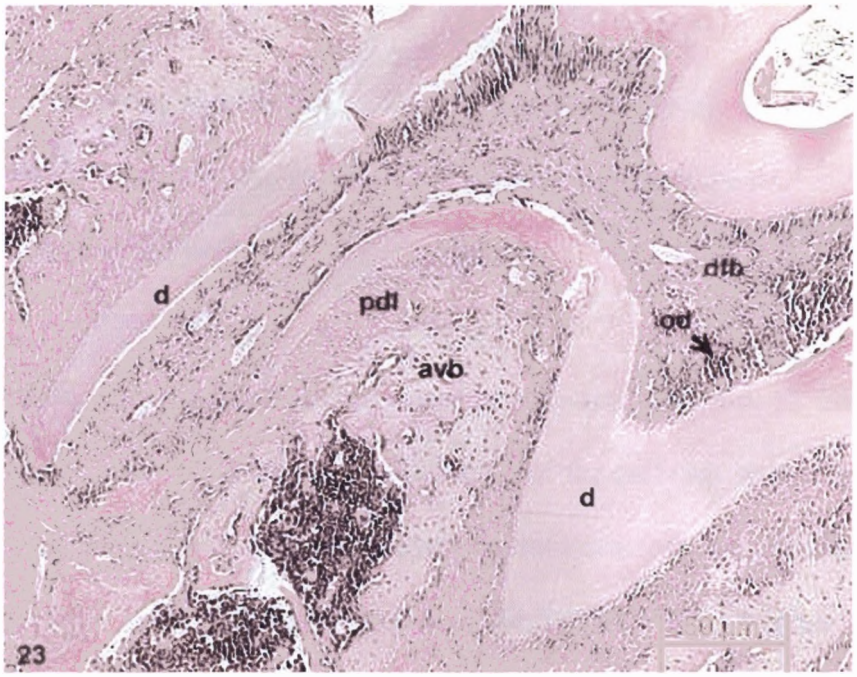
(-): Negative for immunoreactivity for FGF-2

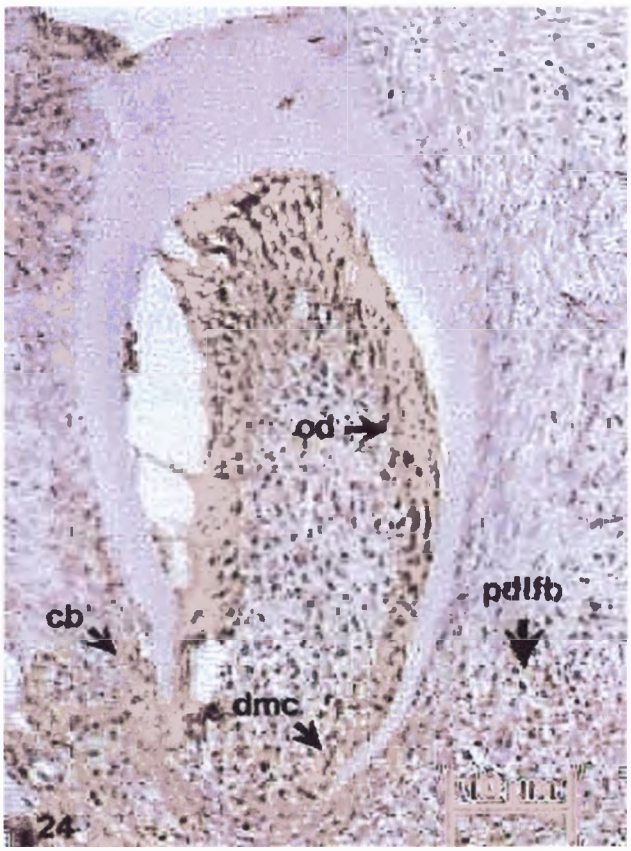
(A): Absent at this stage

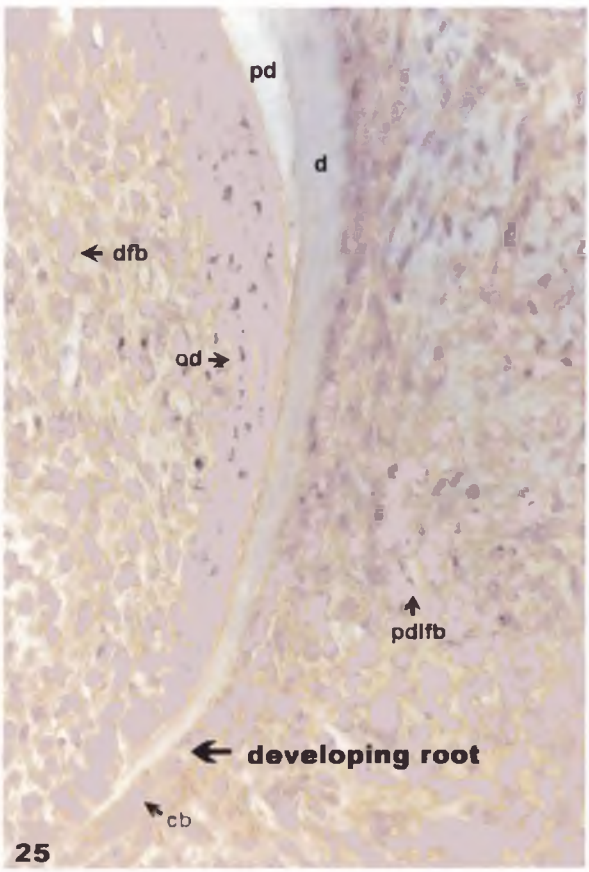
Figure 23 Representative section of the developing mandibular first molar of a 24-day post-natal mouse pup. Note the presence of dentin (d), odontoblasts (od), developing periodontal ligament (pdl), developing alveolar bone (avb) and the differentiating ectomesenchymal cells (dfb) of the pulp [presumptive fibroblasts]. Haematoxylin and eosin stain. Bar: 50 μm.

Figure 24 Representative section of the developing root of a mandibular third molar of a 24-day post-natal mouse pup. Note immunolocalization of FGF-2 in the odontoblasts (od), in the differentiating ectomesenchymal cells (dmc) in the root region [presumptive odontoblasts], in the ectomesenchymal cells (cb) in the zone of cementum formation [presumptive cementoblasts] and in a few differentiating ectomesenchymal cells (pdlfb) [presumptive fibroblasts] of the periodontal ligament. Counterstained with Meyer's haematoxylin. Bar: 10 μm.

Figure 25 Representative section of the developing root of a mandibular first molar of a 24-day post-natal mouse pup. Note immunolocalization of FGF-2 in the odontoblasts (od) in the root region, in the differentiating ectomesenchymal cells (cb) [presumptive cementoblasts] in the zone where cementum will form and in a few presumptive fibroblast of the pulp canal (dfb) and in periodontal ligament (pdlfb). Counterstained with Meyer's haematoxylin. Bar: 20 μm.


d
pdl
avb
dfb
od
d
23


od
cb
pdlfb
dmc
24


pd
d
dfb
od
pdlfb
developing root
cb
25

3.7 Summary of the Results

Immunocytochemical localization of FGF-2 was seen in the cytoplasm, nucleus and in the extracellular matrix of the various tissues and cell types of the root at different times of development of the teeth.

To summarize, the ameloblasts were present on post-natal days 9 to 12, after which they started degenerating. While present, localization of the antibody was seen in almost all of the cells. The cytoplasm in general was immunoreactive at these stages, while the nuclei of some cells also demonstrated affinity for FGF-2. With development, expression of FGF-2 progressed from the crown region to the apical part of the teeth. On post-natal day 12, the intensity of localization of FGF-2 antibody seemed to be decreased in most of the ameloblasts (Fig.15) as compared to the previous two stages of development i.e. post-natal days 9 and 10.

In the odontoblast layer, which was present at all stages examined, homogeneous localization of the antibody was observed in the cytoplasm of most of the cells. Intensity of the FGF-2 appeared to be increased in the cells developing apically at all the stages, although this was not quantified. However, specific mention should be made to the presumptive odontoblasts in the apical region of the developing root that demonstrated nuclear immunoreactivity for FGF-2, in addition to the cytoplasmic affinity.

On post-natal days 9, 10 and 12, only a few differentiating ectomesenchymal cells (presumptive odontoblasts, fibroblasts and osteoblasts) from the dental papilla and dental follicle were immunoreactive for the antibody, and demonstrated more nuclear affinity. With progression in development at later stages i.e. on post-natal days 16, 20 and 24, many cells of the same type were immunoreactive to FGF-2.

The dentin demonstrated a faint "discoloration" at 9 and 10 days postnatal. In the later stages i.e. post-natal days 12, 16, 20 and 24, discoloration of the dentin did not occur.

The stratum intermedium, which was present until postnatal day 12, showed no evidence of immunolocalization at any stage. The predentin, was similarly negative for FGF-2 at all the stages examined.

When the process of cementogenesis started on post-natal day 16, a few differentiating ectomesenchymal cells (presumptive cementoblasts) next to the dentin layer, exhibited immunolocalization. This continued on post-natal days 20 and 24.

At all the stages examined i.e. post-natal days 9, 10, 12, 16, 20 and 24, condensed differentiating ectomesenchymal cells in the area of root development and the presumptive furcation zone exhibited nuclear affinity for FGF-2 protein.

CHAPTER 4

4.0 DISCUSSION

4.1 General

Tooth development is an excellent example of a system in which the mechanisms of early epithelio-mesenchymal interactions and molecular signaling can be studied (Vaino *et al.,* 1993; Chen *et al.,* 1996; Vaahtokari *et al.,* 1996; Neubüser *et al.,* 1997; Thesleff and Sharpe, 1997). It is believed that the same conserved signaling molecules such as fibroblast growth factors (FGF's), bone morphogenetic proteins (BMP's), Hedgehog genes (Hh) and Wnt family members mediate the inductive interactions between the epithelium and mesenchyme (Pispa *et al.,* 1999). These signaling molecules regulate the development of vertebrate organs such as the teeth, the hair and the glands. The present study has investigated the pattern of expression of fibroblast growth factor-2 (FGF-2), in the different cell types and tissues of the developing root of murine teeth (first, second and third molars and incisors) at various stages of development.

Appropriate controls such as the positive, negative and absorption (peptide neutralization) controls were used for the immunocytochemistry. Sections of the submandibular gland of an adult rat were used as a positive control, in which localization of FGF-2 in the epithelial cells lining the striated and excretory ducts and granular convoluted tubules had been previously demonstrated (Amano *et al.,* 1993). In the present study, results of the positive control were the same as described by Amano *et al.* (1993). For the negative controls, sections adjacent to the section on which FGF-2 had been localized were utilized. Minimal or no background staining was observed in these sections. For the absorption control,

sections of the tooth adjacent to a section depicting the localization of the antibody were incubated with a solution of pre-absorbed antibody and antigen (1: 5 incubated overnight at 4° C) substituted for the primary antibody. Once again minimal or no background staining was observed in these sections. This confirmed that, indeed, the primary antibody is responsible for immunolocalization in the test sections.

Consistency of localization of FGF-2 (thus attesting to antibody binding) was noted throughout the serial sections examined within each of the age groups and at all the stages examined. With progression in root development apically, spatial and temporal differences in the expression of FGF-2 in some cell types and tissues were noted. This may be due to differences in the stages of root morphogenesis. Some cells/tissues e.g. ameloblasts, disappeared while some cell types (of ectomesenchymal origin) e.g. presumptive odontoblasts, osteoblasts and fibroblasts, differentiated from the dental papilla and dental follicle as time progressed. However, the pattern of immunolocalization for all cell types studied was consistent for both the mandibular and the maxillary molars and the incisors at all the six stages examined.

4.2 Differential localization of FGF-2 in the cytoplasm and nucleus, and in the extracellular matrix

Immunolocalization of FGF-2 occurred in both parts of the different cell types and tissues namely the cytoplasm and nucleus at different times of development. Cell types and tissues included ameloblasts, odontoblasts, dentin and the differentiating ectomesenchymal cells (presumptive odontoblasts, osteoblasts, fibroblasts and cementoblasts) from the developing dental follicle and the dental papilla.

Some evidence of localization in the extracellular matrix of different cell types was also observed. Recent advances in techniques have helped developmental biologists to understand the protein chemistry and molecular cloning of this family of growth factors. Several studies have elucidated that FGF-2 in mice and humans occurs in four isoforms: a low molecular weight [LMW FGF-2, 18 KDa] isoform and three high molecular weight [HMW FGF-2, 22, 22.5 and 24 KDa] isoforms (Arnaud *et al.,* 1999; Delrieu, 2000; Nugent and Iozzo, 2000; Ornitz, 2000). These different isoforms are generated as a result of initiation of alternative translational sites within a single mRNA species (Arnaud *et al.,* 1999). In the present study, an antibody to FGF-2 was used that has a molecular weight of 17 KDa as per the literature supplied by the manufacturer. This belongs to the low molecular weight isoform of FGF-2. The predominant form of FGF-2 is an 18 KDa protein that is formed due to initiation at the AUG codon (Abraham *et al.,* 1986). However, proteolytic cleavage of the first nine amino acids of this form may even produce shorter forms such as 16 KDa and 17 KDa, which are as active as the 18 KDa form (Klagusbrun *et al.,* 1987). It is believed that the low molecular weight isoform is expressed in the cytoplasm and in the extracellular matrix and primarily functions in an autocrine manner.

However, the recent studies of Arese *et al.* (1999) have shown that due to common amino acid sequence of low and high molecular weight isoforms, LMW FGF-2 is capable for modulation of low serum growth and thus may also act as a biological messenger in both the autocrine/paracrine and intracrine pathways.

On the other hand, the three high molecular weight isoforms are formed due to translation that initiates at CUG codons 5' to the AUG codon. This translation results in two functional domains of HMW isoforms: a complete amino acid sequence of the 18 KDa form and N-terminal extensions of varying lengths. These isoforms are supposed to have a preferential affinity for the nucleus and exert their activities through intracrine, perhaps nuclear, pathways (Arese *et al.,* 1999).

4.2.1 Nuclear localization of FGF-2

In the present study, localization of the 17 KDa (LMW) isoform occurred in the nuclei of various cell types. Although the scientific literature has tried to elucidate the affinity of FGF-2 for the nucleus, the complete mechanism of the actions occurring at cellular and sub-cellular levels is yet to be understood.

It is believed that the members of fibroblast growth factor family mediate their effects via membrane bound receptors (Basilico and Moscatelli, 1992; Klint *et al.,* 1999). But not all of the effects of cellular messengers may be produced in this manner. It is suggested that there exists a direct association of growth factors, namely platelet derived growth factor (PDGF), fibroblast growth factor (FGF), nerve growth factor (NGF), epidermal growth

factor (EGF) and neuro- transmitters or cytokines, with the cell nucleus (Mason, 1994; Wiedlocha *et al.,* 1996; Choi *et al.,* 2000).

To address the issue of nuclear affinity of FGF-2, Bouche *et al.* (1987) and Baldin *et al.* (1990) have reported that when low molecular weight (18 KDa) FGF-2 is added exogenously to synchronized cultures of bovine aortic endothelial cells (ABAE), this protein translocates and accumulates in the nucleolus during the G0/G1 transition cycle and remains upgraded for up to 6 hours. They also observed that up to 50% of total internalized FGF-2 is rapidly targeted to the nucleus in coronary venular endothelial cells. They believed that this nuclear uptake is controlled by the cell cycle, as this was specific only for the late G1 phase of the cell cycle whereas cytoplasmic uptake occurs throughout the cell cycle (Baldin *et al.,* 1990). On the basis of their work, it was suggested that some of the biological activities of this growth factor might be modulated by nuclear FGF-2, after it binds to the cell surface receptors.

In line with the above work, immunofluorescence studies of Tessler and Neufeld (1990), with FGF-2 antibody have demonstrated that FGF-2 exhibits intense nuclear affinity for various endothelial cells known to produce FGF-2 and, in addition, in FGF-2 transfected BHK cells.

Dono and Zeller (1994) have shown that FGF-2 protein expresses in the nuclei of the post-mitotic, terminally differentiating cells during chicken lung morphogenesis. In the

latter study they identified synthesis of three isoforms of FGF-2 by alternate translation initiation. They suggested that out of three isoforms, the low molecular weight isoform signals through the FGF receptor, and is internalized to the nucleus of the responding cells. However, none of these studies provide a satisfactory mechanism to understand the functional aspect of the localization of this growth factor to the nucleus.

To address the nuclear localization of FGF-2, two mechanisms have been put forward:

(1) Internalization of the extracellular 18 KDa FGF-2 isoform.

(2) Preferential nuclear localization of the high molecular weight FGF-2 isoform.

The high molecular weight isoforms are known to contain functional nuclear localization signals (NLSs), whereas the 18 KDa isoforms do not. However, both 18 KDa FGF-2 (low molecular weight) and 24 KDa FGF-2 (high molecular weight) isoforms are capable of binding non-specifically and with high affinity to nuclear chromatin. This is due to the fact that sequences within the 18 KDa protein, but not the amino-terminal extension, are necessary for this binding. This is substantiated by the fact that FGF receptor-1 (FGFR-1) also, which is believed to be the most effective receptor for FGF-2, translocates to the nucleus following internalization. In addition, it is shown that neither the ligand nor the receptor contains a nuclear localization signal (NLS). Nuclear import and the definitive function of this receptor in this cascade were not proven. Recently, however, Reilly and Maher (2001) described that nuclear translocation of FGFR-1 occurs via a mechanism distinct from classical nuclear import but dependent upon importin β, a component of multiple nuclear import pathways.

Another proposal to describe the nuclear localization of the low molecular weight isoform (18 KDa) states that this event correlates with the stimulation of ribosomal gene transcription, whose activation is mediated via the direct interaction of nuclear FGF-2 with the regulatory subunit of the protein kinase CK II (Bonnet *et al.,* 1996).

To further elaborate this aspect of nuclear localization of LMW FGF-2, Arese *et al.* (1999) using contemporary techniques like cell culture, FGF-2 cDNA mutation, immunocytochemistry, western blot analysis, β -Galactosidase activity assays and BrdU incorporation analysis demonstrated that when LMW FGF-2 is artificially fused to a canonical nucleus localization signal (NLS) and targeted to a nucleus in NIH-3T3 cells, it mimics the effects of HMW FGF-2 on low serum growth. This finding supports the concepts that nuclear activity of HMW is due to sequences it shares with LMW FGF-2. Two separate signals, the amino-acid terminal extension of HMW and a particular nucleolar localization sequence within the LMW sequence, are needed for nuclear topogenesis of FGF-2.

4.2.2 Localization of low molecular weight FGF-2 in the cytoplasm and extracellular matrix

Sub-cellular distribution of the four forms of FGF-2 has been examined in different cells which over-express FGF-2, as well as in the non-transfected endothelial, neuronal and neuro-endocrine cells using both immunocytochemistry and sub-cellular fractionation techniques (Rento *et al.,* 1990; Bugler *et al.,* 1991; Yu *et al.,* 1993; Stachowiak *et al.,* 1994).

Regarding the localization of low molecular weight isoforms of FGF-2 in the cytoplasm and in the extracellular matrix, it is proposed that the complex genetic organization of both FGF ligand and receptors present a great number of regulatory mechanisms to mediate the biological effects of this extensive family of growth factors. These biological effects of FGF's (ranging from proliferation and apoptosis to migration and differentiation) are mediated through four high affinity transmembrane kinase receptors, known as fibroblast growth factor receptor 1-4 [FGFR-1 to FGFR-4] (Basilico and Moscatelli, 1992; Klint *et al.,* 1999). These FGFR's structurally resemble other transmembrane kinase receptor and their presence in all contemporary vertebrates is documented (Coulier *et al.,* 1997). These receptors are believed to initiate the signaling cascade after binding with the cell surface and thus mediate gene expression in the nucleus (Stachowiak *et al.,* 1994).

As documented in the literature, two FGF-receptor (FGF-R) binding sites (of high and low molecular affinity) on FGF-2 are believed to act in concert to initiate signal transduction (Springer *et al.,* 1994). These sites are known to be distinct from the heparan sulfate

proteoglycans-binding domain. The high affinity binding sites consist of a solvent exposed hydrophobic amino acids cluster (Tyr-24, Tyr-103, Leu-140 and Met-142) and provide 75% of binding affinity and dominate the primary binding interactions. The low affinity binding sites are composed of amino acids (Lys-110, Tyr-111 and Trp-114) and participate in the secondary binding [which is 250-fold lower in affinity than primary interaction] (Springer *et al.,* 1994). It has been determined that the residues that constitute the primary-receptor binding site of FGF-2 are conserved throughout the FGF family, whereas those of secondary binding sites of FGF-2 are not (Springer *et al.,* 1994). It is suggested that "variable" secondary sites on both FGF as well as FGFR mediate specificity of a given FGF to a given FGFR isoform. The receptors that are monomeric in their native state dimerize after binding with an FGF ligand. This dimerization activates tyrosine kinase and triggers downstream effects through multiple signaling pathways (Springer *et al.,* 1994).

In the above proposed model of FGF-FGFR, the two domains of a single FGFR wrap around a single FGF-2 molecule such that one domain of FGFR binds to the primary receptor binding site of the FGF molecule, while the second domain of the same FGFR binds to the secondary receptor binding site of the same FGF molecule. This binding of FGFR to both FGF-2 surfaces is said to promote growth factor-mediated cell proliferation. This model compensates for not only heparin-like glycosaminoglycan (HLGAG) interactions with FGF and FGFR, but also FGF dimerization or oligomerization mediated by HLGAG (Venkataraman *et al.,* 1999).

In addition to the binding of low molecular weight isoform of FGF-2 with high-affinity FGFR's, localization of FGF-2 in the extracellular matrix may also occur through its binding with heparin sulfate proteoglycans (HSPG's) as well as to a low-affinity, cysteine-rich transmembrane FGF-binding protein (Zhou *et al.,* 1997). These HSPG's are sulfated glycosaminoglycans bound to a core protein, which is localized in the extracellular matrix and are believed to provide a storehouse for FGF-2 in this location. This mechanism not only provides the gradual bioavailability of FGF-2 in response to the internal or external stimuli, but also protects FGF-2 from degradation.

4.3 Immunolocalization of FGF-2 in the odontoblasts and presumptive odontoblasts

In the present study, consistent and uniform expression of FGF-2 was noted in the cytoplasm of most of the odontoblasts from post-natal days 9 to 24. A nuclear affinity for this protein was also noted in some odontoblasts at all the stages examined. On post-natal day 9, when the formation of the root had not yet begun in most of the teeth, odontoblasts located in the crown region of all the teeth were immunoreactive to FGF-2. With the onset of root formation from post-natal day 10, odontoblasts in the root region started differentiating. This process continued until the mature length of the root was observed, in most of the teeth, by post-natal day 24. Concurrent with progressive odontoblast differentiation, FGF-2 immunoreactivity appeared to progress from the cervical to the apical region of the developing root. Specific mention should be made of the differentiating ectomesenchymal cells (presumptive odontoblasts) of the developing root. These cells exhibited specific nuclear affinity for this protein at all the stages examined. It has been shown that nuclear localization of FGF-2 is specific for the late G1 phase of the cell cycle as described in section 4.2.1 (Baldin *et al.,* 1990). In the present study, this may account for the localization of FGF-2 in the nuclei of the presumptive odontoblasts with their successive differentiation from the ectomesenchymal cells of the dental papilla.

Previous studies using immunocytochemical techniques by Cam *et al.* (1992) and Russo *et al.* (1997) have reported expression of FGF-2 in the cytoplasm of the odontoblasts in early tooth morphogenesis. These pre-natal developmental studies were carried out in mice up to day 18 of the embryonic stage (E18). Cam *et al.* (1992) and Russo *et al.* (1997) demonstrated that FGF-2 expression in the cytoplasm of the odontoblasts is first evident at

the cap stage and continues until the late bell stage, with intense localization of this growth factor in the late bell stage of the developing crown. These investigators suggested that FGF-2 might be involved in the control of polarization and differentiation of the odontoblasts. They have also reported the expression of FGF-2 in the dental basement membrane and the stellate reticulum in early stages of odontogenesis. It is possible that expression of this growth factor in the structures of epithelial origin may play a role in the sequential events of the epithelio-mesenchymal interactions in the developing crown. Also, Unda *et al.* (2000) have shown that FGF-2 and FGF-receptors (FGFR) are expressed in the basement membrane in the early bell stage (E-17) of molars, and have assigned a role to FGF-2 in cell polarization. In addition, it is established that transcripts of the FGF receptor-1 (FGFR-1 also called *flg*) are detected in the odontoblasts (Orr-Urtreger *et al.*, 1991; Peters *et al.*, 1992) and recently the IIIc splice form of FGFR-1, which binds FGF-1 and FGF-2, has been found in the dental epithelium, pre-odontoblasts and differentiated odontoblasts (Kettunen *et al.*, 1998). Studies by Lesot *et al.* (1992) and Tziafas *et al.* (1992) also support localization of FGF-2 in the odontoblasts. These investigators have demonstrated that fibronectin (a component of the extracellular matrix) plays a role in the induction of odontoblast differentiation. FGF-2 is proposed to bind to type-IV collagen, laminin and fibronectin.

FGF-2 may play a similar role in odontoblast differentiation in root development. *In vitro* and *in vivo* studies have shown that the process of odontoblast differentiation is a key feature in root morphogenesis (Ruch, 1985). This comprises of sequential and highly integrated mechanisms that involve a series of cytological and functional changes. This cascade of events mediates sequential interactions of pre-odontoblasts with the adjacent

inner dental epithelium, through the specific basement membrane (Thesleff *et al.,* 1996; Thesleff and Nieminen, 1996; Thesleff and Sharpe, 1997; Avery, 2001). Reciprocally, the inner dental epithelium promotes the terminal differentiation of the pre-odontoblasts into post-mitotic, cytologically differentiated odontoblasts (polarized odontoblasts). Newly secreted extracellular matrix at the epithelio-mesenchymal interface plays an important role in this interaction. This is followed by terminal differentiation (i.e. cytoplasmic polarization, including changes in the distribution of microtubules, microfilaments and intermediate elements) of odontoblasts. Finally, odontoblasts become fully differentiated in structure and function and secrete the components of the predentin and dentin at their apical pole (Ruch 1998).

The importance of many growth factors either at the mRNA level or protein level that may play a critical role in the onset of odontoblast differentiation has been elucidated. These include: FGF-1 and FGF-2 (Cam *et al.*, 1992), IGF-I and –II (Martin *et al.*, 1998), PDGF (Chai *et al.,* 1998) and polypeptides belonging to the transforming growth factor-β (TGF-β) superfamily, which include BMP-2, -4, -6, and -7 (Cam *et al.*, 1990; D'Souza *et al.*, 1990; Heikinheimo *et al.*, 1997; Thomadakis *et al.,* 1999). It is observed that these growth factors and/or their mRNA transcripts, demonstrate a specific pattern of localization both spatially and temporally. Based on this specificity, these studies have suggested that these growth factors may play a role in odontoblast differentiation.

It is believed that synergistic interactions between members of different families of growth factors mediate the cascade of events for the induction of proliferation and differentiation in

different cell types and organs (Frenz *et al.,* 1994 and Li *et al.,* 2000). No single "standalone" growth factor can undertake this mammoth task. This fact holds true for FGF-2. Studies by Martin *et al.* (1998) has shown that the culture of the dental papilla of the mandibular first molar, treated with FGF-2 alone induces cell polarization at the periphery of the explants. This FGF-2 induced polarization occurs in the restricted areas corresponding to the cusps with no accumulation of extracellular matrix. However, FGF-2 in combination with transforming growth factor β-1 (TGFβ-1) and insulin like growth factor-I (IGF-I) induces intense cell polarization. Since TGFβ-1 alone has not been shown to be capable of promoting cell differentiation in dental papillae cultured *in vitro* (Unda *et al.,* 2001), the inductive effect may be attributed to synergistic and specific interactions between TGF β-1 with FGF-2.

4.4 Immunolocalization of FGF-2 in the cementoblasts, periodontal ligament and alveolar bone

The present study has reported the localization of FGF-2 in the differentiating ectomesenchymal cells of the periodontal ligament. These included the presumptive cementoblasts located in the innermost part of the ligament, presumptive fibroblasts of the periodontal ligament located centrally and presumptive osteoblasts lining the developing alveolar bone in the outermost part, in relation to the developing root.

4.4.1 Immunolocalization of FGF-2 in presumptive cementoblasts

On post-natal days 16, 20 and 24, the presence of presumptive cementoblasts was noted along the coronal-to-apical gradient of the root. This coincides with the time when the process of cementogenesis begins in the mouse root. Cho and Garrant (1988) have suggested that these cementoblast precursors arise from the ectomesenchymal cells of the dental follicle and migrate towards the root dentin surface following disruption of the intervening epithelial root sheath. Immunolocalization of FGF-2 was observed in the cytoplasm and nuclei of the cementoblasts at all stages of presence. Expression was evident in some of the cells. This may be due to the different stages of differentiation of the cementoblasts from the dental follicle and may vary with the time of "recruitment" of the cells from the follicle.

The presence of FGF-2 in the fibroblasts of mature human periodontal ligament is well documented in the literature (Gao *et al.,* 1996). This may support the expression of this growth factor in the presumptive cementoblasts also, as both are of the same embryonic origin. Murakami *et al.* (1999; 2003) have suggested that local application of FGF-2 in experimental models increases the number of undifferentiated mesenchymal cells of the periodontal ligament. These undifferentiated mesenchymal cells differentiate into cementoblasts and osteoblasts and thus induce the process of cementogenesis and osteogenesis. Thus, the presence of FGF-2 in the developing root suggests that this growth factor may play a role in the differentiation of cementoblasts.

4.4.2 Immunolocalization of FGF-2 in presumptive fibroblasts of the periodontal ligament

The periodontal ligament (PDL) develops from the central layer of the dental follicle shortly after root development is initiated (Ten Cate, 1998). The development and maturation of the PDL is dependent on the formation of the root dentin and the synthesis of its investing mineralized tissue, the cementum. Fibroblasts are the most predominant cells of the PDL. They synthesize and remodel extracellular matrices that include collagen fibres and a large component of the nonfibrillar glycoproteins. They play an important role in the development, structure and function of the supporting apparatus of the tooth (Ten Cate, 1998).

In the present work, immunolocalization of FGF-2 was detected in the cytoplasm and nucleus of some presumptive fibroblasts of the periodontal ligament (PDL), as well as in the extracellular matrix (ECM) at all the stages examined. Fibroblasts were identified on the basis of their morphology by light microscopy. Localization of FGF-2 progressed in many fibroblasts with progression in root development.

On post-natal day 9, only a few differentiating ectomesenchymal cells of the periodontal ligament (presumptive fibroblasts) were immunoreactive. The expression of FGF-2 was concentrated more in the nuclei than in the cytoplasm of these cells. This probably was due to the internalization of FGF-2 in the nuclei during the early stage of the cell cycle as described in section 4.2.1. With progression in the development of the periodontal ligament from post-natal days 10 to 24, many differentiating presumptive fibroblasts in the

same region demonstrated the affinity for FGF-2 in the cytoplasm and in the nucleus, as well as in their extracellular matrix.

Expression of FGF-2 in the cytoplasm and nucleus as well as in the extracellular matrix is well documented in the literature. Aktas and Kayton (2000) reported cytoplasmic and nuclear expression of FGF-2 in fibroblasts of the adrenal gland and kidney of adult rats and in the human lung and dermal tissue in normal healthy conditions. Using pre-embedding labeling methods, expression of FGF-2 in fibroblasts and in the extracellular matrix has been reported by Ohtani *et al.* (1993) and Yabu *et al.* (1993). Another study by Gao *et al.* (1996) on mature human periodontal ligament has immunolocalized FGF-2 in the cytoplasm and extracellular matrix of the fibroblasts of the adult periodontal ligament i.e. in some fibrocytes, endothelial cells of the blood vessels and in the extracellular matrix. These authors have suggested that FGF-2 is released from fibroblasts and endothelial cells and is stored in the extracellular matrix (ECM) where it may have a role in the stimulation of fibroblasts. It was suggested that FGF-2 binds with the other components of the extracellular matrix, namely the basement membrane, collagen, fibronectin, glycoproteins and proteoglycans and may play a crucial role in cell adhesion, migration and morphology, differentiation and proliferation (Baum *et al.,* 1980).

According to McNeil *et al.* (1989) and Clark *et al.* (1993), FGF-2 may be secreted from fibroblasts and transported to the extracellular matrix [which has been shown to be a storehouse for the bioactive form of FGF-2]. These investigators suggest that endogenous

FGF-2 released from fibroblasts to the ECM may result from cell lysis, as reported in endothelial cells, or secretion via a non-traditional mechanism. This feature of FGF-2 is attributed to the unusual property of this growth factor for lacking a signal peptide sequence (Mignatti *et al.*, 1991).

Based on these studies, it is suggested this FGF-2 stored in the extracellular matrix may influence the proliferation and differentiation of the immature multipotent cells of the periodontium in the later stages of root development.

Takayama *et al.* (1997), using a cell culture technique, thymidine incorporation assay, reverse transcription polymerase chain reaction (RT-PCR) and an alkaline phosphatase (ALPase) assay, demonstrated the effects of FGF-2 on the cellular functions of PDL cells. Their studies have shown that FGF-2 induces the proliferation of PDL cells but inhibits the induction of ALPase activity in a dose dependent manner. In addition, FGF-2 enhances the synthesis of type-I collagen, which is one of the most common extracellular fibres in the periodontal ligament, and is essential for calcified nodule formation. Similar effects were observed on osteoblast cell lines. In addition, FGF-2 enhances the proliferative response of PDL cells in a dose-dependent manner in beagle dogs.

Recently, several polypeptide growth factors such as fibroblast growth factor-2 (FGF-2), transforming growth factor ß-1 (TGF ß-1) and bone morphogenetic protein-2 (BMP-2) have received attention as they regulate migration, attachment, proliferation and/or differentiation of the PDL cells (Terranova *et al.,* 1987; Matsuda *et al.,* 1992; Sigurdsson *et*

al., 1995; Mohammed *et al.,* 1998; Murakami *et al.,* 1999). These factors may thus enhance the healing process by periodontal regeneration. The goal of PDL regeneration is to reconstitute the periodontal tissue onto a root surface. In this regenerative process, progenitor PDL cells need to migrate to the root surface, attach to it, proliferate and differentiate into an organized and functional fibrous attachment apparatus (Takayama *et al.,* 1997).

The presence of FGF-2 in the developing root calls for further studies to investigate if this growth factor is present in the adult root. This may prove an important tool for PDL regeneration by employing recombination techniques. This suggestion is based on the findings that FGF-2 application is of great advantage for active induction of not only connective tissue regeneration, but also osteogenesis and cementogenesis (Murakami *et al.,* 1999).

4.4.3 Immunolocalization of FGF-2 in presumptive osteoblasts

In the present study, immunolocalization of FGF-2 in the presumptive osteoblasts was evident at all the developmental stages studied i.e. from post-natal days 9 to 24. Localization of this growth factor was mainly in the cytoplasm with some evidence in the extracellular matrix, but some cells also demonstrated nuclear affinity for this protein. On post-natal day 9, most of the presumptive osteoblasts (which were recognized by their location around the surface of developing alveolar bone) were immunoreactive. With the progression of development, the number of FGF-2 positive cells surrounding the developing alveolar bone increased. This was probably due to the differentiation of more undifferentiated mesenchymal cells of the dental follicle at the surface of developing bone.

It is demonstrated that bone is a storehouse for growth factors that are capable of stimulating both osteoblast cell proliferation and differentiation (Hauschka *et al.,* 1986; Ogawa *et al.,* 1992). It has also been established that osteoblasts, which are responsible for bone formation, secrete a number of growth factors, namely fibroblast growth factors (FGF's), transforming growth factor-β (TGF-β), bone morphogenetic proteins (BMP's) and insulin-like growth factors (IGF's). These secreted polypeptides are said to be mitogenic to bone cells *in vitro* (Hauschka *et al.,* 1986).

Studies of FGF signaling in human and mouse have shown a pivotal role of this family of cytokines in the development of bone including growth, remodeling and repair. FGF family members control osteoblast gene expression in a biphasic fashion. However, conflicting reports on the exact nature of these effects exist in the literature. These differential effects

of FGF's depend upon the stage of osteoblast maturation (Rodan *et al.,* 1989; Pitaru *et al.,* 1993; Liang *et al.,* 1999; Mansukhani *et al.,* 2000; Zeng *et al.,* 2003).

In vitro studies have demonstrated that FGF-2 in particular, is capable of stimulating the proliferation of osteoblasts, chondrocytes and periosteal cells, and can also stimulate the formation of mineralized bone-like nodules in cultures of bone marrow stromal cells (Martin *et al.,* 1997). It is believed that osteoblasts synthesize FGF-2 and store it in a bioactive form in the extracellular matrix (Rodan *et al.,* 1989; Hurley *et al.,* 1993).

Mansukhani *et al.* (2000) using a number of novel techniques namely immortalization of osteoblasts, DNA synthesis assays, alkaline phosphatase staining, apoptosis assay, immunoprecipitation and western blot analysis have shown that FGF-2 induces the proliferation of the immature phenotype of the osteoblasts. In the same context, it inhibits the proliferation of differentiating osteoblasts that are producing matrix and steadily upregulating alkaline phosphatase (ALP) and gradually promotes their apoptosis. Apoptosis results in repression of alkaline phosphatase (ALP) expression and further leads to the inhibition of DNA synthesis at a later time during differentiation. The main signaling cascade of FGF-2 responsible for cell proliferation and differentiation is known to be a RAS/MAP kinase dependent pathway (Klint *et al.,* 1995; Kouhara *et al.* 1997). In addition, other pathways such as FRS2, Shp2, protein kinase C, cytoplasmic tyrosine kinase Src and protein kinase C-independent p 70S60 kinase are also suggested (Zhan *et al.,* 1994; Kanda *et al.,* 1997). Mansukhani *et al.* (2000) have shown that FGF signaling activates the FRS2, Shp2 and MAP kinase pathways in osteoblasts, while the STAT1 pathway is inhibited. It is suggested that during these pathways, differentiating osteoblasts could be expressing

adapters or substrates that are different from those expressed in the immature phenotype and which could direct FGF signaling in novel, yet different pathways.

Mansukhani *et al.* (2000) also describe the dual role of FGF signaling in intramembranous ossification. It is thought that initially FGF stimulates the proliferation of immature osteoblasts, thus increasing the pool of osteoblast progenitors, and then acts as a "brake". This would control the number of osteoblasts that undergo terminal differentiation. In addition, *in vitro* studies have shown that FGF-2 either stimulates or inhibits the production of type-I collagen (depending upon, the duration of FGF exposure and maturational stages of the cells being treated), the major differentiated product of osteoblasts (McCarthy *et al.,* 1989). Short term (24 h) treatment with FGF-2 resulted in the stimulation of the collagen gene (Hurley *et al.,* 1993).

In contrast to the above studies, Debiais *et al.* (1998) have reported that FGF-2 reduces the expression of osteoblast markers in the less mature cells, while increasing osteocalcin production and matrix maturation in more mature cells. These observations indicate that FGF-2 may not have a consistent effect on osteoblast differentiation *in vitro*. This inconsistent effect of FGF-2 on osteoblast differentiation is attributed to the redundancy of FGF signaling. This is substantiated by the fact that the upregulated activation of FGF-signaling leads to bone morphogenetic defects namely achondroplasia and various craniosynostosis syndromes (McIntosh *et al.,* 2000). However, overexpression of FGF-2 in transgenic mice results in premature mineralization, achondroplasia and shortening of long

bones (Coffin *et al.,* 1995) as well as the disruption of the FGF-2 gene that decreases the bone mass and bone formation (Montero *et al.,* 2000).

Several studies have shown that FGF-2, while acting in concert with other growth factors, produces different effects at different developmental stages. Hanada *et al.* (1997) and Fujimura *et al.* (2002) using recombinant application of FGF-2 and BMP-2 in adult Wister rats have shown that low doses of FGF-2 with BMP-2 increase the osteoinductive activity (by inducing osteocalcin mRNA expression), while high doses result in inhibition of the activity. FGF-2 in synergism with TGF-β has also been shown to promote proliferation of chondrocytes and osteoblasts (Nakamura *et al.,* 1995).

In line with the above investigations, it is believed that FGF-2 demonstrates strong anabolic effects (in the rat model), namely an increase in the number and activity of osteoblasts along the surfaces of cancellous and endochondral bone surface. This results in the accumulation of osteoid and the formation of cancellous and cortical bone mass. The anabolic effect of FGF-2 is also substantiated by *in vivo* treatment studies of fractured tibias in rabbits where it was shown that single injections of FGF-2 at 100 μg or above in a 3-mm bone defect results in increased volume and mineral content of the newly made bone after 5 weeks (Kato *et al.,* 1998). A similar model in dogs using FGF-2 (200 μg) also shows increased intramembranous ossification, osteoclast number in the periosteal callus and fracture strength (Nakamura *et al.,* 1998; Kawaguchi *et al.,* 2001).

In the present study, localization of FGF-2 in osteoblasts, in successive morphogenetic stages of root development indicates that this growth factor may play a role in the differentiation of osteoblasts. It is possible that this growth factor may be deployed post-developmentally in regeneration of the alveolar bone if applied in low doses.

4.5 Immunolocalization of FGF-2 in presumptive fibroblasts of the pulp

The present study demonstrates the immunolocalization of FGF-2 in presumptive fibroblasts of the pulp at important stages during the development of the root. Expression of FGF-2 was first evident in the cytoplasm and nucleus as well as in the surrounding extracellular matrix of presumptive fibroblasts of the pulp on post-natal day 9. With developmental progression and root elongation in the later stages i.e. from post-natal days 10 to 24, many cells in this region showed the same pattern of expression of FGF-2.

The presence of FGF-2 in the presumptive fibroblasts of the pulp, in the present study, is in line with a previous study by Cam *et al.* (1992), who reported the presence of this growth factor in the dental papilla of the developing crown during the late bell stage (mouse). These investigators defined the expression of FGF-2 in successive morphogenetic stages of the tooth crown, but did not describe any functional role for FGF-2. In addition, *in situ* hybridization studies by Peters *et al.* (1992) have indicated the presence of FGF receptor-1 (FGFR-1/*flg*) but not FGF receptor-2 (FGFR-2/*bek*) [known to bind with FGF-2], in odontoblasts and in the underlying dental papilla in embryonic mice. However, they also could not co-relate the expression of FGFR-1 with its function. Thesleff *et al.* (1995) reported that FGF family members, including FGF-2 and FGF-receptors are co-expressed in the dental mesenchyme and dental epithelial cells when epithelio-mesenchymal signaling regulates the inductive events of tooth morphogenesis. Kettunen *et al.* (2000) using tissue recombination, bead experiments, *in situ* hybridization and cell proliferation assays have detected the presence of mRNA transcripts of two other members of the FGF family, namely FGF-3 and FGF-10, in the dental papilla of molars and incisors during early stages

of mouse tooth odontogenesis. Kettunen *et al.* (2000) suggest that FGF-3 and FGF-10 are expressed when the differentiation of ectomesenchymal cells is regulated at the interface between the epithelium and mesenchyme. Niswander and Martin (1992) suggest that the members of FGF family of ligands in general, play an important role in the control of proliferation, condensation and odontogenic differentiation of the dental mesenchymal cells by autocrine and paracrine mechanisms.

In the present work, expression of FGF-2 in some presumptive fibroblasts of pulp of the developing root may indicate that this growth factor is expressed at a time when the undifferentiated mesenchymal cells of the dental papilla become committed/determined to be presumptive fibroblasts, which are the predominant cell type of the pulp. In line with the previous documented work, it is possible that with initiation of differentiation of presumptive fibroblasts, FGF-2 and its receptors are expressed in these cell types and thus facilitate the cascade of events for differentiation.

Based on the expression of FGF-2, its related peptides and FGF-receptors in the mesenchymal cells of the dental papilla, several studies have suggested that this family of growth factor could be involved in the development and regeneration of the dentin, if employed therapeutically. These suggestions are based on findings that exogenous FGF-2 added to cultured human pulp cells demonstrates higher levels of expression of osteonectin/SPARC (secreted protein, acidic and rich in cysteine) and SPARC transcripts (a major non-collagenous matrix protein in bone and dentin; abundant in the odontoblasts, but

absent in the pulp cells) in the later stages, when the proliferation of the pulp cells is minimal. Also, this growth factor enhances alkaline phosphatase (ALPase) activity (Takano-Yamamoto *et al.,* 1994; Unda *et al.,* 2001). The marked expression of osteonectin/SPARC with increased alkaline phosphatase (ALPase) activity is believed to participate in the cascade of events that finally leads to the formation of dentin (Yoshiki and Kurahashi, 1971). These studies suggest that isolated pulp cells may be able to differentiate into odontoblasts *in vitro* when exposed to exogenous FGF-2.

4.6 Immunolocalization of FGF-2 in the dentin

In the present work, faint discoloration of the dentin was observed on post-natal days 9 and 10. In the later stages, no immunoreactivity in the dentin was observed at all. This immunolocalization, when it occurred was not convincing and definitive. It is possible that this may not be a true immunoreactivity at these particular developmental stages, but rather background staining or immunolocalization of FGF-2 in odontoblastic processes extending into the dentin.

Previous work by Russo *et al.* (1997) reported an intense expression of FGF-2 in the dentin matrix in late bell stage [which corresponds approximately to embryonic day-20 (E-20)] of mouse tooth development. This coincides with the time when the odontoblasts begin secreting the dentin matrix. Their studies also indicate the presence of FGF-2 in differentiating odontoblasts during crown morphogenesis. However, these investigators did not explain the presence of FGF-2 in the dentin and its possible role therein. In their studies, it may be possible that FGF-2 present in the differentiated coronal odontoblasts is transported to the dentin matrix, as the extracellular matrix is believed to be storehouse for the growth factors. Another possibility in line with the work of Russo *et al.* (1997) may be that in the present study, a fraction of FGF-2 is still present in the dentin or in the odontoblastic processes on post-natal days 9 and 10. This may exhibit immunoreactivity in the dentin matrix. In the later stages, when the mineralization of dentin occurs, this growth factor is not expressed.

Roberts-Clark and Smith (2003) recently measured the concentration of angiogenic factors in human dentin matrix. Using an enzyme-linked immunosorbent assay (ELISA) they measured the concentration of FGF-2 and other factors in the soluble and insoluble matrix fractions isolated from human dentin. It was observed that while high concentrations of platelet-derived growth factor (PDGF-AB) and low concentrations of vascular endothelial growth factor (VEGF), placenta growth factor (PlGF) and FGF-2 are present in the EDTA-soluble matrix fraction, low concentration of epidermal growth factor (EGF) and no FGF-2 or PlGF was detected in the insoluble matrix fractions.

It is proposed that, in the present work, a definitive presence of FGF-2 in the dentin in these two developmental stages can only be defined with further studies using other molecular techniques such as *in situ* hybridization and RT-PCR.

4.7 FGF-2 and the stratum intermedium

In the present study, the presence of the stratum intermedium was observed only on post-natal days 9, 10 and 12, in the crown of the maxillary and mandibular molars and the incisors. The stratum intermedium, which is a subtle and transient epithelial structure associated with the inner dental epithelium, though present at these stages, was not distinct. It appears as a layer of stratified epithelial cells (surrounding the ameloblasts proximally). In the later stages, as root development progressed apically, this stratified epithelial layer disappears. However, while present, no immunolocalization of FGF-2 was observed in the stratum intermedium.

Russo *et al.* (1997) reported the presence of FGF-2 in the stratum intermedium during the early and late bell stages (approximately E18-20) in early mouse tooth morphogenesis. This is prior to the stages used in the present study. Russo *et al.* (1997) also reported that the signal for FGF-2 shifts from the epithelium to the mesenchyme in later stages i.e. the late bell stage of crown development.

The present study supports the above findings of Russo *et al.* (1997). It was observed that with the progression of root morphogenesis, the expression of FGF-2 disappears in the structures of epithelial origin e.g. the stratum intermedium and ameloblasts (with progressive disappearance of these structures) and appeared in the structures derived from ectomesenchymal cells e.g. odontoblasts.

4.8 Immunolocalization of FGF-2 in the ameloblasts

Presence of ameloblasts, in the maxillary and mandibular molars and incisors was observed on post-natal days 9, 10 and 12. On post-natal day 9, a homogeneous immunoreactivity of FGF-2 in the cytoplasm of most of the ameloblasts and in the nuclei of a few ameloblasts was noted. The same pattern of expression continued on post-natal day 10 as more ameloblasts differentiated apically. However, on post-natal day 12 the signal seemed to disappear in most of the cells. In the later stages of development i.e. post-natal days 16, 20 and 24, ameloblasts were absent. This was probably due to the fact that with elongation in the root apically followed by eruption of various teeth in the oral cavity, these epithelial derivates i.e. the ameloblasts formed part of the outer layer of the crown that is "peeled" away.

Expression of FGF-2 in ameloblasts in the present study is in line with the studies of Cam *et al.* (1992) and Russo *et al.* (1997) who have reported the presence of FGF-2 in pre-ameloblasts and ameloblasts in mouse crown morphogenesis. However, these investigators did not comment on the pattern of localization of FGF-2 in the cytoplasm and nucleus. Although not a scope of this project, the present study also describes stage specific expression of FGF-2 in the cytoplasm and nucleus of the ameloblasts, as occurred in different stages of development.

Orr-Urtreger *et al.* (1991) and Peters *et al.* (1992) using *in situ* hybridization techniques with local application of the FGF-2 on agarose beads on isolated dental mesenchyme have demonstrated the intense expression of FGF receptor-1 (FGFR-1) in ameloblasts and odontoblasts and FGF receptor –2 IIIb (FGFR-2 IIIb) in the ameloblasts, both known to bind with FGF-2. Both these studies have suggested that FGF-2 may participate in

regulation of differentiation and/or secretion of the ameloblasts in early crown morphogenesis.

Expression of other members of the FGF family, namely FGF-3, -4, -9 and –10, is also known in tooth development (Kettunen and Thesleff, 1998; Kettunen *et al.,* 2000). These studies also elucidate that ligand members of the FGF family act as epithelial signals and thereby mediate inductive interactions between dental epithelium and mesenchyme during successive stages of tooth formation.

It is possible that the early expression of FGF-2 in the epithelial derivates i.e. ameloblasts and stratum intermedium followed by progressive expression of FGF-2 in the odontoblasts (ectomesenchymal derivatives) of the root in later stages, plays an important role in successive development of the tooth. However, a definitive role for FGF-2 in ameloblasts could not be determined in the present study.

CHAPTER FIVE

5.0 CONCLUSION

Fibroblast growth factor-2 (FGF-2) was localized in the odontoblasts, presumptive odontoblasts and cementoblasts of the developing root of both the maxillary and the mandibular molars and incisors of the mouse. Also, immunolocalization of FGF-2 occurred in the presumptive fibroblasts of the pulp and the presumptive osteoblasts, cementoblasts and fibroblasts of the periodontal ligament. In addition, immunolocalization of FGF-2 also occurred in the ameloblasts from post-natal days 9 to 12. Although the presence of FGF-2 was also noted in the dentin on post-natal days 9 and 10, this needs further investigation to support its presence. Localization of FGF-2 occurred in the cytoplasm and the nucleus as well as in the extracellular matrix of various structures as described. Minimal or no background staining was observed in the sections incubated with FGF-2.

Reciprocal and sequential epithelio-mesenchymal interactions are said to be important regulators of organ/tissue development. These interactions are mediated by growth factors such as BMP's, TGF ß-1 and FGF's. FGF-2, a ligand member of FGF family of growth factors is also considered as a common molecular signal for embryonic development.

Immunolocalization of FGF-2 at different morphogenetic stages, spatially and temporally, in different structures may indicate its participation in root development. Although present at different developmental stages, localization of a specific growth factor such as FGF-2, spatially and temporally does not signify a particular function. Its

role in the mechanisms of cascades of events and interactions requires further investigation.

It is believed that if the action of these factors is mimicked and reproduced post-developmentally, these molecules may be utilized for the purpose of regeneration (Sigurdsson *et al.,* 1995; Murakami *et al.,* 1999).

APPENDIX

APPENDIX I

Calcium oxalate test for completion of decalcification

Solutions:

(A) Concentrated ammonia (S.G. 0.880)

(B) Saturated aqueous ammonium oxalate

Protocol:

(1) Take approximately 5.0 ml of used decalcifying fluid and add a small piece of litmus paper.

(2) Add strong ammonia drop by drop till the solution is neutral to litmus.

(3) Shake after the addition of each drop.

(4) Add approximately 5.0 ml saturated ammonium oxalate.

(5) Shake well.

(6) Allow to stand for 30 minutes.

Result Interpretation:

(a) If a precipitate (calcium hydroxide) forms after addition of ammonia, a considerable amount of calcium is present. Tissue should be kept for a longer period of time to precede further decalcification.

(b) If precipitation occurs after the addition of ammonium oxalate, less calcium is present. Tissue should be kept for a further period of time to precede decalcification.

If the fluid remains clear for 30 minutes, it is safe to assume that decalcification is complete.

APPENDIX II

Aminoalkylsilane treatment of slides

Clean slides by leaving them in 10% Extran MA 01(Merck Darmstadt, Germany) overnight.

1. Wash in hot running water for minimum of 2 hours
2. Dry at 60°C in an oven overnight and cool to room temperature.
3. Immerse slides for one minute in a freshly prepared 2% solution of 3-aminopropyltriethoxysilane (Sigma Code A 3648) in dry acetone. The capacity of this solution is sufficient for the preparation of 200-250 slides.
4. Give slides two short washes in dry acetone.
5. Give slides short wash in distilled water.
6. Dry at 42°C overnight.
7. Store slides at room temperature.

APPENDIX III

Haematoxylin and eosin staining solutions:

(A) Acid Haemalum (Modified Mayer's)

Haematoxylin	1.00 gm
Sodium Iodate	0.20 gm
Potassium Alum	50.00 gm
Citric Acid	1.00 gm
Chloral Hydrate	50.00 gm
Distilled water	1.000 litre

Method

1. Allow haematoxylin, alum and sodium iodate to dissolve overnight.
2. Add chloral hydrate and citric acid and bring to the boil.
3. Continue boiling for 5 minutes.
4. Allow solution to cool down at bench side.

(B) Stock eosin

Eosin	8.00 gm
Erythrosine	2.00 gm
Distilled water	1000 ml

(C) Eosin working solution

Stock eosin	250.00 ml
Distilled water	750.00 ml
Calcium chloride	20.00 gm

APPENDIX IV

Phosphate Buffered Saline (PBS) and 0.1% Tween -20

(1) Sodium chloride 16.00 gm

(2) Potassium chloride 0.40 gm

(3) Disodium hydrogen phosphate 2.88 gm

(4) Potassium dihydrogen ortho phosphate 0.48 gm

(5) Distilled water 1600 ml

Method:

(1) Put all four reagents in distilled water together

(2) Mix well on a stir plate

(1) Adjust the pH to 7.4 with hydrochloric acid.

(2) Make up to 2.0 litres with distilled water.

(3) Add 2ml of Tween-20 and shake well.

APPENDIX V

ImmunoCruz™ staining system (Cat #sc-2051)

Reagents in ImmunoCruz™ staining system

Negative control (normal rabbit IgG)	15 ml
Peroxidase block	15 ml
Serum block (5% normal goat serum)	15 ml
Biotinylated secondary antibody	15 ml
HRP-strept-avidin reagent	15 ml
50X peroxidase substrate	5 ml
50X DAB chromogen	5 ml
10X substrate buffer	5 ml

APPENDIX VI

10 mM sodium citrate buffer

(4) Add 2.1 gm of citric acid in 1.0 litre of distilled water.

(5) Adjust pH to 6.0 with 2 M sodium hydroxide (Na OH).

APPENDIX VII

HRP substrate mixture

Use reagents from ImmunoCruz™ staining system (Cat #sc-2051)

Method:

(1) Place 1.6 ml de-ionized water in the substrate-mixing bottle.

(2) Add 5 drops of the 10X substrate buffer.

(3) Add 1 drop of the 50X DAB chromogen.

(4) Add I drop of the 10X peroxidase substrate.

(5) Mix well on a stir plate.

This mixture should be sufficient for 15 to 20 slides.

REFERENCES

Abraham, J.A., Mergia, A., Whang, J.L., Tumolo, A., Friedman, J., Gospadarowicz, D. & Fiddes, J.C. 1986. Nucleotide sequence of a bovine clone encoding the angiogenic protein, basic fibroblast growth factor. *Science*, vol. 233, pp. 545-548.

Aktas, G. & Kayton, R. 2000. Ultrastructural immunolocalization of basic fibroblast growth factor in fibroblasts and extracellular matrix. *Histochemistry and Cell Biology*, vol.113, no. 3, pp. 227-33.

Amano, O., Yoshitake, Y., Nishikawa, K. & Iseki, S. 1993. Basic fibroblast growth factor in rat salivary glands. *Cell and Tissue Research,* vol. 273, pp. 467-474.

Amaya, E., Stein, P.A., Musci, T.J. & Kirschner, M.W. 1993. FGF signalling in the early specification of mesoderm in Xenopus. *Development,* vol. 118, no. 2, pp. 477-87.

Arese, M., Chen, Y., Florkiewicz, R.Z., Gualandrris, A., Shen, B. & Rifkin, D.B. 1999. Nuclear activities of basic fibroblast growth factor: potentiation of low-serum growth mediated by natural or chimeric nuclear localization signals. *Molecular Biology of the Cell,* vol. 10, pp.1429-1444.

Armelin, H.A. 1973. Pituitary extracts and steroid hormones in the control of 3T3 cell growth. *Proceedings of the National Academy of Sciences of the United States of America,* vol. 70, pp. 2702-2707.

Arnaud, E., Touriol, C., Boutonnet, C., Gensac, M.C., Vagner, S., Prats, H., & Prats, A.C. 1999. A new 34-kilodalton isoform of human fibroblast growth factor 2 is cap dependently synthesized by using a non-AUG start codon and behaves as a survival factor. *Molecular Biology of the Cell,* vol. 19, no.1, pp. 505-514.

Arnim, S.S. 1935. A method of preparation of serial sections of teeth and surrounding structures of the rat. *The Anatomical Record,* vol. 62, pp. 32.

Avery, J.K. 2001. Development of teeth: root and supporting structures. In: *Oral Development and Histology.* Ed. Avery, J. K. 4th Ed. Williams & Wilkins publishers, Baltimore, USA, pp.102-105

Aviezer, D., Levy, E., Safran, M., Svahn, C., Buddecke, E., Schmidt, A., David, G., Vlodavsky, I., & Yayon, A. 1994. Differential structural requirements of heparin and heparan sulfate proteoglycans that promote binding of basic fibroblast growth factor to its receptor. *The Journal of Biological Chemistry,* vol. 269, no. 1, pp.114-121.

Baldin, V., Roman, A.M., Bosc-Bierne, I., Amalric, F., & Bouche, G. 1990. Translocation of bFGF to the nucleus is G1 phase cell cycle specific in bovine aortic endothelial cells. *The EMBO Journal,* vol. 9, no. 5, pp.1511-1517.

Barasch, J. 1999. FGF-2: specific activity in kidney? *Kidney International,* vol. 56, pp. 1156-1157.

Bashkin, P., Doctrow, S., Klagusbrun, M., Svahn, C.M., Folkman, J. & Vlodavsky, I. 1989. Basic fibroblast growth factor binds to subendothelial extracellular matrix and is released by hepaitinase and heparin-like molecules. *Biochemistry,* vol. 28, pp. 1737-1743.

Basilico, C. & Moscatelli, D. 1992. The FGF family of growth factors and oncogenes. *Advances in Cancer Research,* vol. 59, pp. 115-165.

Baum, B.J. & Wright, W.E. 1980. Demonstration of fibronectin as a major extracellular protein of human gingival fibroblasts. *Journal of Dental Research,* vol. 59, pp. 631-637.

Bean, A.J., Elde, R., Cao, Y., Oellig, C., Tamminga, C., Goldstein, M., Pettersson, R.F. & Hokfelt, T. 1991. Expression of acidic and basic fibroblast growth factors in the substantia nigra of rat, monkey, and human. *Proceedings of National Academy of Sciences of the United States of America,* vol. 88, pp. 10237-10241.

Bei, M. & Maas, R. 1998. FGF's and BMP4 induce both Msx-1-independent and Msx-1 dependent signaling pathways in early tooth development. *Development,* vol. 125, pp. 4525-4533.

Bonnet, H., Filhol, O., Truchet, I., Brethenou, P., Cochet, C., Amalric, F. & Bouche, G. 1996. Fibroblast growth factor-2 binds to the regulatory beta subunit of CK2 and directly stimulates CK2 activity toward nucleolin. *The Journal of Biological Chemistry*, vol. 271, no. 40, pp. 24781-24787.

Bouche, G., Gas, N., Prats, H., Baldin, V., Tauber, J.P., Teissie, J. & Amalric, F. 1987. Basic fibroblast growth factor enters the nucleolus and stimulates the transcription of ribosomal genes in ABAE cells undergoing G0-G1 transition. *Proceedings of the National Academy of Sciences of the United States of America,* vol. 84, no. 19, pp. 6770-6774.

Bratthauer, G.L. 1994. Immunocytochemical Methods and Protocols. In: *Methods in Molecular Biology.* Ed. Javois, L.C. Humana Press Inc; Totowa, NJ, vol. 34, pp. 175-193

Burgess, W.H. & Maciag, T. 1989. The heparin-binding fibroblast growth factor (FGF) family of proteins. *Annual Review of Biochemistry,* vol. 58, pp. 575-606.

Cam, Y., Neumann, M.R. & Ruch, J.V. 1990. Immunolocalization of transforming growth factor β1 epitopes in mouse incisors and molars with a demonstration of *in vitro* production of transforming activity. *Archives of Oral Biology,* vol. 35, pp. 813-822.

Cam, Y., Neumann, M. R., Oliver, L., Raulis, D., Janet, T. & Ruch, J. V. 1992. Immunolocalization of acidic and basic fibroblasts growth factors during mouse odontogenesis. *The International Journal of Developmental Biology,* vol. 36, pp. 381-389.

Cancilla, B., Ford-Perriss, M.D. & Bertam, J.F. 1999. Expression and localization of fibroblast growth factors and fibroblast growth factor receptors in the developing rat kidney. *Journal of Nephrology,* vol. 56, pp. 2025-2039.

Cauchi, J., Alcorn, D., Cancilla, B., Key, B., Berka, J., Nurcombe, V., Ryan, G.B. & Bertram J.F. 1996. Light–microscopic immunolocalization of fibroblast growth factor-1 and –2 in adult rat kidney. *Cell and Tissue Research,* vol. 285, pp. 179-187.

Chai, Y., Bringas, P. Jr., Mogharei, A., Shuler, C.F. & Slavkin, H.C. 1998. PDGF-A and PDGFR-alpha regulate tooth formation via autocrine mechanism during mandibular morphogenesis in- vitro. *Developmental Dynamics*, vol. 213, no. 4, pp. 500-511.

Chen, Y., Bei, M., Woo, I., Satokata, I. & Maas, R. 1996. Msx1 controls inductive signaling in mammalian tooth morphogenesis. *Development,* vol. 122, no. 10, pp. 3035-3044.

Cho, M.J. & Garant, P.R. 1988. Ultra structural evidence of directed cell migration during initial cementoblast differentiation in root formation. *The Journal of Periodontal Research,* vol. 3, pp. 268-276.

Choi, J., Ko, M.K. & Kay, E.P. 2000. Subcellular localization of the expressed 18 KDa FGF-2 isoform in corneal endothelial cells. *Molecular Vision [Electronic Resource],* vol. 6, pp. 222-231.

Clarke, M.S., Khakee, R. & McNeil, P.L. 1993. Loss of cytoplasmic basic fibroblast growth factor from physiologically wounded myofibers of normal and dystrophic muscle. *Journal of Cell Science,* vol. 106, pt. 1, pp. 121-133.

Clayden, E.C. 1952. A discussion on the preparation of bone sections by the paraffin wax method with special reference to control of decalcification. *The Journal of Medical Laboratory Technology,* vol. 10, pp. 103.

Coffin, J.D., Florkiewicz, R.Z., Neumann, J., Mort-Hopkins, T., Dorn, G.W., Lightfoot, P., German, R., Howles, P.N., Kier, A. & O'-Toole, B.A. 1995. Abnormal bone growth and selective translational regulation in basic fibroblast growth factor (FGF-2) transgenic mice. *Molecular Biology of Cell,* vol. 6, pp. 1861-1873.

Cohn, S.A. 1956. Development of the molar teeth in the albino mouse. *Acta Anatomica,* vol. 28, pp. 295-310.

Cohn, M.J., Izpisúa-Belmonte, J.C., Abud, H., Heath J.K. & Tickle, C. 1995. Fibroblast growth factors induce additional limb development from the flank of chick embryos. *Cell,* vol. 80, pp. 739-746.

Coulier, P., Pontarotti, P., Roubin, R., Hartung, H., Goldfarb, M. & Birnbaum, D. 1997. Of worms and men: An evolutionary perspective on fibroblast growth factor (FGF) and FGF receptor families. *Journal of Molecular Evolution,* vol. 44, pp. 43-56.

D'Souza, R.N., Happonen, R.P., Ritter, N.M. & Butler, W.T. 1990. Temporal and spatial patterns of transforming growth factor ß1 expression in developing rat molars. *Archives of Oral Biology,* vol. 35, no. 12, pp. 957-965.

Dapson, R.W. 1993. Fixation for the 1990's: a Review of needs and accomplishments. *Biotechnic and Histochemistry,* vol. 68, no. 2, pp. 75-82.

Dassule, H.R., Lewis, P., Bei, M., Maas, R. & McMahon, A.P. 2000. Sonic hedgehog regulates growth and morphogenesis of the tooth. *Development,* vol. 127, no. 22, pp. 4775-4785.

Debiais, F., Hott, A., Graulet, M. & Marie P.J. 1998. The effects of fibroblast growth factor-2 on human neonatal calvaria osteoblastic cells are differentiation stage specific. *Journal of Bone and Mineral Research,* vol. 13, pp. 645-654.

Delrieu, I. 2000. The high molecular weight isoforms of basic fibroblast growth factor (FGF-2): an insight into an intracrine mechanism. *FEBS Letters,* vol. 468, no. 1, pp. 6-10.

Dickson, C. & Peter, G. 1987. Potential oncogens product related to growth factors (letter). *Nature,* vol. 326, pp. 833.

Dionne, C.A., Crumley, G., Bellot, F., Kaplow, J.M., Searfoss, G., Ruta, M., Burgess, W.H., Jaye, M & Schlessinger, J. 1990. Cloning and expression of two distinct high-affinity receptors cross –reacting with acidic and basic fibroblast growth factors. *The EMBO Journal,* vol. 2, pp. 2685-2692.

Dono, R. & Zeller, R. 1994. Cell-type-specific nuclear translocation of fibroblast growth factor-2 isoforms during chicken kidney and lung morphogenesis. *Developmental Biology,* vol. 163, pp. 316-330.

Du-cros, D.L., Isaacs, K. and Moore, G.P.M. 1993. Distribution of acidic and basic fibroblast growth factors (FGF) in ovine skin during follicle morphogenesis. *Journal of Cell Science,* vol. 105, pp. 667-674.

Fannon, M. & Nugent, M.A. 1996. Basic fibroblast growth factor binds its receptors, is internalized, and stimulates DNA synthesis in Balb/c3T3 cells in the absence of heparan sulfate. *The Journal of Biological Chemistry,* vol. 271, pp. 17949-17956.

Feldman, B., Poueymirou, W., Popaioannou, V.E., Dchaiara, T.M. & Goldher, M. 1995. Requirement of FGF-4 for postimplantation mouse development. *Science,* vol. 267, pp. 246-249.

Finch, P.W., Rubin, J.S., Miki, T., Ron, D. & Aaronson, S.A. 1989. Human KGF is FGF-related with properties of a paracrine effector of epithelial cell growth. *Science,* vol. 245, pp. 75-755.

Finch, P.W., Cunha, G.R., Rubin, J.S., Wong, J. & Ron, D. 1995. Patterns of Keratinocyte growth factor and Keratinocyte growth factor receptor expression during mouse fetal development suggest a role in mediating morphogenetic mesenchymal-epithelial interactions. *Developmental Dynamics,* vol. 203, pp. 223-240.

Floege, J., Hudkins, K.L., Eitner, F., Cui, Y., Morrison, R.S., Schelling, M.A. & Alpers, C.E. 1999. Localization of fibroblast growth factor-2 (basic FGF) and FGF receptor-1 in adult human kidney. *Kidney International,* vol. 56, pp. 883-897.

Ford, M.D., Cauchi, J., Greferath, U. & Bertram, J.F. 1997. Expression of fibroblast growth factors (FGF) and their receptors in rat glomeruli. *Kidney International,* vol. 51, pp. 1729-1738.

Frenz, D. A., Liu, W., Williams, J.D., Hatcher, V., Galinovic-Schwartz, V., Flanders, K.C. & Van De Water, T.R. 1994. Induction of chondrogenesis requirement for synergistic interaction of basic fibroblast growth factor and transforming growth factor-beta. *Development,* vol. 120, pp. 415-424.

Fujimura, K., Bessho, K., Okubo, Y., Kusumoto, K., Segami, N. & Iizuka T. 2002. The effect of fibroblast growth factor-2 on the osteoinductive activity of recombinant human bone morphogenetic protein-2 in rat muscle. *Archives of Oral Biology,* vol. 47, pp. 577-584.

Gao, J., Jordan, T.W. & Cutress, T.W. 1996. Immunolocalization of basic fibroblast growth factor (bFGF) in human periodontal ligament (PDL) tissue. *Journal of Periodontal Research,* vol. 31, pp. 260-264.

Gospodarowicz, D. 1975. Purification of a fibroblast growth factor from bovine pituitary. *The Journal of Biological Chemistry,* vol. 250, pp. 2515-2520.

Gritli-Linde, A., Bei, M., Mass, R., Zhang, X.M., Linde, A. & McMahon, A.P. 2002. Shh signaling within the dental epithelium is necessary for cell proliferation, growth and polarization. *Development,* vol. 129, no. 23, pp. 5323-5337.

Grobstein, C. 1953. Morphogenetic interactions between embryonic tissues separated by a membrane filter. *Nature,* vol. 172, pp. 869-871.

Grothe, C. & Unsicker, K. 1990. Immunocytochemical mapping of basic fibroblast growth factor in the developing and adult rat adrenal gland. *Histochemistry,* vol. 94, no. 2, pp. 141-147.

Guillonneau, X., Régnier-Ricard, F., Laplace, O., Jonet, L., Bryckaertt, M., Courtois, Y. & Mascarelli, F. 1998. Fibroblast growth factor (FGF) soluble receptor 1 acts as a natural inhibitor of FGF-2 neurotropic activity during retinal degeneration. *Molecular and Cellular Biology,* vol. 9, pp. 2785-2802.

Hanada, K., Dennis, J.E. & Caplan, A.I. 1997. Stimulatory effect of basic fibroblast growth factor and bone morphogenetic protein-2 on osteogenic differentiation of rat bone marrow-derived mesenchymal stem cells. *Journal of Bone and Mineral Research,* vol. 12, pp. 1606-1614.

Hauschka, A.E., Mavrakos, M.D., Iafrati, S.E., Doleman, S.E. & Klagusbrun, M. 1986. Growth factors in bone matrix: Isolation of multiple types by affinity chromatography on heparin-sephrose. *The Journal of Biological Chemistry,* vol. 261, pp. 12665-12674.

Heikinheimo, K., Bègue-Kirn, C., Ritvos, O., Tuuri, T. & Ruch, JV. 1997. The activin-binding protein follistatin is expressed in developing murine molar and induces odontoblast-like cell differentiation in vitro. *Journal of Dental Research,* vol. 76, no. 10, pp. 1625-1636.

Hogan, B.L.M. 1999. Morphogenesis. *Cell,* vol. 96, pp. 225-233.

Hsu, S.M., Raini, L. & Fanger, H. 1981. Use of avidin-biotin-peroxidase complex (ABC) and unlabelled antibody (PAP) procedures. *The Journal of Histochemistry and Cytochemistry,* vol. 29, pp. 577-580.

http://honeybee.helsinki.fi/toothexp: Gene expression in tooth. Edited by Nieminen, P. 2001. Last edited: 25-01-2001. (Accessed 19-08-2001)

Hurley, M.M., Aberu, C., Harrison, J.R., Lichtler, A.C., Raisz, L.G. & Kream, B.E. 1993. Basic fibroblast growth factor inhibits type I collagen gene expression in osteoblastic MC3T3-E1 cells. *The Journal of Biological Chemistry,* vol. 268, pp. 5588-5593.

Jackson, A., Fridman, S., Zhan, X., Engleka, K.A., Forough, R. & Maciag, T. 1992. Heat shock induces the release of fibroblast growth factor-1 (FGF-1) from NIH3T3 cells. *Proceedings of National Academy of Sciences of the United States of America,* vol. 89, pp. 10691-10695.

Jernvall, J. & Thesleff, I. 2000. Reiterative signaling and pattering during mammalian tooth morphogenesis. *Mechanisms of Developement,* vol. 92, pp. 19-29.

Kan, M., Wu, X., Wang, F. & McKeehan, W.L. 1999. Specificity for fibroblast growth factors determined by heparan sulfate in a binary complex with the receptor kinase. *The Journal of Biological Chemistry,* vol. 274, no. 22, pp. 15947-15952.

Kanda, S., Hodgkin, M.N., Woodfield, R.J., Wakelam, M.J., Thomas, G. & Claesson-Welsh, L. 1997. Phosphatidylinositol 3'-kinase-independent p70 S6 kinase activation by fibroblast growth factor receptor-1 is important for proliferation but not differentiation of endothelial cells. *The Journal of Biological Chemistry,* vol. 272, no. 37, pp. 23347-23353.

Karabagli, H., Karabagli, P., Ladher, R.K. & Schoenwolf, G.C. 2002. Survey of fibroblast growth factor expression during chick organogesis. *The Anatomical Record,* vol. 268, pp. 1-6.

Karavanova, I., Vainio, S. & Thesleff, I. 1992. Transient and recurrent expression of the Egr-1 gene in epithelial ad mesenchymal cells during tooth morphogenesis suggests involvement in tissue interactions and in determination of cell fate. *Mechanisms of Development,* **vol**. 39, pp. 41-50.

Kato, T., Kawaguchi, H., Hanada, K., Aoyama, I., Hiyama, Y., Nakamura, T., Kuzutani, K., Tamura, M., Kurokawa, T. & Nakamura, K. 1998. Single local injection of recombinant fibroblast growth factor-2 stimulates healing of segmental bone defects in rabbits. *Journal of Orthopedic Research,* vol. 16, pp. 654-659.

Kawaguchi, H., Nakamura, K., Tabata, Y., Ikada, Y., Aoyama, I., Anzai, J., Nakamura, T., Hiyama, Y. & Tamura, M. 2001. Acceleration of fracture healing in nonhuman primates by fibroblast growth factor-2. *The Journal of Clinical Endocrinology and Metabolism,* vol. 86, no. 2, pp. 875-880.

Kettunen, P. & Thesleff, I. 1998. Expression and function of FGFs-4, -8, and –9 suggest functional redundancy and repetitive use as epithelial signals during tooth morphogenesis. *Developmental Dynamics,* vol. 211, no. 3, pp. 256-268.

Kettunen, P., Karavanova, I. & Thesleff, I. 1998. Responsiveness of developing dental tissues to fibroblast growth factors: expression of splicing alternatives of FGFR1, -2, -3, and of FGFR4; and stimulation of cell proliferation by FGF-2, -4, -8, and -9. *Developmental Genetics,* vol. 22, no. 4, pp. 374-85.

Kettunen, P., Laurikkala, J., It ranta, P., Vaino, A. & Itoh, N. 2000. Associations of FGF-3 and FGF-10 with signalling networks regulating tooth morphogenesis. *Developmental Dynamics,* vol. 219, pp. 322-332.

Kimelman, D., Abraham, J.A., Haaparanta, T., Palisi, T.M. & Kirschner, M.W. 1988. The presence of fibroblast growth factor in the frog egg: its role as a natural mesoderm inducer. *Science,* vol. 18, pp. 1053-1056.

Klagusbrun, M., Smith, S., Sullivan, R., Shing, Y., Davidson, S., Smith, J. A. & Sasse, J. 1987. Multiple form of basic fibroblast growth factor: amino-terminal cleavages by tumor cells- and brain cell-derived acid proteinases. *Proceedings of National Academy of Sciences of United States of America,* vol. 84, pp. 1839-1843.

Klint, P. & Claeson-Welsh, L. 1999. Signal transduction by fibroblast growth factor receptors. *Frontiers in Bioscience: A Journal and Virtual Library,* vol. 4, pp. 165-177.

Kollar, E.J. & Baird, G.R. 1970. Tissue reactions in embryonic mouse tooth germs II. The inductive role of the dental papilla. *Journal of Embryology and Experimental Morphology,* vol. 24, pp. 173-186.

Kouhara, H., Hadari, Y.R., Spivak-Kroizman, T., Schilling, J., Bar-Sagi, D., Lax, I. & Schlessinger, J. 1997. A lipid-anchored Grb2-binding protein that links FGF-receptor activation to the Ras/MAPK signaling pathway. *Cell,* vol. 89, no. 5, pp. 693-702.

Kuhn, H.G., Winkler, J., Kempermann, G., Thal, L.J. & Gage, F.H. 1997. Epidermal growth factor and fibroblast growth factor-2 have different effects on neural progenitors in the adult rat brain. *The Journal of Neuroscience,* vol. 17, no. 15, pp. 5820-5829.

Kuzis, K., Reed, S., Cherry, N.J., Woodward, W.R. & Eckenstein, F.P. 1996. Developmental time course of acidic and basic fibroblast growth factors' (FGF) expression in distinct cellular populations of the rat central nervous system. *The Journal of Neuroscience,* vol. 358, pp. 142-153.

LaBonne, C. & Whitman, M. 1994. Mesoderm induction by activin requires FGF-mediated intracellular signals. *Development,* vol. 120, no. 2, pp. 463-472.

Lee, P.L., Johnson, D., Cousens, L., Fried, A. & Williams, L.T. 1989. Purification and complementary DNA cloning of a receptor for basic fibroblast growth factor. *Science,* vol. 245, pp. 57-60.

Lesot, H., Fausser, J.L., Ariyana, S.C., Staub, A., Black, D., Kubler, M.D. & Ruch, J. V. 1992. The carboxy-terminal extension of the collagen binding domain of fibronectin mediates interactions with a 165 KDa membrane protein involved in odontoblasts differentiation. *Differentiation,* vol. 49, pp. 109-118.

Liang, H., Pun, S. & Wronski, T.J. 1999. Bone anabolic effects of basic fibroblast growth factor in ovariectomized rats. *Endocrinology,* vol. 140, no. 12, pp. 5780-5788.

MacKenzie, A., Ferguson, M.W. & Sharpe, P.T. 1992. Expression patterns of the homeobox gene, Hox-8; in the mouse embryo suggest a role in specifying tooth initiation and shape. *Development,* vol. 115, no. 2, pp. 403-420.

Mansukhani, A., Bellosta, P., Sahni, M. & Basilico, C. 2000. Signalling by fibroblast growth factors (FGFs) and fibroblast growth factor receptor-2 (FGFR-2) - activating mutations blocks mineralization and induces apoptosis in osteoblasts. *The Journal of Cell Biology,* vol. 149, no. 6, pp. 1297-1308.

Martin, I., Muraglia, A., Campanile, G., Cancedda, R. & Quarto, R. 1997. Fibroblast growth factor-2 supports ex vivo expansion and maintenance of osteogenic precursors from human bone marrow. *Endocrinology,* vol. 138, no. 10, pp. 4456-4462.

Martin, A., Unda, F.J., Bègue-Kirn, C., Ruch, J.V. & Arechaga, J. 1998. Effects of aFGF, bFGF, TGFbeta1 and IGF-I on odontoblast differentiation in vitro. *European Journal of Oral Sciences,* vol. 106, suppl 1, pp. 117-121.

Masahiro, A., Atsuko, Y. & Toru, I. 2001. Engineering of Heparin-binding growth factor with heparan sulfate sugar chains. *Trends in Glycoscience and Glycotechnology: TIGG,* vol. 13, no. 72, pp. 385-394.

Mason, I.J. 1994. The ins and outs of fibroblast growth factor. *Cell*, vol. 78, pp. 547-552.

Matsuda, N., Lin, W.L., Kumar, N.M., Cho, M.I. & Genco, R.J. 1992. Mitogenic, chemotactic, and synthetic response of rat periodontal ligament fibroblast cells to polypeptide growth factors *in vitro*. *The Journal of Periodontology,* vol. 63, pp. 515-525.

McCarthy, T.L., Centrella, M. & Canalis, E. 1989. Effects of fibroblast growth factors on deoxyribonucleic acid and collagen synthesis in rat parietal bone cells. *Endocrinology,* vol. 125, pp. 2118-2126.

McFarlane, S., Zuber, M.E. & Holt, C.E. 1998. A role for the fibroblast growth factor receptor in cell fate decisions in the developing vertebrate retina. *Development,* vol. 125, no. 20, pp. 3967-3975.

McIntosh, I., Bellus, G.A. & Jabs, E.W. 2000. The Pleotropic Effects of Fibroblast Growth Factor Receptors in Mammalian Development. *Cell Structure and Function*. Vol. 25, no. 2, pp. 85-96.

McNeil, P.L., Muthukrishnan, L., Warder, E. & D'Amore, P.A. 1989. Growth factors are released by mechanically wounded endothelial cells. *The Journal of Cell Biology,* vol. 109, pp. 811-822.

Mignatti, P., Morimoto, T. & Rifkin, D.B. 1991. Basic fibroblast growth factor released by single, isolated cells stimulates their migration in an autocrine manner. *Proceeding of National Academy of Sciences of United States of America,* vol. 88, no. 24, pp. 11007-11011.

Miki, T., Fleming, T.P., Crescenzi, M., Molloy, C.J., Blam, S.B., Reynolds, S.H. & Aaronson, S.A. 1991. Development of a highly efficient expression cDNA cloning system: application to oncogene isolation. *Proceedings of National Academy of Sciences of United States of America,* vol. 88, no. 12, pp. 5167-5171.

Mina, M. & Kollar, E.J. 1987. The induction of odontogenesis in non-dental mesenchyme combined with early murine mandibular arch epithelium. *Archives of Oral Biology,* vol. 32, pp. 123-127.

Miyamoto, M., Nauro, K., Seko, C., Matusmoto, S., Kondo, T. & Kurokawa, T. 1993. Molecular cloning of a novel cytokine cDNA encoding the ninth member of the fibroblast growth factor family, which has a unique secretion property. *Molecular and Cellular Biology,* vol. 13, pp. 4251-4259.

Mohammed, S., Pack, A.R.C. & Kardos, T.B. 1998. The effect of transforming growth factor beta one (TGF-ß 1) on wound healing, with or without barrier membrane, in a class II furcation defects in sheep. *Journal of Periodontal Research,* vol. 33, pp. 335-344.

Montero, A., Okada, Y., Tomita, M., Ito, M., Tsurukami, H., Nakamura, T., Doetschman, T., Coffin, J.D. & Hurley, M.M. 2000. Disruption of the fibroblast growth factor-2 gene results in decreased bone mass and bone formation. *The Journal of Clinical Investigation,* vol. 105, no. 8, pp. 1085-1093.

Moy, F.J., Seddon, A.P., Bolhen, P. & Powers, R. 1996. High resolution structure of basic fibroblast growth factor determined by multidimensional hetronuclear magnetic resonance microscopy. *Biochemistry,* vol. 35, pp. 13552-13561.

Murakami, S., Takayama, S., Shimabukuro, Y., Kitamura, M. & Nozaki, T. 1999. Regeneration of periodontal tissues by basic fibroblast growth factor. *Journal of Periodontal Research,* vol. 34, pp. 425-430.

Murakami, S., Takayama, S., Kitamura, M., Shimabukuro, Y., Ikezawa, K., Nozaki, T. & Okada, H. 1999. Recombinant human basic fibroblast growth factor (bFGF) stimulates periodontal regeneration in class II furcation defects created in beagle dogs. *Journal of Periodontal Research,* vol. 38, pp. 97-103.

Nakamura, T., Hara, Y., Tagawa, M., Tamura, M., Yuge, T., Fukuda, H. & Nigi H. 1998. Recombinant human basic fibroblast growth factor accelerates fracture healing by enhancing callus remodeling in experimental dog tibial fracture. *Journal of Bone and Mineral Research,* vol. 13, pp. 942-949.

Neubüser, A., Peters, H., Balling, R. & Martin, G.R. 1997. Antagonistic interactions between FGF and BMPs signaling pathways: a mechanism for positioning the sites of tooth information. *Cell,* vol. 90, pp. 247-255.

Niswander, L. & Martin, G.R. 1992. FGF-4 expression during gastrulation, myogenesis, limb and tooth development in the mouse. *Development,* vol. 114, pp. 755-768.

Nugent, M.A. & Iozzo R.V. 2000. Fibroblast growth factor-2. *Immunology and Cell Biology,* vol. 32, no. 2, pp. 115-120.

Ogawa, Y., Schmidt. J.R., Dasch, J.R., Chang, R.J. & Glaser, C.B. 1992. Purification and characterization of transforming growth factor-beta 2.3 and –beta 1, 2 heterodimers from bovine bone. *The Journal of Biological Chemistry,* vol. 267, pp. 2325-2328.

Ohbayashi, N., Hoshikawa, M., Kimura, S., Yamasaki, M., Fukui S. & Itoh, N. 1998. *The Journal of Biological Chemistry,* vol. 273, pp. 18161-18164.

Ohtani, H., Nakamura, S., Watanabe, Y., Mizoi, T., Saku, T. & Nagura, H. 1993. Immunocytochemical localization of basic fibroblast growth factor in carcinomas and inflammatory lesions of the human digestive tract. *Laboratory Investigations; A Journal of Technical Methods and Pathology,* vol. 68, no. 5, pp. 520-527.

Okada-Ban, M., Theiry, J.P. & Jouanneau, J. 2000. Fibroblast growth factor-2. *Immunology and Cell Biology,* vol. 32, no. 3, pp. 263-267.

Ornitz, D.M. 2000. FGFs, heparan sulfate and FGFRs: complex interactions essential for development. *BioEssays: News and Reviews in Molecular, Cellular and Developmental Biology,* vol. 22, no. 2, pp. 108-112.

Ornitz, D.M., Xu, J., Colvin, J.S., McEwen, D.G., MacAurther, C.A., Courlier, F., Gao, G. & Goldfarb, M. 1996. Receptor specificity of the fibroblast growth factor family. *The Journal of Biological Chemistry,* vol. 271, pp. 15292-15297.

Ornitz, D.M. & Itoh, N. 2001. Fibroblast growth factors. *Genome Biology,* vol. 2, no. 3, pp. 3005.1-3005.12.

Orr-Urtreger, A., Givol, D., Yayon, A., Yarden, Y. & Lonai ,P. 1991. Developmental expression of two murine fibroblast growth factor receptors, flg and bek. *Development,* vol. 113, no. 4, pp. 1419-1434.

Paria, B.C., Ma, W., Tan, J., Raja, S., Das, S.K., Dey, S.K. & Hogan, B.L.M. 2001. Cellular and molecular responses of the uterus to embryo implantation can be elicited by locally applied growth factors. *Proceedings of National Academy of Sciences of United States of America,* vol. 98, no. 3, pp. 1047-1052.

Pasumarthi, K.B., Kardami, E. & Cattini, P.A. 1996. High and low molecular weight fibroblast growth factor-2 increase proliferation of neonatal rat cardiac myocytes but have differential effects on binucleation and nuclear morphology. Evidence for both paracrine

and intracrine actions of fibroblast growth factor-2. *Circulation Research,* vol. 78, no. 1, pp. 126-36.

Patel, A. & McFarlane, S. 2000. Overexpression of FGF-2 alters cell fate specification in the developing retina of *Xenopus laveis*. *Developmental Biology,* vol. 222, pp. 170-180.

Peters, H. & Balling, R. 1999. Teeth: Where and how to make them. *Trends in Genetics,* vol. 15, no. 2, pp. 59-65.

Peters, K. G., Werner, S., Chen, G. & Williams, L.T. 1992. Two FGF receptor genes are differentially expressed in epithelial and mesenchymal tissues during limb formation and organogenesis in the mouse. *Development,* vol. 114, pp. 233-243.

Pispa, J., Jung, H-S., Jernvall, J., Kettunen, P., Mustonen, T., Tabata, M.J., Kere, J. & Thesleff, I. 1999. Cusp patterning defects in Tabby mouse teeth and its partial rescue by FGF. *Developmental Biology,* vol. 216, pp. 521-534.

Pitaru, S., Hekmati, M., Geiger, S. & Savion, N. 1988. The effects of partial demineralization and fibronectin on migration and growth of gingival epithelial cells on cementum in vitro. *Journal of Dental Research,* vol. 67, no. 11, pp. 1386-1391.

Poole, T.J., Finkelstein, E.B. and Cox, C.M. 2001. The role of FGF and VEGF in angioblast induction and migration during vascular development. *Developmental Dynamics,* vol. 220, pp. 1-17.

Rapraeger, A.C. 2000. Syndecan-regulated receptor Signaling. *The Journal of Cell Biology,* vol. 149, no. 5, pp. 995-998.

Reiland, J. & Rapraeger, A.C. 1993. Heparan sulfate proteoglycans and FGF receptor target basic FGF to different intracellular destinations. *Journal of Cell Science,* vol. 105, pt. 4, pp. 1085-1093.

Reilly, J.F. & Maher, P.A. 2001. Importin beta-mediated nuclear import of fibroblast growth factor receptor: role in cell proliferation. *The Journal of Cell Biology,* vol. 152, no. 6, pp. 1307-1312.

Rentrop, M., Knapp, B., Winter, H. & Schweizer, J. 1986. Aminoalkylsilane-treated slides as support for in situ hybridization of keratin cDNAs to frozen tissue sections under varying fixation and pretreatment conditions. *Histochemical Journal* vol. 18, pp. 271-276.

Rifkin, D.B. & Moscatelli, D. 1989. Recent developments in the cell biology of basic fibroblast growth factor. *The Journal of Cell Biology,* vol. 109, no. 1, pp. 1-6.

Roberts-Clark, D.J. & Smith, A.J. 2003. Angiogenic growth factors in human dentine matrix. *The Journal of Biological Chemistry,* vol. 278, no. 1, pp. 479-485.

Rodan, S.B., Wesolowski, G., Yoon, K. & Rodan, G.A. 1989. Opposing effects of fibroblast growth factor and pertussi toxin on alkaline phosphatase, osteopontin, osteocalcin, and type I collagen mRNA levels in ROS 17/2.8 cells. *The Journal of Biological Chemistry,* vol. 264, pp. 19934-19941.

Roghani, M., Mansukhani, A., Dell' Era, P., Bellosta, P., Bascilico, C., Rifkin, D.B. & Moscatelli, D. 1994. Heparin increases the affinity of basic fibroblast growth factor for its receptors but is not required for binding. *The Journal of Biological Chemistry,* vol. 269, pp. 3976-3984.

Ruch, J.V. 1985. Odontoblast differentiation and the formation of the odontoblast layer. *Journal of Dental Research,* vol. 64, pp. 489-498.

Ruch, J.V. 1998. Odontoblast commitment and differentiation. *Biochemistry and Cell Biology,* vol. 76, no. 6, pp. 923-938.

Ruoslahti, E. & Yamaguci, Y. 1991. Proteoglycans as modulators of growth factor activities. *Cell,* vol. 64, pp. 867-869.

Russo, G. L., Maharajan, P. & Maharajan, V. 1997. Basic fibroblast growth factor (FGF-2) in mouse tooth morphogenesis. *Growth Factors (Chur, Switzerland),* vol. 15, pp. 125-133.

Sarkar, L. & Sharpe, P.T. 1999. Expression of Wnt signalling pathway genes during tooth development. *Mechanisms of Development,* vol. 85, no. 1-2, pp. 197-200.

Sarkar, L., Cobourne, M., Naylor, S., Smalley, M., Dale, T. & Sharpe, P.T. 2000. Wnt/Shh interactions regulate ectodermal boundary formation during mammalian tooth development. *Proceedings of National Academy of Sciences of United States of America,* vol. 97, no. 9, pp. 4520-4524.

Sato, Y. & Rifkin, D.B. 1988. Autocrine activities of basic fibroblast growth factor (FGF), regulation of endothelial cell movement, Plasminogen activator synthesis, and DNA synthesis. *The Journal of Cell Biology,* vol. 107, pp. 1129-1205.

Saxen, L., Karkien-Jaaskelainen, M., Nordling, S. & Wartiovaara, J. 1976. Are morphogenetic tissue interaction mediated by transmissible signal sequences or through cell contacts? *Nature,* vol. 259, pp. 662-663.

Sigurdsson, T.J., Lee, M.B., Kubota, K., Turok, T.J., Wozney, J.M. & Wikesjö, U.M.E. 1995. Periodontal repair in dogs: recombinant human bone morphogenetic protein-2 significantly enhances periodontal regeneration. *The Journal of Periodontology,* vol. 66, pp. 131-138.

Slavkin, H.C. & Bringas, P. Jr. 1976. Epithelial-mesenchyme interactions during odontogenesis. Morphological evidence for direct heterotypic cell-cell contacts. *Developmental Biology,* vol. 50, no. 2, pp. 428-442.

Smallwood, P.M., Munoz-Sanjuan, I., Tong, P., Macke, J.P., Hendry, S.H., Gilbert, D.J., Copeland, N.G., Jenkins, N.A. & Nathans, J. 1996. Fibroblast growth factor (FGF) homologous factors: New members of the FGF family implicated in nervous system development. *Proceedings of the National Academy of Sciences of the United States of America,* vol. 93, pp. 9850-9857.

Springer, B.A., Pantoliano, M.W., Barbera, F.A., Gunyuzlu, P.L., Thompson, L.D., Herblin, W.F., Rosenfeld, S.A. & Book, G.W. 1994. Identification and concerted function of two receptor binding surfaces on basic fibroblast growth factor required for mitogenesis. *The Journal of Biological Chemistry,* vol. 269, no. 43, pp. 26879-26884.

Stachowiak, M.K., Moffett, J., Joy, A., Puchacz, E., Florkiewicz, R. & Stachowiak, E.K. 1994. Regulation of bFGF gene expression and subcellular distribution of bFGF protein in adrenal medullary cells. *The Journal of Cell Biology,* vol. 127, no. 1, pp. 203-223.

Stern, H.M., Lin-Jones, J. & Hauschka, S.D. 1997. Synergistic interactions between bFGF and a TGF-β family member may mediate myogenic signals from the neural tube. *Development*, vol. 124, pp. 3511-3523.

Steven, A; & Wilson, I. 1996. The haematoxylin and eosin. In: *The Theory and Practice of Histological Techniques.* Eds. Bancroft, J. D; & Stevens, A. 4th ed. Churchill Livingstone, pp. 99-112.

Stevens, A., Lowe, J. & Bancroft, J.D. 1996. Bone. In: *The Theory and Practice of Histological Techniques.* Eds. Bancroft, J. D. & Stevens, A. 4th Ed. Churchill Livingstone, pp. 309-339.

Sutherland, D., Samakovlis, C. & Krasnow, M.A. 1996. Branchless encodes a drosophila FGF homolog that controls tracheal cell migration and the patterns of branching. *Cell,* vol. 87, pp. 1091-1101.

Takano-Yamamoto, T., Takemura, T., Kitamura, Y. & Nomura, S. 1994. Site-specific expression of mRNAs for osteonectin, osteocalcin, and Osteopontin revealed by in situ hybridization in rat periodontal ligament during physiological tooth movement. *The Journal of Histochemistry and Cytochemistry,* vol. 42, pp. 885-896.

Takayama, S., Murakami, S., Miki, Y., Ikezawa, K., Takasa, S., Terashima, A., Asano, T. & Okada, H. 1997. Effects of basic fibroblast growth factor on human periodontal ligament cells. *Journal of Periodontal Research,* vol. 32, pp. 667-675.

Tanaka, A., Miyamoto, K., Minamino, N., Takeda, M., Sato, B., Matsuo, H. & Matusmoto, K. 1992. Cloning and characterization of an androgen-induced growth factor essential for the androgen-dependent growth of mouse mammary carcinoma cells. *Proceedings of the National Academy of Sciences of the United States of America,* vol. 89, pp. 8928-8932.

Ten Cate, A.R. 1998. Development of the tooth and its supporting tissues. In: *Oral Histology: Development, Structure, and Function.* Ed. Ten Cate, A.R. 5th ed. St Louis: Mosby publishers, USA, pp. 78-104.

Terranova, V.P. & Wikesjö, U.M.E. 1987. Extracellular matrices and polypeptide growth factors as mediators of functions of cells of the periodontium. *Journal of Periodontology,* vol. 6, pp. 371-380.

Tessler, S. & Neufeld, G. 1990. Basic fibroblast growth factor accumulates in the nuclei of various b-FGF producing cell types. *Journal of Cellular Physiology,* vol. 145, pp. 310-317.

Thesleff, I. 2000. Genetic basis of tooth development and dental defects. *Acta Odontologica Scandinavica,* vol. 58, pp. 191-194.

Thesleff, I., Vaahtokari, A., & Partanen, A.M. 1995. Regulation of organogenesis: Common molecular mechanisms regulating the development of teeth and other organs. *The International Journal of Developmental Biology,* vol. 39, pp. 35-50.

Thesleff, I. & Nieminen, P. 1996. Tooth morphogenesis and cell differentiation. *Current Opinion in Cell Biology,* vol. 8, pp. 844-850.

Thesleff, I., Vaahtokari, A., Vainio, S. & Jowett, A. 1996. Molecular mechanisms of cell and tissue interactions during early tooth development. *The Anatomical Record,* vol. 245, pp. 151-161.

Thesleff, I. & Sharpe, P. 1997. Signalling networks regulating dental development. *Mechanisms of Development,* vol. 67, pp. 111-123.

Thomadakis, G., Ramoshebi, L.N., Crooks, J., Rueger, D.C. & Ripamonti, U. 1999. Immunolocalization of bone morphogenetic protein-2 and -3 and osteogenic Protein-1 during murine tooth root morphogenesis and in other craniofacial structures. *European Journal of Oral Sciences,* vol. 107, no. 5, pp. 368-377.

Thomas, B.L., Tucker, A.S., Qiu, M., Ferguson, C.A., Hardcastle, Z., Rubenstein, J.L.R. & Sharp, P.T. 1997. Role of Dix-1 and Dix-2 genes in pattering of the murine dentition. *Development,* vol. 124, pp. 4811-4818.

Tucker, R.P., Hammarback, J.A., Jenrath, D.A., Mackie, E.J. & Xu, Y. 1993. Tenascin expression in the mouse: in situ localization and induction in vitro by bFGF. *Journal of Cell Science,* vol. 104, pt. 1, pp. 69-76.

Tziafas, D., Alvanou, A. & Kidaglou, K. 1992. Dentinogenic activity of allogenic plasma fibronectin on dog dental pulp. *Journal of Dental Research,* vol. 71, pp. 1189-1195.

Unda, F. J., Martin, A., Hilario, E., Bègue-Kirn, C., Ruch, J. V. & Arèchaga, J. 2000. Dissection of the odontoblast differentiation process In Vitro by a combination of FGF1, FGF2, and TGFß1. *Developmental Dynamics,* vol. 218, pp. 480-489.

Unda, F.J., Martin, A., Hernandez, C., Perez-Nanclares, G., Hilario, E. & Arechaga, J. 2001. FGFs-1 and -2, and TGF beta 1 as inductive signals modulating in vitro odontoblast differentiation. *Advances in Dental Research,* vol. 15, pp. 34-37

Vaahtokari, A., Vainio, S. & Thesleff I. 1991. Association between transforming growth factor β1, RNA expression and epithelial-mesenchymal interactions during tooth morphogenesis. *Development,* vol. 113, pp. 985-994.

Vaahtokari, A., Aberg, T. & Thesleff, I. 1996. Apoptosis in the developing tooth: association with an embryonic signaling center and suppression by EGF and FGF-4. *Development,* vol. 122, pp. 121-129.

Vainio, S., Karavanova, I., Jowett, A. & Thesleff, I. 1993. Identification of BMP-4 as a signal mediating secondary induction between epithelial and mesenchymal tissues during early tooth development. *Cell,* vol. 75, no. 1, pp. 45-58.

Venkataraman, G., Raman, R., Sasisekharan, V. & Sasisekharan, R. 1999. Molecular characteristics of fibroblast growth factor-fibroblast growth factor receptor-heparin-like glycosaminoglycan complex. *Proceedings of the National Academy of Sciences of the United States of America,* vol. 96, pp. 3658-3663.

Wiedlocha, A., Falnes, P.O., Madshus, I.H., Sandvig, K. & Olsnes, S. 1994. Dual mode of signal transduction by externally added acidic fibroblast growth factor. *Cell,* vol. 76, no. 6, pp. 1039-1051.

Wiedlocha, A., Falnes, P.O., Rapak, A., Munoz, R., Klingenberg, O. & Olsnes S. 1996. Stimulation of proliferation of a human osteosarcoma cell line by exogenous acidic fibroblast growth factor requires both activation of receptor tyrosine kinase and growth factor internalization. *Molecular and Cell Biology,* vol. 16, pp. 270-280.

Wilkinson, D.G., Bhatt, S. & McMahon, A.P. 1989. Expression pattern of FGF-related proto-oncogene int-2 suggests multiple roles in foetal development. *Development,* vol. 105, pp. 131-136.

Wolpert, L. 1989. Positional information revisited. *Development,* vol. 107, suppl, pp. 3-12.

Yabu, M., Shinmura, Y., Minami, T. & Matsuzara, Y. 1993. Immunohistochemical localization of basic fibroblastic growth factor in the healing stages of mouse gastric ulcer. *Histochemistry,* vol. 100, pp. 409-413.

Yayon, A., Klagusbrun, M., Esko, J.D., Leder, P. & Ornitz, D.M. 1991. Cell surface, heparin-like molecules are required for binding of basic fibroblast growth factor to its high affinity receptor. *Cell,* vol. 64, no. 4, pp. 841-848.

Yu, Z.X., Biro, S., Fu, Y.M., Sanchez, J., Smale, G., Sasse, J., Ferrans, V.J. & Casscells, W. 1993. Localization of basic fibroblast growth factor in bovine endothelial cells: immunohistochemical and biochemical studies. *Experimental Cell Research,* vol. 204, pp. 247-259.

Zeng, H., Du, J.Y., Zheng, Q.X., Duan, D.Y., Liu, Y., Xiong, A., Kang, B. & Liu, G.P. 2003. Effects of basic fibroblast growth factor on biological characteristics of osteoblasts. *Chinese Journal of Traumatology,* vol. 6, no. 4, pp. 229-33.

Zhan, X., Plourde, C., Hu, X., Friesel, R. & Maciag, T. 1994. Association of fibroblast growth factor receptor-1 with c-Src correlates with association between c-Src and cortactin. *The Journal of Biological Chemistry 12,* vol. 269, no. 32, pp. 20221-20224.

Zhou, Z., Zuber, M.E., Burrus, L.W. & Olwin, B.B. 1997. Identification and characterization of a fibroblast growth factor (FGF) binding domain in the cysteine-rich FGF receptor. *The Journal of Biological Chemistry,* vol. 272, no. 8, pp. 5167-5174.

University
of the Witwatersrand,
Johannesburg

Faculty of Health Sciences

7 York Road, Parktown 2193 South Africa • Fax: +27-11-643 4318 • Telephone:+27-11-717-2000 • Telegrams: 'Witsmed'

11 October 2001 Ref: 0105655K

Professor B Kramer
Department of Anatomical Sciences

Dear Professor Kramer

Approval of change in title for MSc(Med)(research)

Your request to change the title of the MSc(Med)(research) dissertation for Dr Madan has been approved by the Chair of the Postgraduate Committee.

Old title: **'Immunolocalization of bone morphogenetic protein-5 (BMP-5) in the developing root of the murine tooth'**

New title: **'Immunolocalization of fiboblast growth factor-2 (FGF-2) in the developing root of the murine tooth'**

Many thanks for your assistance.

Yours sincerely

Jill Mainwaring

Jill Mainwaring
Postgraduate Officer
Faculty of Health Sciences

cc: Dr Madan

AESC 3

STRICTLY CONFIDENTIAL

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

ANIMAL ETHICS SCREENING COMMITTEE

CLEARANCE CERTIFICATE NO:

2001	01	2A

APPLICANT: Dr Anil Kumar Madan

DEPARTMENT: Anatomical Sciences

PROJECT TITLE: Immunocytochemical Localization Of Bone Morphogenetic Protein 5 (BMP-5) In Murine Tooth Root Morphogenesis

Species	Number	Expiry Date
Mouse Pups	36	23 January 2003

i) Approval is hereby given for the experiment described in the above application.

The use of these animals is subject to AESC Guidelines for the use and care of animals, is limited to the procedures specified in the application form, and to:

Euthanasia Procedure To Be Discussed With The CAS

SIGNED D. A. Gray DATE: 23 January 2001

(Chairman: ~~Animal~~ Ethics Screening Committee)

ii) I am satisfied that the persons listed in this application are competent to perform the procedures therein, in terms of Section 23(1)(c) of the Veterinary and Para-veterinary Professions Act (19 of 1982)

SIGNED _____ DATE: 23 January 2001

(Registered Veterinarian)

NOTE:

First-time users of the CAS should contact the Director of the CAS in order to familiarise themselves with the facilities available, and the procedures required by the CAS for the carrying out of experiments.

Please note that only typewritten applications will be accepted. Should additional space be required for section "i" and/or "j", please use the back of this form.

ANIMAL ETHICS SCREENING COMMITTEE

MODIFICATIONS AND EXTENSIONS TO EXPERIMENTS

a. Name: Dr Anil Kumar Madan

b. Department: Anatomical Sciences

c. Experiment to be modified / extended

AESC NO:		
2001	01	2A

d. Project Title: Immunocytochemical Localization of Bone Morphogenetic Protein 5 (BMP-5) In Murine Tooth Root Morphogenesis

e. Number and species of animals **originally approved**:

36	Mouse Pups

f. Number of additional animals **previously allocated** on M&Es:

0	Nil

g. Total number of animals **allocated** to the experiment to date:

2	MF1 Mice

h. Number of animals **used** to date:

2	MF1 Mice

i. Specific modification / extension requested: 2 Pregnant 18d mice

j. Motivation for modification / extension: E18 mice required as a positive control for BMP-5 antibody. (known to be positive for BMP-5)

Date: 07- MAY 2001 Signature: [signature]

RECOMMENDATIONS:

Approved: 2 pregnant (18d) mice

Date: 8th May 2001 Signature: D. A. Gray

Chairman, AESC

November 2000