GROWTH FACTOR EXPRESSION ASSOCIATED WITH REGULATION OF OLFATORY NEUROGENESIS

By

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Abstract

Olfactory neurons arise from the division of a stem cell in the basal area of the epithelium. After dividing asymmetrically and symmetrically, the stem cell gives rise to many immature olfactory receptor neurons that gradually differentiate into mature neurons as they migrate away from the basement membrane. The neurogenesis in the olfactory epithelium takes place throughout adult life, which makes the olfactory epithelium system a useful model with which to study the mechanisms that direct neural development.

Olfactory neurogenesis is highly regulated for the need of maintaining the equilibrium between the basal cell mitosis, cell death and cell survival in olfactory epithelium, for which many growth factors have been reported to play roles in regulating olfactory neurogenesis. Many reports observed the proliferative role of TGFα and EGF in the olfactory neurogenesis and the expression of their receptors in horizontal basal cells, suggesting their signaling pathways for proliferation were mediated by a common receptor on the horizontal basal cells. FGF2 was reported to induce proliferation in a mouse embryo explant, a basal cell line, and in our laboratory, a basal cell culture. However, the target cells of FGF2 in the olfactory epithelium were not clear at the time of the research. TGFβ-2 was observed to stimulate differentiation in semi-dissociated olfactory tissues, in basal cell cultures and a basal cell line. Some of the receptors for TGFβ growth factors were found to be expressed in the olfactory epithelium but the identity of the target cells of TGFβ growth factors and the cells expressing them remained largely unknown. PDGF was observed in our laboratory to promote survival of immature neurons but there was no evidence to show the existence of its receptors on the immature neurons or to locate its source in the olfactory epithelium.

This project aimed to identify and characterize the cells expressing TGFβ-2, PDGF and FGF growth factors and receptors in the olfactory epithelium using techniques of RT-PCR, immunohistochemistry, and in situ hybridisation. Our results have shown most
members of TGFβ-2 superfamily were expressed in the olfactory epithelium in that TGFβ growth factors 1, 2, 3 and TGFβ receptor type 1, 2 and 3 were expressed extensively in superficially located basal cells, immature and mature neurons. FGF1 was expressed in olfactory epithelium. FGF2 and FGFr-1 were expressed by neurons and presumed globose basal cells. The supporting cells were like to express FGF2 mRNA. PDGF A and PDGF receptor α had similar expression patterns in the olfactory epithelium. In support of previous studies, this project has provided in vivo evidence for the cells expressing the growth factors of importance and for the target cells these growth factors might act on. In addition to these, this project also investigated an unknown gene, 16b5, which was previously found to be upregulated by differentiation of an olfactory cell line, and has provided the in vivo evidence to support the finding.
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<tr>
<td>ADP</td>
<td>Adenosine Diphosphate</td>
</tr>
<tr>
<td>ActR</td>
<td>Activin Receptor</td>
</tr>
<tr>
<td>AMP</td>
<td>Adenosine Monophosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>BDNF</td>
<td>Brain-derived Neurotrophic Factor</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone Morphogenetic Protein</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>cAMP</td>
<td>Adenosine 3',5'-cyclic Monophosphate</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>CDK</td>
<td>Cyclin Dependent Kinase</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>DAB</td>
<td>3,3-diaminobenzidine</td>
</tr>
<tr>
<td>dCTP</td>
<td>Deoxy-Cytidine Triphosphate</td>
</tr>
<tr>
<td>ddATP</td>
<td>Dideoxy-Adenosine Triphosphate</td>
</tr>
<tr>
<td>ddCTP</td>
<td>Dideoxy-Cytidine Triphosphate</td>
</tr>
<tr>
<td>ddGTP</td>
<td>Dideoxy-Guanosine Triphosphate</td>
</tr>
<tr>
<td>ddTTP</td>
<td>Dideoxy-Tyrosine Triphosphate</td>
</tr>
<tr>
<td>dGTP</td>
<td>Deoxy-Guanosine Triphosphate</td>
</tr>
<tr>
<td>dH$_2$O</td>
<td>distilled water</td>
</tr>
<tr>
<td>DEPC</td>
<td>Diethyl Polycarbonate</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>dNTP</td>
<td>Deoxyribonucleotide</td>
</tr>
<tr>
<td>ds cDNA</td>
<td>Double Stranded cDNA</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
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<tr>
<td>dTTP</td>
<td>Deoxy-Tyrosine Triphosphate</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene Diamine Tetra-Acetic Acid</td>
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<td>EGF</td>
<td>Epidermal Growth Factor</td>
</tr>
<tr>
<td>EGFr</td>
<td>Epidermal Growth Factor Receptor</td>
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<td>ethanol</td>
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<td>EtBr</td>
<td>ethidium bromide</td>
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<tr>
<td>Abbreviation</td>
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<tr>
<td>FCS</td>
<td>Fetal Calf Serum</td>
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<td>GAP-43</td>
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<td>GAPDH</td>
<td>Glyceraldehyde-3-Phosphate Dehydrogenase</td>
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<td>GBC</td>
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<td>GDF</td>
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<td>GDNF</td>
<td>Glial-derived Neurotrophic Factor</td>
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<td>GFAP</td>
<td>Glial Fibrillary Acidic Protein</td>
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<tr>
<td>G_{olf}</td>
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<td>GS domain</td>
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<td>GTP</td>
<td>Guanosine Triphosphate</td>
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<tr>
<td>h</td>
<td>hours</td>
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<tr>
<td>HBC</td>
<td>horizontal basal cell</td>
</tr>
<tr>
<td>hrs</td>
<td>hours</td>
</tr>
<tr>
<td>INP</td>
<td>Immediate Neuronal Precursor</td>
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<td>IGF-1</td>
<td>Insulin-like Growth Factor 1</td>
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<td>ISH</td>
<td>In Situ Hybridization</td>
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<tr>
<td>kD</td>
<td>Kilo Dalton</td>
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<td>L</td>
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<tr>
<td>LIF</td>
<td>Leukaemia Inhibitory Factor</td>
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<td>Mammalian Achaete-scute Homolog-1</td>
</tr>
<tr>
<td>Min</td>
<td>minute</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger Ribonucleic Acid</td>
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<tr>
<td>N-CAM</td>
<td>Neural Cell Adhesion Molecule</td>
</tr>
<tr>
<td>NCBI</td>
<td>National Center of Biotechnology Information</td>
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<tr>
<td>NGF</td>
<td>Nerve Growth Factor</td>
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<tr>
<td>NIC</td>
<td>Neuroblast in Culture</td>
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<td>NTP</td>
<td>Ribonucleotide</td>
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<td>OE</td>
<td>olfactory epithelium</td>
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<tr>
<td>OB</td>
<td>olfactory bulb</td>
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OMP  Olfactory Marker Protein
ORF  Open Reading Frame
P27Kip1  CDK-inhibitory Protein
PAGE  PolyAcrylamide Gel Electrophoresis
PBS  Phosphate Buffered Saline
PCR  Polymerase Chain Reaction
PDGF  Platelet-derived Growth factor
PLP  Periodate-Lysine-Paraformaldehyde Fixative
PVP  Polyvinly Pyrolidone
RNA  Ribonucleic Acid
rpm  Revolutions Per Minute
RT  Reverse Transcription
RT-PCR  Reverse Transcriptase Polymerase Chain Reaction
sdH2O  Sterile Deionized Water
SDS  Sodium Dodecyl Sulphate
sec  second
S/P  Streptomycin
SPARC  Secreted Protein Acidic and Rich in Cysteine
SSC  150 mM NaCl, 15 mM sodium citrate, pH 7.2
SUS-1  Monoclonal Antibody to Supporting Cells
TBE  Tris, Borate And EDTA
TBS  Tris-Buffered Saline
TCA  Trichloroacetic Acid
TE  Tris and EDTA
TEA  Triethanolamine
TGF  Transforming Growth Factor
TGFα  Transforming Growth Factor alpha
TGFβ1  Transforming Growth Factor beta one
TGFβ2  Transforming Growth Factor beta two
TGFβr  Transforming Growth Factor beta receptor
Trk  Tyrosine kinase
Tris  Tris (hydroxymethyl) aminomethane
TTP  Tyrosine Triphosphate
Tubulin  β-tubulin Type III
UTP  Uridine Triphosphate
V  Volt
ACKNOWLEDGEMENT

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There are many other people for me to appreciate. It is too long a list to thank everyone individually. For all who I knew during my research, I would like to say, “Thank you for being a part of my research life. I enjoyed working with you!”
STATEMENT OF ORIGINALITY

The work described in this thesis is original and was carried out in the School of Biomolecular and Biomedical Science, Faculty of Science, Griffith University, under the supervision of Dr. Alan Mackay-Sim and Dr. Gillian Bushell. This work has not previously been submitted for a degree or diploma in any university. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made in the thesis itself.


Pi-En Hsu
Chapter one

General introduction
1.1 The significance of olfactory system

The olfactory epithelium is the site of smell, providing a powerful discriminating system to differentiate (depending upon training) around 10,000 odorous chemicals in tiny concentrations (Axel, 1995). Because of several advantages the olfactory system has been widely used to investigate events involved in neurogenesis and neural differentiation. An invaluable merit of the system is that neurogenesis continually occurs in the olfactory epithelium throughout adult life but is easily accessible for studies. The continual occurrence of neurogenesis in the olfactory system has been proposed to respond to constant neurodegeneration resulting from direct exposure of sensory neurons to environmental toxins or physical stresses (Crews & Hunter, 1994). The olfactory epithelium system is structurally simple but its development involves similar or equally complex events to those in the developing central nervous system. For example, the basal location of mitotic cells and their differentiation into neurons are features shared by the olfactory epithelium and many developing central nervous structures (Crews & Hunter, 1994). With these advantages the olfactory system is therefore able to provide an accessible and comparable model with which to study mechanisms that regulate neurodevelopment.

1.2 Structure and function of the olfactory system

1.2.1 Olfactory mucosa

Olfactory mucosa covers an area of 2.5 cm² mainly situated at the roof of each nasal cavity consisting of two tissue layers: olfactory epithelium and lamina propria, lying over and around the nasal turbinate and the nasal septum within the nasal cavity (Barr and Kiernan, 1983). The septum separates the nose into two nasal cavities. The inferior and the middle turbinate constrict the cross sectional area of the nasal cavity, thus enlarging the mucosal surface considerably (Barr and Kiernan, 1983). Olfactory epithelium was found with the highest possibility in the dorsoposterior regions of the nasal septum and the superior turbinate, although it was also found in the anterior and ventral regions of the septum and the turbinate (Feron et al., 1998).
1.2.1.1 Olfactory epithelium

The olfactory epithelium system (OE) is a pseudostratified columnar neuroepithelium constructed by three cell types. From nasal surface to basement membrane, these cell types are supporting/sustentacular cell, olfactory receptor neuron, and basal cell (Figure 1-1).

The supporting/sustentacular cell is a columnar cell shaped like a wineglass with the nucleus closest to nasal lumen and a stemlike basal projection to the basement membrane (Figure 1-1). The function of supporting cells is not clearly understood. They may help regulate ions and oxidize toxic compounds on the mucosal surface (Crews & Hunter, 1994; Krishna et al., 1994) or may have a role in chemoreception (Snyder et al., 1991).
The olfactory receptor neuron is the functional cell in the olfactory epithelium responsible for the sense of smell. An olfactory receptor neuron is a bipolar cell with a small ovoid body, located in the middle region of olfactory epithelium, and a long slender apical dendrite that extends to the mucosal surface where it enlarges into a dendritic knob or olfactory vesicle with cilia (Greer, 1991; see Figure 1-1). The plasmalemma of the cilia studded with numerous intramembrane particles has been suggested as the putative site of olfactory receptor molecules (Menco & Farbman, 1985a and 1985b). The thin, non-myelinated, axonal process extends from the pole of the receptor cell somata and come together to form axon bundles (fascicles) that are enveloped by olfactory ensheathing cells (OEC's), a special type of glial or neuronal support cell that guides the axon and supports its elongation or promotes axon regeneration in spinal cord injury (Lu, et al, 2002). The bundles travel to the base of the tissue and cross over to the cranial cavity through a perforated area of bone named the cribriform plate (Figure 1-2). They then enter the olfactory bulb, a relay station where they form synapses in glomeruli with
dendrites of mitral, tufted, and periglomerular cells (Crews & Hunter, 1994), which eventually lead to other brain areas via the olfactory tract (Figure 1-2).

The basal cell is a mitotic cell with its cell body located nearest the basement membrane. According to cell morphology, location, and biological marker expressed, the basal cell is further subdivided into two cell types: horizontal basal cell (HBC) that is ovoid, cytokeratin-positive, lying nearest the basement membrane, and globose basal cell (GBC) that is spherical, neural cell adhesion molecule (NCAM) positive, and located more superficially (Crews & Hunter, 1994). The lineage relationships between HBC and GBC populations are not clearly understood. Many believe HBC is the self-renewing stem cell responsible for olfactory neurogenesis (Calof & Chikaraishi, 1989; Mackay-Sim & Kittel, 1991a; Satoh & Takeuchi, 1995) but this is disputed (Chen et al., 2004).

1.2.1.2 Lamina propria

The lamina propria contains Bowman’s glands, collagenous connective tissue, olfactory nerve bundles and numerous lymphoid elements (Farbman, 1992). The Bowman’s glands, also named olfactory glands, extend narrow ducts perpendicular to the layer of olfactory and supporting cells to the epithelial surface (Farbman, 1992; Greer, 1991; see Figure 1-1) producing serous, watery secretory components of the mucus that dissolve odorous substances and present them to receptors (Greer, 1991). The viscous component of the mucus is believed to originate from supporting cells (Greer, 1991).
1.3 Cell markers specific to the olfactory system

There are many biochemical markers useful for identification of cells in the olfactory epithelium (Table 1-1). Many proteins act as cell/stage specific markers, as their expression is limited to a subset of cells within an organism, for example, GBC-1 monoclonal antibody for globose basal cells (Goldstein & Schwob, 1996), and olfactory marker protein (OMP) for mature olfactory receptor neuron (Margolis, 1972). Cells can be classified and identified on the basis of the expression of these cell/stage specific proteins (Zehntner, 1998). Some biochemical markers commonly used to identify cells in olfactory epithelium are listed in table 1-1.

<table>
<thead>
<tr>
<th>Target cell</th>
<th>Cell marker</th>
<th>References</th>
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<tbody>
<tr>
<td>Horizontal basal cell</td>
<td>Cytokeratin 5</td>
<td>Holbrook et al., 1995</td>
</tr>
<tr>
<td></td>
<td>Cytokeratin 14</td>
<td>Suzuki &amp; Takeda, 1991</td>
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<td></td>
<td>EGF-r</td>
<td>Holbrook et al., 1995</td>
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<td>Globose basal cell</td>
<td>BrdU incorporation</td>
<td>Suzuki and Takeda, 1991</td>
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<tr>
<td></td>
<td>N-CAM</td>
<td>Miragall et al., 1988; Calof &amp; Chikaraishi, 1989; Carr et al., 1989; Satoh &amp; Takeuchi, 1995</td>
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<tr>
<td></td>
<td>β-tubulin (type III)</td>
<td>Pixley, 1992; Lee and Pixley, 1994</td>
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<td></td>
<td>GBC-1</td>
<td>Goldstein &amp; Schwob, 1996</td>
</tr>
<tr>
<td>Immature olfactory neuron</td>
<td>GAP 43/B50</td>
<td>Verhaagen et al., 1989; Benowitz et al., 1988; Pellier et al., 1994</td>
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<td></td>
<td>N-CAM</td>
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<td>Pixley, 1992; Lee &amp; Pixley, 1994</td>
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<tr>
<td></td>
<td>MAP 5</td>
<td>Viereck et al., 1989</td>
</tr>
<tr>
<td>Mature olfactory neuron</td>
<td>OMP</td>
<td>Farbman &amp; Margolis, 1980; Chuah &amp; Farbman, 1983; Chuah &amp; Au, 1988</td>
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<td></td>
<td>MAP5</td>
<td>Viereck et al., 1989</td>
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<tr>
<td>Ensheathing cell</td>
<td>GFAP</td>
<td>Barber &amp; Lindsay, 1982; Pixley, 1992</td>
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<td>Supporting cell</td>
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<td>Hempstead and Morgan, 1983</td>
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<td>UDP-Glucuronyl transferase</td>
<td>Lazard et al., 1990</td>
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<td></td>
<td>Cytokeratin 18</td>
<td>Suzuki &amp; Takeda, 1991; Pixley, 1992; Schwob et al., 1994</td>
</tr>
</tbody>
</table>
1.4 Neurogenesis

Neurogenesis is the process whereby neuronal progenitor cells proliferate and differentiate into postmitotic neurons (Shou et al., 1999). Production of most neurons takes place during embryogenesis, gradually slowing and in most cases permanently ceasing toward the end of development (Calof et al., 1998b).

The majority of neurogenesis in the central nervous system ceases at birth or soon after except for sparse occurrence in some regions such as the forebrain including the ependymal, subependymal and the telencephalon (Reviewed by Alvarez-Buylla & Lois, 1995), the striatum of the adult mouse brain (Reynolds & Wiess, 1992), the olfactory bulb (Kaplan et al., 1985), and the hippocampus (Bayer et al., 1982).

In vertebrates, the nervous system develops from the dorsal ectoderm of the early embryo where nerve cells, together with neuroglial or interstitial cells, are derived from the outer ectodermal layer, similar to the cells of the epidermis covering the body surface (Barr and Kiernan, 1984). When a human embryo is about 18 days old, the ectoderm differentiates and thickens along the future midline of the back to form neural plate from which neural tube, placodes, and neural crests are derived. The neural tube is the primordial structure for the central nervous system (brain and spinal cord) including all neurons in central nervous system (Demarest, 1981). Sensory cells in the head, including the olfactory epithelium, are derived from placodes.

1.5 Olfactory neurogenesis

The olfactory epithelium in the nose develops bilaterally from thickened patches of ectoderm called olfactory placodes (Crews and Hunter, 1994). In mouse, cell division in the olfactory epithelium initially is restricted to the apical zone but after embryonic day 12 (E12), proliferating cells become more prevalent in the basal layers (Crews and Hunter, 1994). The mitotic figures in the developing olfactory epithelium dramatically
fall before emergence of axons. The division is not synchronous, so cells in all stages are present, reflecting in the adult tissue where the neurons continually grow throughout adult life (Chuah and Farbman, 1995; Farbman, 1992).

An early event in receptor cell development is the genesis of the axon (Figure 1-1), a narrow process that grows out of the basal pole of the receptor cell and penetrates the basal lamina of the epithelium to grow toward the olfactory bulb (Farbman, 1991). In both rats and mice, growing axons first reach the presumptive olfactory bulb region soon after the start of axon genesis and soon establish contact with target cells (Farbman, 1991). Axonal projection through the lamina propria occurs at about day 13 in the rat and the first arrival of nerve fibers formed by axons occurs at about day 15 (Chuah and Farbman, 1995). Following axon formation, dendrite formation starts and the primitive dendrites terminate at the surface of the olfactory epithelium at E13 (Menco et al., 1994).

The olfactory bulb develops in rats on E14 to E15, slightly after the growing olfactory nerves reach the presumptive bulb (Farbman, 1991). At early stages of olfactory bulb development, there are only two layers formed in the region: the cellular layer and the acellular layer. On E15, the mitral cell layer appears in the primordial bulb. The larger tufted cells are next to form followed by the smaller tufted cells in the more superficial parts of external plexiform layer (Figure 1-3). Most small periglomerular and granule cells are formed after birth in mice and rats. Neurogenesis in this population reaches a peak between the second postnatal week and the third week. Bulbar glial cells are formed throughout the fetal and postnatal periods (Farbman, 1991).
Figure 1-3. Olfactory bulb and laminar distribution of its cells. Top: longitudinal section of olfactory bulb observed under low power of magnification (x10); lines indicates the field observed under high power of magnification. Bottom: The sectioned field from the top figure, observed under high power of magnification (x40). ONL=olfactory nerve layer; GL=glomerular layer; EPL=external plexiform layer; MCL=mitral cell layer; IPL=internal plexiform layer; GCL=granular cell layer. Arrows indicate periglomerular cells. (Figure source: In situ hybridisation for PDGF α expression in mouse olfactory bulb, which shows the olfactory bulb and laminar distribution of its cells).
1.6 Regulation of olfactory neurogenesis

An olfactory neuron arises from division of stem cell close to or on the basement membrane (Mackay-Sim and Kittel, 1991b). The division undergoes two phrases: slow and rapid (Mackay-Sim and Kittel, 1991b). In the slow phrase, the stem cell divides slowly (about once every 50 days) and asymmetrically into another stem cell, which remains close to the basement membrane, and a neuronal precursor, which divides rapidly and symmetrically for two to three times giving rise to many immature receptor neurons migrating away from basement membrane and differentiating into mature neurons (Newman et al., 2000; Mackay-Sim and Kittel, 1991b). The initial differentiation is proposed to involve the transition from the stem cell into an ‘immediate neuronal precursor’ (INP) which exists in the globose basal cell population (Calof, 1995). The identity of the stem cell remains controversial up to date. Some believe the multipotent stem cell is the horizontal basal cell (Mackay-Sim and Kittel, 1991b) but some believe it is the globose basal cell (Schwob et al., 1994; Goldstein et al., 1997; Chen et al., 2004).

Although the olfactory basal mitosis continually occurs throughout adult life, the surface density of olfactory neurons does not change with epithelial thickness and this was found true in all species studied (Mackay-Sim and Kittel, 1991b; Mackay-Sim and Patel, 1984). For a pseudo-columnar epithelium like olfactory epithelium, this means the number of mature receptor neurons must be highly regulated so as to remain constant in a dynamic equilibrium between basal cell birth, neuronal differentiation and apoptosis of basal cells and neurons (Newman et al., 2000). Presumably, the equilibrium is under control of autocrine and paracrine signals that stimulate or inhibit proliferation, differentiation and cell survival (Mackay-Sim and Chuah, 2000). Among all signaling molecules, peptide growth factors are possibly the most noticeable.
1.7 Growth factors regulating olfactory neurogenesis

A number of growth factors have been implicated to be involved in the regulatory mechanism of olfactory neurogenesis. Of these, some with well-documented findings are discussed as below:

1.7.1 Epidermal growth factor family

Epidermal growth factor (EGF) and transforming growth factor α (TGFα), both acting through the same EGF receptor, are highly potent in promoting the proliferation of progenitors (Farbman et al. 1994; Farbman & Ezeh, 2000). In situ RT-PCR evidence has indicated the expression of EGF receptor in basal cells suggesting the EGF receptor mediates the mitogenic effect of TGFα and/or EGF on the quiescent basal cells, particularly horizontal basal cells, initiating the cell cycle (Krishna et al., 1996). TGFα has been reported to induce the EGF receptor expression (Farbman et al., 1994). Although EGF has an outstanding role in promoting basal cell proliferation, long-term OE cultures containing EGF appear not to cause basal cells to generate immediate neuronal precursors (INPs). This indicates that EGF may inhibit the transition from basal cell to INPs and eventually cause a net decrease in neurogenesis (Calof et al., 1991). Farbman and Buchholz (1996) reported that TGFα, a member of EGF family, is most effective mitogen among other factors including EGF, insulin-like growth factor (IGF) β, and platelet-derived growth factor AB (PDGF AB), TGFβ-1 and -2 (Farbman & Buchholz, 1996).

1.7.2 Transforming growth factor beta family

TGFβ-2 has been reported to be expressed in the epithelial components of sense organs in vivo (Millan et al., 1991), to stimulate terminal differentiation of olfactory neurons in vitro and promote the survival of N-CAM positive cells hence facilitating neurogenesis (Mahanthappa and Schwarting, 1993). Using cultures composed of only basal and
supporting cells of OE, TGFβ-2 has been found to induce differentiation of some basal cells which are postulated to be GBCs (Newman et al, 2000). TGFβ-1 and -3 appear to be expressed only slightly in nervous tissues of mouse embryos during early developing stages (Millan et al., 1991). In situ hybridisation has demonstrated the TGFβ growth factors 1, 2 and 3 are expressed in the vomeronasal organ and high levels of TGFβ 3 expressed in the olfactory epithelium (Pelton et al., 1990). At the time of the present research, no information as to the cells expressing TGFβ 2 and TGFβ receptors in the olfactory epithelium are available.

Bone morphogenetic proteins (BMPs) are members of TGFβ superfamily. BMP2, BMP4, and BMP7 were expressed by olfactory lamina propria (Shou et al., 2000). During mouse embryogenesis, BMPr 1B has been found in situ to be strongly expressed in the olfactory epithelium (Dewulf et al., 1995). At later stages of development of many systems, BMP2 and BMP4 have been shown to inhibit proliferation and/or induce apoptosis of neural progenitor cells (Furuta et al., 1997; Golden et al., 1999; Mabie et al., 1999; Shou et al., 1999; Song et al., 1998). BMP6 and BMP7, have been reported to have both positive (Furuta et al., 1997; Jordan et al., 1997) and negative (Shou et al., 1999) effects on neurogenesis. BMP4 was reported to stimulate neurogenesis at a low concentration but to inhibit neurogenesis at a high concentration (Shou et al., 2000).

1.7.3 Fibroblast growth factor family

Fibroblast growth factors (FGFs) are a family including at least 23 peptide growth factors which interact with four tyrosine kinase receptors, FGFr 1-4 (Wiedlocha & Sorensen, 2004; Ohbayashi et al., 1998; Ornitz el al., 1996; Ozawa et al., 1996), of which FGF1 is the first to be studied and FGF2 is most well documented (Hsu et al., 2001). In peripheral and central nervous systems or other tissues, FGF2 functionally acts as the mitogen and morphogen which stimulate proliferation and induce/delay the differentiation of neurons and their precursors (Gensburger et al., 1987; Unsicker et al., 1987; Caday et al., 1990; Murphy et al., 1990; Dehamer et al., 1994; Calof, 1995; Murrell et al., 1996). In the olfactory epithelium system, a previous study observed that FGF2 stimulates proliferation
of the unidentified “immediate neuronal precursor” (Dehamer et al., 1994). Newman et al. (2000), demonstrated that FGF2 alone does not induce differentiation but increases the number of GBCs for later differentiation induced by TGFβ2. A separate in vitro result revealed that FGF2 suppresses neurogenesis of the neuroblasts in culture (NIC) cell line derived from a postnatal rat (Goldstein et al., 1997). Later studies found that both FGF receptor 1 (FGFr-1) and FGF receptor 3 (FGFr-3) are expressed by olfactory neurons and neural precursors and, FGF2, the ligand for FGFr-1 and –3, is expressed in supporting cells (Yu et al., 1998).

1.7.4 Neurotrophin growth factor family

The neurotrophin growth factor family has been found to play a role in the olfactory epithelium system. Nerve growth factor (NGF) has been found important for neuronal maturation and differentiation in OE and necessary for neuronal survival and neurite extension in culture (Ronnett et al., 1991). Trk A has been found in sparse basal cells on normal conditions and in the entire basal cells after OE lesion (Roskams et al., 1996). Brain-derived neurotrophic factor (BDNF) was reported to be expressed by mitral and periglomerular cells in the olfactory bulb, and was capable to enhance neuronal differentiation and neuron survival, both in vitro and in vivo, in the OE (Liu et al., 1998). Trk B was found to be expressed by immature neurons (Roskams et al., 1996). Roskams et al., (1996) has demonstrated that Trk C is expressed throughout the OE. Neurotrophin-3 (NT-3) has been shown to promote survival of embryonic olfactory neuronal cells in vitro and Trk C was found to be expressed predominantly in the receptor neurons in neonatal OE (Holcomb et al., 1995). Trk C was also found to be expressed in the progenitors of the OE receptor neurons implying that NT-3 may be an important survival factor in the OE (Calof et al., 1998),
1.7.5 Platelet-derived growth factor family

Platelet-derived growth factor (PDGF) was first identified as a product of platelets which stimulated the proliferation in vitro of connective tissue cell types, indicating a role in wound healing. Later findings suggested PDGF was able to stimulate directed migration and enhance modulated extracellular matrix production by fibroblast and smooth muscle cells. It has subsequently become clear that there are a magnitude of sources for PDGF in addition to platelets cells (Reviewed by Betsholtz, 1995).

PDGF is a family of dimers of at least four gene products, PDGF-A, PDGF-B, PDGF-C and PDGF-D, whose biological activities are mediated through two receptor types, PDGF receptor α and PDGF receptor β (Betsholtz et al., 2001). The platelet-derived growth factor gene is thought to be expressed in olfactory supporting cells (Lee et al., 1990). PDGF receptor α has been observed to be expressed in the non-neural cells of the OE as well as in the olfactory bulb (Lee et al., 1990). PDGF-B was found to be expressed in the olfactory nerve and nerve glia, suggesting a possible role as a stimulating or regulating factor in both the developing and mature neurons (Sasahara et al., 1992). In cell culture, the dimer ligand, PDGF-AB, was found to promote survival of TGFβ2-induced immature neurons to enhance olfactory neurogenesis (Newman et al, 2000). Nevertheless, at the time of the research, no in situ studies of expression of PDGF ligands and receptors have been performed in the olfactory epithelium.

1.7.6 Growth factor control of olfactory neurogenesis

Based on the current findings including the work done in our laboratory, a working model of the growth factors regulating olfactory neurogenesis has been constructed (Newman et al., 2000; Figure 1-4). In brief, FGF2 stimulates proliferation of globose basal cells, TGFβ2 induces differentiation of the globose basal cells into immature neurons, and PDGF-AB promotes the survival of the immature neurons to enhance neurogenesis. Since these findings were obtained in vitro and since actions of these
growth factors might not reflect the complicated events of signaling mechanisms in vivo, an in vivo investigation of the cells expressing these growth factors in olfactory epithelium has become of great importance. For example, showing that the globose basal cells indeed express receptors for TGFβ−2 will support the finding that TGFβ-2 stimulates FGF2-induced differentiation of globose basal cells in vivo. The identification of the cells expressing PDGF and FGF2 growth factors and their receptors in OE will clarify the hypothetical signaling mechanisms in OE.

Figure 1-4. The working model of the growth factors regulating olfactory neurogenesis and their sites of action on identified cell types (Newman et al., 2000). The lineage relation between horizontal basal cells and globose basal cells is not clear (dotted arrows). GBC, globose basal cell; HBC, horizontal basal cell; IN, immature neuron; MN, mature neuron. Figure source: Professor Alan Mackay-sim
1.8 Project aims

The aims of this project are to identify and characterize the cells expressing TGFβ, FGF and PDGF growth factors and their receptors in the olfactory epithelium system, and to explore the growth factor expression and receptor expression in an olfactory cell line, OLF442. Techniques involved will be largely based on reverse-transcriptase polymerase chain reaction, in situ hybridization, and immunohistochemistry. Technical issues of some of these investigations will be discussed in appropriate parts of the following chapters. The investigations will also include a novel gene, 16b5, previously identified and isolated from OLF442, and found to be upregulated by differentiation.
Chapter two

Identification and characterization of TGF β–2 growth factors and receptors in olfactory mucosa
2.1 Introduction

2.1.1 Transforming growth factor β superfamily

2.1.1.1 Structural studies of transforming growth factor β superfamily

2.1.1.1.1 Transforming growth factor ligand proteins

The transforming growth factor β (TGF-β) superfamily consists of groups of isoforms that progressively diverge from bone morphogenetic protein 2 (BMP2) and their Drosophila counterpart, Decapentaplegic (DPP) (Massague, 1996). The complexity of this family is reflected not only in its diverging signaling pathways but also in its naming system. Listed in Table 2-1 are documented TGFβ growth factors and receptors and their known interacting properties (Refer to Caestecker, 2004).

TGFβ family proteins are homo- or heterodimeric proteins synthesized as larger precursors that undergo a proteolytic cleavage releasing a pro-domain from the smaller, active, receptor binding, carboxy-terminal region sized 110-140 amino acids. The mature region is highly conserved between family members and distinctively has 7 cysteine residues. Six of the characteristic cysteine residues form three disulfide bonds within each monomer subunit (Kinsley, 1994). The threaded ring configuration containing the three disulfide bonds is known as “cysteine knot” that provides a firm core of the ligand (Massague, 1996).

2.1.1.1.2 Transforming growth factor receptors

All plasma membrane serine/threonine kinases described to date in animal cells are TGFβ receptors. The characteristic structural feature of the TGFβ superfamily of receptors is the three-finger toxin fold in the ligand-binding extracellular domain, a single transmembrane domain (Caestecker, 2004). There are two major types of TGFβ receptor family: type 1 and type 2 receptors. Type 1 and type 2 receptors are sized about 53 kD and 70-85 kD, respectively (Kinsley, 1994). Both types of receptors are transmembrane proteins with an amino-terminal signal peptide, a small extracellular region, a single hydrophilic transmembrane helix, and a cytoplasmic domain predicted to have
Type 1 receptors differ from type 2 receptors in the sequence of the kinase domain, the presence of a short series of tandem Ser/Gly residues (also known as GS domain) and other conserved amino acids immediately preceding the kinase domain (Kingsley, 1994; Massague, 1996). Other than the two major types of receptors, some accessory receptors have been identified, one of which is high molecular weight TGFβ receptor 3 (or betaglycan) which is required for binding TGFβ–2 and TGFβr-2 and now known also to be a co-receptor for Inhibin, promoting interactions between Inhibin and the type 2 receptors ActR-2, ActR-2B and BMPR-2 (reviewed by Caestecker, 2004).

2.1.1.2 Functional studies of transforming growth factor β superfamily

In association with TGFβ signaling, one unique feature of the family is the dual requirement of type 1 and type 2 receptors (Massague, 1996). Ligand binding was presumed to stimulate the serine/threonine kinase activity of the receptors, perhaps by inducing the formation of receptor dimers or multimers. A series of ligand-receptor binding and mutation studies demonstrated that type 2 receptors act upstream of the type 1 receptors and the central event in the generation of signals by the ligand-receptor complexes is phosphorylation of the type 1 receptors (Massague and Weis-Garcia, 1996; Feng et al., 1995; Wieser et al., 1995). In the combinatorial model, the type 1 receptors, particularly their GS domain in the serine/threonine kinase, are essential in determining the nature of signal regardless of the choice of the type 2 receptor and the ligand. For example, TGFβr 1 and ActR 1B, which have nearly identical kinase domains, can generate the same set of responses such as growth inhibition even though their respective ligands, type 2 receptors, extracellular domains are quite different (Massague, 1996).

However, a recent study has suggested that interaction of ligands with preformed BMP receptor complexes activates distinct downstream signals to BMP receptor complexes that are recruited only after addition of the ligand (Nohe et al., 2002), which has contradicted the signal-determining role of type 1 receptor in the signaling pathway (Massague, 1996). Another example is Inhibins; like the Activin dimer, Inhibins have the capacity to interact with Activin type 2 receptors, but they failed to recruit an active
Alk4/ActR2 signaling complex, thus promoting competitive inhibition of Activin-dependent signaling (Chapman et al., 2002; Lewis et al., 2000). The complexity of the TGFβ receptor system is likely to explain the diversity of downstream responses that can be generated by engagement of these receptors in different cell types (Caestecker, 2004).

### Table 2-1 Known ligand-binding properties and nomenclature for the TGFβ superfamily ligands and receptors

<table>
<thead>
<tr>
<th>Receptor types</th>
<th>Receptor subgroups</th>
<th>Receptors</th>
<th>Alternative names for the receptors</th>
<th>Ligands</th>
<th>Alternative names for the ligands</th>
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<td></td>
<td></td>
<td>GDF1</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Nodal (with EGF-CFC)</td>
<td>Ndr</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>GDF11</td>
<td>BMP11</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Alk5</td>
<td>TGFβe-1/SKR4</td>
<td>TGFβ</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>Alk7</td>
<td>Nodal</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>MISr2 (Mullerian inhibitory substance type 2 receptor)</td>
<td></td>
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<td></td>
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<tr>
<td>Type 2 receptors</td>
<td>TGFβr 2</td>
<td>TGFβ</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>BMPr 2</td>
<td>Brk3/T-Alk</td>
<td>Inhibin A (with TGFr 3)</td>
<td>Inhibin α-βA subunits</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>BMP2/4/6/7</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>GDF5/6/9b</td>
<td></td>
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<tr>
<td></td>
<td>ActR 2 (also known as ActR2A)</td>
<td>ACVR2</td>
<td>Activin A</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Inhibin A</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Inhibin B</td>
<td>α-βA</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>GDF1/5/9b</td>
<td></td>
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<td>Nodal (with EGF-CFC)</td>
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<td>BMP2/6/7</td>
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<td>GDF8</td>
<td>Myostatin</td>
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<td></td>
<td>GDF11</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>ActR2B</td>
<td>ACVR2B</td>
<td>Activin A</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Inhibin A/B</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Nodal</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>BMP2/6/7</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>MISr2</td>
<td>AMHR2</td>
<td>MIS</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note that both type 1 and type 2 receptors are required for TGFβ action in mammalian cells (Wrana et al., 1994). TGFβ ligands against TGFβ receptors 1 and 2 are TGFβ 1, 2 and 3. The TGFβ receptor type 3 (TGFβr 3 or betaglycan) acts as an accessory receptor for regulation of signaling (Caestecker, 2004) and is not listed in the table.
2.1.1.3 Current findings

TGFβ family members have been found to be involved in many developmental events in other tissues. For instance, BMPs were reported to play a role in normal bone formation (Wozney et al., 1988), mesoderm induction (Dale et al., 1992), neurulation (Wilson & Hemmati-Brivanlou, 1995), dorsoventral patterning of the neural tube (Liem et al., 1995; Arkell & Beddington, 1997), apoptosis (Graham et al, 1994) and neural differentiation (Mehler et al., 1997). Drosophila Decapentaglegic was found to be required for embryonic dorsal-ventral patterning, development of the eye, and the differentiation of the larval midgut (Liu, et al., 1995). Dorsalin, another member of TGFβ family, was reported to promote the outgrowth of neural crest cells but inhibit the formation of motor neuron cells at the same time suggesting it may play an important role in neural patterning (Basler, et al., 1993). Functionally, at the presence or absence of exogenous serum, TGFβ 2 was found to be able to stimulate or inhibit neurogenesis of rat cerebellar granule cells in culture (Kane, et al., 1996). BMP 2 and 4 proteins were reported to be upregulated in vitro to 4-5 folds by growth hormone and insulin-like growth factor 1 (IGF-1) in human dental fibroblasts (Li, et al, 1998), which implies the growth factors BMP2 and 4 may be regulated by IGF-1 in the olfactory epithelium as it is in other tissues.

In the olfactory mucosa, in addition to the findings in our lab, BMPs have been functionally shown to inhibit olfactory neurogenesis via degradation of a transcriptional factor, Mash 1 (Shou et al, 1999). BMP4 was reported to stimulate neurogenesis at a low concentration but to inhibit neurogenesis at a high concentration (Shou et al., 2000). During mouse embryogenesis, BMPr 1B has been found in situ to be strongly expressed in the olfactory epithelium (Dewulf et al., 1995). In vivo studies have demonstrated that some BMP receptors, Activin receptors and GDF-11 are expressed in the olfactory epithelium (Nakashima et al., 1999; Dewulf et al., 1995). Despite these findings, the expression of most of the other members of the large TGFβ superfamily in the olfactory system remains largely unknown. The documented evidence with important implications and deficiency of related information has therefore raised our desire to study the
expression of TGFβ family in the olfactory system and to characterize its role in olfactory neurogenesis.

2.1.2 Chapter aims

This chapter aims to identify and characterise the cells expressing TGF β receptors (TGFβr) and the cells expressing the TGFβ growth factors in the adult mouse olfactory mucosa, and to identify whether they are expressed in an olfactory cell line, OLF442, in order to characterize the signaling pathway of TGFβ growth factor family. The receptors to be studied include BMPr-1A, 1B, and 2, TGFβr-1, -2 and -3, and ActR-1A, -1B, -2A and -IIB. The growth factor ligands to be studied include BMP-2, -4 and -7, TGFβ-1, -2 and -3 and Activins. In addition, the chapter also aims to determine whether the expression of BMP2 and BMP4 is altered by IGF-1 in human olfactory epithelium. The major techniques involved are RT-PCR, competitive RT-PCR, in situ hybridization and immunocytochemistry.
2.2 Methods and materials

2.2.1 Tissue preparation from mice

Adult Quackenbush or HOMPLacZ transgenic mice (The latter was originally a gift of Frank Magolis) from Animal House, Griffith University, Brisbane, weighing approximately 30g, were sacrificed in 100% CO\textsubscript{2} in accordance with the guidelines and recommendations of Griffith University and the National Health and Medical Research Council of Australia. After decapitation and dissection, the whole nose including olfactory epithelium was fixed in 4% paraformaldehyde under vacuum for 2 hours to overnight, cryoprotected with 30% sucrose, sectioned at ~10 um onto slides coated with aminopropylethysilane (Superfrost Plus, Menzel-Glaser, Germany), and stored at -80\textdegree C. Before use the tissue sections were placed at room temperature for 30 min and then dried at 40\textdegree C for 1.5 hours.

The fixation time at room temperature for the dissected tissues was estimated according to the following formula (Bancroft & Stevens, 1982):

\[ d = K \sqrt{T} \]

where \( d \) (mm) = depth penetrated, \( T \) (hour) = time of fixation, and \( K \) = constant of fixative diffusibility, e.g., for 4% formaldehyde \( K = 0.78 \).

Two temperatures were attempted in tissue fixation: 4\textdegree C and room temperature. The low temperature of 4\textdegree C was used in fixation to slow down autolytic processes and preserve the tissue close to the ‘true’ conditions in vivo. However, because the low temperature can slow down all chemical reactions including the fixation at the same time, a longer fixation time is required for 4\textdegree C. The additional time was determined on the basis of a general rule that an increase in temperature of 10\textdegree C doubles the reaction rate (Masterton, et al., 1981). Thus the actual fixation time used was four times longer than the time
predicted by above formula, as the fixation temperature was lowered from room temperature to 4°C.

For RT-PCR, the olfactory mucosa was separated from the nasal septum after the mice were decapitated. The olfactory tissue was washed twice in Dulbecco’s Modified Eagles Medium (DMEM, Gibco-BRL) and then incubated for 60 min at 37°C / 10% CO₂ in a 4.8 units/ml Dispase II solution (Boehringer Mannheim). The olfactory epithelium was carefully separated from the underlying lamina propria under the dissection microscope. Both olfactory epithelium and lamina propria were kept in DMEM ready for immediate use.

2.2.2 Preparation of OLF442 cells (an olfactory cell line created by MacDanold et al., 1996; see Chapter Five for details)

All the following procedures were performed with sterile reagents in a biohazard hood.

2.2.2.1 Frozen cells

Frozen cells were warmed at room temperature for 20 min, added with 5 ml 37°C prewarmed Glutamine-incorporated DMEM containing 10% FCS and 1/20 streptomycin (P/S), and then centrifuged at 300 g (1500 rpm) for 5 min. The pellet was resuspended in 1 ml DMEM medium, the suspension was transferred to a 25 cm² flask containing 4 ml DMEM-10% FCS medium and incubated at 37°C for 24-48 hours.

2.2.2.2 Passage of cell line

The medium in the flask with confluent OLF442 cells was aspirated. The cells were washed three times with 3ml PBS and then detached from the flask base by 0.5 – 1 ml Trypsin-Versine (CSL) at 37°C for 3-5 min. Following that, 5 ml DMEM-10% FCS was added to the flask and the suspension was transferred to a centrifuge tube. After centrifugation at 1500 rpm for 5 min, the pellet was resuspended in 2 ml DMEM-10% FCS. The cells were counted in a haemocytometer and the cell suspension was equally
dispensed into two 25 cm\(^2\) flasks (~1 ml each) containing 4 ml DMEM-10% FCS. Cells were maintained in the flasks at 37°C and passaged at 1-2 day intervals.

2.2.2.3 Depletion of serum medium (differentiated OLF442 cell preparation)
OLL442 cells were transferred into 80 cm\(^2\) flasks and when the cells reached confluence, they were washed in DMEM once and then washed another time in DMEM containing 10% FCS and 1% BSA. After aspiration of the medium, cells were left to grow in DMEM-1%BSA at 37°C for 48 hours when the cells differentiated (Zehntner, 1998, unpublished observations).

2.2.2.4 Storage of cell line
Cells were harvested by trypsinisation, tritutated and centrifuged for 5 min. The pellet was resuspended with 1 ml 90% FCS, 10% dimethyl sulfoxide (DMSO, Sigma, St Louis, MO, USA, v/v). The cell suspension was equally dispensed into two cryotubes (Nunc) and the tubes were then packed in cotton wool stored at -70°C overnight, after which they were transferred to liquid nitrogen for longer storage.

2.2.3 Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

2.2.3.1 Primer selection
The optimal specificity of a single primer is a balance amongst considerations of GC content, melting temperature, annealing temperature, length of the primer, disturbing tandem, whether the sequence selected overlaps an exon of the genomic DNA and so on. It is always desirable for a researcher to select a primer whose sequence crosses exon boundaries in order to eliminate questions about genomic DNA contamination. However, because the exon and intron information of the target genes was not always available in literature and because our RT-PCR approaches were in part to analyze the value of further investigations on many growth factors and receptors, our primer design was therefore oriented by the considerations of cost and time where the specificity of primers was maintained by optimising PCR conditions and including positive and negative controls. For example, for the multiplex RT-PCR attempt (Table 2-2), the primers for
TGFβ and BMP receptors were designed from published sequences and their similar GC contents and melting temperatures (Tm) were intentionally selected to accommodate a set of RT-PCR conditions.

Table 2-2  Primers for TGFβ and BMP receptors

<table>
<thead>
<tr>
<th>Locus</th>
<th>Nucleotide sequence (5’ → 3’)</th>
<th>GC contents (%)</th>
<th>Tm (°C)</th>
<th>Genbank ID</th>
</tr>
</thead>
</table>
| TGFbr-1 | Forward: TTACAGTGTTTCTGCCACCTCTGTAC  
          | Reverse: GACTGCTTTTCTGTAGTTGGGAGTTC | 46   | 62.9  | D25540     |
| TGFbr-1 | Forward: AGTACTCCTCGTGGAAAACAGAGAAG  
          | Reverse: CTAGGATTTAGCTTCCTGTGAGTCC  | 50   | 62.1  | S69114     |
| TGFbr-3 | Forward: GATATCGGAGAAGATGTCCTTCTCTG  
          | Reverse: TAGTTGAGTTGCTTCTGACTAAGCC | 46   | 62.9  | AF039601   |
| BMPriA  | Forward: ACACCTATCATCAGAATCTGGGAGCC  
          | Reverse: GTACCTCCTCTGCTTGCTAAGACTCC | 50   | 62.9  | Z23154     |
| BMPriB  | Forward: TGTCACCTCTGGATGTCTAGGACTAG  
          | Reverse: GAGGACCAAGAGTAAACTACAGACAGTC | 50   | 62.2  | Z23143     |
| BMP2    | Forward: AGAGTGTGTAGTAACTACCACCCCAC  
          | Reverse: CTTTATATACTGCTCCGTACTGACC   | 50   | 61.0  | U78048     |

Primers for Activin receptors 1, 1B, 2, and 2B in mouse tissues were designed using the ANGIs online program. Primers for TGFβ-1, -2 and -3 in mouse tissues were available in a published paper (Shaddy et al., 1996). They are listed in the Table 2-3 below. The primer pair for the housekeeping gene, GAPDH, is also included, working as a positive control in the following RT-PCR experiments.

Table 2-3  Primers for Activin receptors, TGFβ ligands, and GAP-DH

<table>
<thead>
<tr>
<th>Locus</th>
<th>Nucleotide sequence (5’ → 3’)</th>
<th>Product size (bp)</th>
<th>Genbank</th>
</tr>
</thead>
</table>
| ActR-1(A) | Forward: TTCTTCTCTGAGCATCTACGATGG  
          | Reverse: GTGATGTTCCTGTTACACACGCTCC | 150   | L15436   |
| ActR-1B  | Forward: ATATTGACTTCTGCAACAAGATTGAC  
          | Reverse: GCCGATAATTTCTGTGAAAACAATG  | 353   | Z31663   |
| ActR-2   | Forward: TTACCATATATGATGTTAGCTTCAGAC  
          | Reverse: TACAGCTGTTGCTAGGGCTTCA  | 180   | NM_007396 |
| ActR-2B  | Forward: GAGCGTTCACACCACCTTGCCGGA  
          | Reverse: CAGCAGCTGTTGCTAGGGCTTCA  | 321   | NM_007397 |
| TGFβ-1   | Forward: CTAATGGTGGACCGCAACAAC  
          | Reverse: CGGTTGAGATATCTGATGGATTG   | 431   |        |
| TGFβ-2   | Forward: ACAAATGCATCCCCGCAACTTT  
          | Reverse: CATCAATACTGCAACAGCTCG  | 420   |        |
| TGFβ-3   | Forward: TTGGGAGACACACTAGACTGG  
          | Reverse: CCTTTGAAATTTGATTTCCTAGTC | 415   |        |
| GAPDH    | Forward: ACAGTCCATGCGATCATGACTGG  
          | Reverse: CCTGCTTCCACACCTTCTTG  | 265   | J04038   |
All oligonucleotides were synthesized by Pacific Oligo Pty. Ltd. and their concentrations were re-measured by the following formula (Sambrook et al., 1989) to optimize the RT-PCR conditions, particularly the primer/template ratio that significantly determines the success rate of the PCR reaction.

\[ A_{260} = \varepsilon \times C \]

where \( A_{260} \) = Optic Density (OD) reading at the wavelength of 260 nm, \( C \) = oligonucleotide concentration (mmole/L) before adjustment with dilution factor, and \( \varepsilon \) (L/mmole.cm) = (no. of nucleotide A) x 15.4 + (no. of nucleotide T) x 8.8 + (no. of nucleotide G) x 11.7 + (no. of nucleotide C) x 7.3.

### 2.2.3.2 Extraction of total RNA from mouse tissues or cell lines

(Chomczynski & Sacchi, 1987)

100 mg of each mouse tissue (liver, spleen, kidney, heart, lung, brain, muscle, olfactory epithelium and olfactory bulb) was removed from an adult mouse, placed into a 1.5 microcentrifuge tube containing 1 ml solution D (4 M quanidinium thiocyanate, 25 mM sodium citrate pH 7, 0.5% Sarkosyl and 0.72% \( \beta \)-mercaptoethanol) and immediately homogenised in a glass-teflon homogeniser at room temperature (note that for OLF442 confluent cells grown on a 80 cm\(^2\) flask were denatured directly on the flask with 1 ml solution D). The homogenate obtained was equally dispensed into two 1.5 microcentrifugal tubes, to each of which 50 \( \mu \)l 2M sodium acetate pH 4, 500 \( \mu \)l water saturated phenol, and 200 \( \mu \)l chloroform-isoamyl (49:1) alcohol mixture were added in order. The contents of each tube was mixed well by inverting several times after each addition. The suspension was shaken vigorously for 10 sec, cooled on ice for 15 min, and centrifuged at 13000 rpm for 20 min at room temperature. The RNA was present in the aqueous supernatant, which was carefully transferred to a clean microcentrifuge tube and precipitated with 500 \( \mu \)l of isopropanol overnight at -20°C. Sedimentation was performed at 13000 rpm at 4°C for 20 min. The pellet was dissolved in 150 \( \mu \)l of denaturing solution D and precipitated with 1 volume of isopropanol at -20°C for 3 hours. After
2.2.3.3 **Purity determination and quantification of total RNAs**

The purity and concentration of total RNA were determined by spectrophotometry. An optical density of 1 at 260 nm is equivalent to 40 ng/ml for single stranded RNA (Bradley et al., 1995). To exclude protein contamination the absorbance reading was taken at 280 nm. An \( A_{260}/A_{280} \) ratio greater than 1.8 indicates a protein-free sample (Sambrook et al., 1989).

2.2.3.4 **Quality of total RNAs – electrophoresis of RNA on formaldehyde-containing gels** (Sambrook et al., 1989)

10 μg (or 20 μg) of each RNA extract was mixed with 2 μl 5x formaldehyde gel-running buffer, 3.5 μl formaldehyde (pH < 4), and 10 μl deionised formamide. The samples were incubated at 65°C for 15 min. After addition of 2 μl DEPC-treated formaldehyde gel loading buffer, the sample were loaded onto a 1% agarose gel containing 1x formaldehyde gel-running buffer (MNE buffer) at room temperature at 80V. When the bromophenol blue migrated to two thirds along the gel, the gel was removed from the electrophoresis apparatus and stained with 0.5 μg/ml ethidium bromide (EtBr) in DEPC-treated water for 2 hours. The gel was then visualized and photographed under UV light for the distinctive bands of 28S and 18S rRNAs (see Appendix 1).

**Notes:**

a. **5x MNE buffer:** 0.1 M MOPS pH 7.0 [3-(N-morpholino)propanesulfonic acid], 40 mM sodium acetate, and 5 mM EDTA (pH 8.0).

b. **Formaldehyde gel-loading buffer:** 50% glycerol, 1 mM EDTA (pH 8.0), 0.25% bromophenol blue, and 0.25% xylene cyanol FF.
2.2.3.5 RT-PCR

Using 1\textsuperscript{st} Strand cDNA Synthesis Kit (Boehringer), RNA was reverse transcribed into single-stranded cDNA. Before the reverse transcription (RT), the total RNAs previously extracted were incubated at 65°C for 5 min to loosen their possible secondary structures, and then cooled on ice for 5 min. A 20 μl RT reaction mix was prepared including 2 μl 10x reaction buffer, 4 μl 25 mM MgCl\textsubscript{2}, 2 μl 10 mM dNTP, 1 μl RNase inhibitor, 0.8 μl AMV reverse transcriptase, 5 μl total RNA (~1 μg total RNA) and distilled water. The reaction mix was vortexed and left to stand at room temperature for 10 min. After that the mix was incubated at 42°C for 1 hour, the AMV reverse transcriptase denatured at 95°C for 15 min, and the denatured solution cooled down on ice for 5 min. The 1\textsuperscript{st} strand cDNA obtained was either used in the following PCR reaction immediately or stored at -20°C for a period of time.

For the polymerase chain reaction (PCR), a 25 μl reaction mix was prepared for a set of PCR thermal conditions. Standard components in the mix were 2 μl 10x reaction buffer, 1.5 μl 25 mM MgCl\textsubscript{2}, 5 pmole of each primers, 0.625 units of Taq polymerase, 6 μl 1\textsuperscript{st} strand cDNA and distilled water. Following that, the reaction mix underwent a set of standard thermal cycles: 1 cycle of initial denaturation at 95°C for 5 min, 35 amplifying cycles of 95°C for 30 seconds, 55°C for 1 min, and 72°C for 1 min, and 1 cycle of final extension at 72°C for 7 min. The annealing temperature of 55°C was subject to change depending on the primers used. The Table below shows the optimized conditions in the RT-PCR study for the TGF\textbeta and BMP receptors in various mouse tissues. Note that a multiplex RT-PCR was introduced to overcome a problem arising from the low yield of total RNAs from mouse OE and lamina propria allowing multiple, simultaneous analyses on the same mRNA. In the multiplex RT-PCR approach, the annealing temperature was increased and the primer concentration was reduced to inhibit non-specific amplification (Table 2-4).
Table 2-4 Optimized RT-PCR conditions for detecting TGFb and BMP receptors

<table>
<thead>
<tr>
<th>Reagents</th>
<th>RT-PCR</th>
<th>Multiplex PCR</th>
<th>GAPDH for quality control</th>
</tr>
</thead>
<tbody>
<tr>
<td>10x reaction buffer</td>
<td>2 µl</td>
<td>2.5 µl</td>
<td>2 µl</td>
</tr>
<tr>
<td>25 mM MgCl₂</td>
<td>1.5 µl</td>
<td>1.5 µl</td>
<td>1.5 µl</td>
</tr>
<tr>
<td>10 mM dNTP</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Primers (10 pmol/µl)</td>
<td>0.5 µl each (x2)</td>
<td>0.35 µl each (x6)</td>
<td>0.5 µl each (x2)</td>
</tr>
<tr>
<td>Taq DNA polymerase</td>
<td>0.25 µl</td>
<td>0.4 µl</td>
<td>0.25 µl</td>
</tr>
<tr>
<td>Distilled water</td>
<td>14.25 µl</td>
<td>12.5 µl</td>
<td>14.25 µl</td>
</tr>
<tr>
<td>1st strand cDNA</td>
<td>6 µl</td>
<td>6 µl</td>
<td>6 µl</td>
</tr>
</tbody>
</table>

PCR thermal specification:
- @ Denaturation
  - 95°C – 5 min
  - 95°C – 5 min
  - 95°C – 5 min
- @ Annealing
  - 95°C – 30 sec
  - 95°C – 30 sec
  - 95°C – 30 sec
- @ Extension
  - 55°C – 1 min
  - 60°C – 1 min
  - 60°C – 1 min
  - 72°C – 1 min
  - 72°C – 1 min
  - 72°C – 1 min
  - 72°C – 7 min
  - 72°C – 7 min
  - 72°C – 7 min

@ A 35 cycles of denaturation, annealing, and extension was adapted in all the RT-PCR experiments for TGFb and BMP receptors.

After the 1st cDNAs were amplified with primers, the PCR products were electrophoresed on a 2% agarose along with a 100 bp ladder (Gibco). The specificity of the primers was determined by the gel pattern and product size in general. Two PCR products of the same size (i.e., PCR products for TGFbr-3 and BMP1A) were sequenced to confirm the corresponding primer specificity with confidence.

2.2.3.6 Sequencing

In accordance with the ABI sequencing protocol (ABI PRISM Dye Terminator Cycle Sequencing: Ready Reaction Kit), the reaction mix prepared for ABI PRISM sequencing system contained 8 µl Terminator Ready Reaction Mix, 3 µl of 50 ng/µl PCR product for TGFbr III or BMP1A study, 4 µl of 0.8 pmole/µl M13(-21) primer and 5 µl dH₂O in a PCR tube. The mixture was overlaid with one drop of mineral oil (~40µl) and then processed in the PCR thermal cycler (Hybaid Omniplate 96 Thermal Cycler) for 1 cycle of 96 °C for 5 min at a RAMP of 1 sec, and then 25 cycles of 96°C for 1 min, 50°C for 15 sec and 60°C for 4 min at a RAMP of 1 sec. PGEM 3Zf(+) was prepared and run in parallel as a positive control.
The PCR products were transferred to a 1.5 ml microcentrifuge tube containing 2.0 μl 3 M sodium acetate, pH4.6 and 50 μl 95% ethanol, vortexed well and then placed on ice for 10 min. Following a 30 min centrifugation at 14000 rpm, the ethanol was decanted and the pellet was washed with 250 μl of 70% ethanol. The ethanol was decanted again and the pellet was vacuum dried and sent to ABI-PRISM autoanalyser for sequencing analysis. The target sequences of the PCR product for TGFbr III or BMPr 1A assays were confirmed using the sequencing method (results not shown).

2.2.4 Immunohistochemistry

2.2.4.1 Isolation of proteins from mouse olfactory epithelium

Olfactory mucosa tissues including turbinate were removed from Quackenbush mice as previously described. The tissues were homogenized in ice cold 0.32 M sucrose solution containing Protease Inhibitor Cocktail (Boehringer Mannheim), using a Potter-Elvehjem grinder. The concentrations of the homogenates were estimated using Bradford protein estimation method.

2.2.4.2 Bradford protein estimation

Protein concentration of the olfactory tissue homogenate was determined according to the Bradford dye-binding procedure (Bradford, 1976) using a 96 microtitre plate. The standard curve was established using BSA (Bovine Serum Albumin) ranging in concentration from 0.125 mg/ml to 2 mg/ml (0.125, 0.25, 0.5, 1, and 2 mg/ml). 10 μl of the sample proteins were added in triplicate to wells of the plate, followed by 200 μl of Bradford solution (20% Bio-Rad Protein Assay Dye Reagent Concentrate, 10% 3M NaCl, 70% ddH2O). The samples were mixed well and their optical absorbances were determined at 595 nm using a Spectra Max 250 plate reader. The sample protein concentrations were estimated by comparing their readings with the standard curve.

2.2.4.3 TCA precipitation

Before electrophoresis, samples were concentrated by trichloroacetic acid (TCA). 80 μl 72% TCA was added to a volume for 150 μg of each sample. The samples were put on
ice for 10 min, centrifuged for 1 min and supernatant discarded. The pellets were washed
with 1 ml ice cold acetone, dried and resuspended with 10 μl distilled water and 10 μl 3x
SDS sample buffer (30% glycerol (v/v), 6% SDS (w/v), 15% 2-β-mercaptoethanol,
0.00125% bromophenol blue (w/v), 187.5 mM Tris-HCl (pH 6.8).

2.2.4.4 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-polyacrylamide gel electrophoresis was performed using the Bio-Rad Mini-Protean
II Electrophoresis cell system (Bio-Rad). All proteins and a prestained low molecular
weight marker (Benchmark Prestained Protein Ladder, 9-173 kDa, Life Technology)
were run through a 4% stacking gel and then separated in a 10% separation gel. The
samples [100 μl (~3 μg/μl) in 200 μl 3X SDS sample buffer ≅ 15 μg/lane] and the
standard marker (10 μl) were loaded and run at 200V in the running buffer. When the
bromophenol blue reached the end of the gel, the system was disconnected from the
power source and the gel was used for Western Blotting. Protein gels were stained for a
minimum of 4 hours with 0.25% (w/v) Coomassie brilliant blue R in 50% methanol/10%
acetic acid (v/v). The gels were destained using 5% methanol/10% acetic acid (v/v).

Notes:

10% separation gel (10 ml): 3.3 ml 30% Acrylamide, 3.75 ml 1M Tris-HCl (pH 8.8), 33 μl 10% APS
(ammonium persulphate, 0.3 g in 2.5 ml ddH2O), 10 μl TEMED, and 2.9 ml H2O.

4% stacking gel (5 ml): 665 μl 30% Acrylamide, 625 μl 1M Tris-HCl (pH 6.8), 15 μl 10% APS, 5 μl
TEMED, and 3.690 ml H2O.

2.2.4.5 Western Blotting

Following electrophoretic separation on a 10% SDS-polyacrylamide gel, proteins were
transferred onto a PVDF (polyvinylidene fluoride, Millipore) membrane using Bio-Rad
Mini Transfer Blot apparatus and cold Transfer buffer [10 mM NaHCO3, 3mM Na2CO3,
pH 9.9 in 20% (v/v) methanol]. The PVDF membrane was prepared about 10 min before
the electrophoresis was completed. The membrane was first immersed in 100% methanol
for 30 sec to render the membranes hydrophilic, and then washed ddH2O for 2 min. After
that, the membranes together with 4 filter papers and 4 sponge pads were soaked in
chilled transfer buffer (prechilled at ~30 degrees). Removing the gels from
electrophoretic cell, a ‘sandwich’ transfer unit was made in order of sponge, filter paper, gel, PVDF membrane, filter paper, and sponge, with the PVDF membrane on the side of the anode of the system. The transfer was then carried out at 150 mA overnight at 4°C.

Proteins were detected using immunochemistry. The membranes were blocked at room temperature with agitation at least for 2 hours in 10% blocking solution [10% non-fat milk in TBST (25 mM Tris-HCl, pH 7.4, 137 mM NaCl, 3mM KCl 0.1% Tween 20 (v/v)]. The primary antibodies in a series of dilutions with 10% blocking solution were applied, and the membrane was incubated at room temperature overnight. The blots were then washed for 5 minutes in TBST five times. The membrane was incubated in the secondary antibody, a horseradish peroxidase-conjugated antibody in a proper dilution with blocking solution (1/7500), for 1 hour at room temperature, and washed with TBST five times to remove any traces of excess antibody.

Chemiluminescence was introduced to detect the horseradish peroxidase-conjugated antibodies and was performed using the ECL-detection system (Amerrham Pharmacia Biotech, UK). The blot was exposed to X-ray film, the film developed and distinctive dark bands observed under light.

The Western blotting technique was employed in our laboratory to validate the available antisera for TGFβ receptors-1, -2, -3, BMP receptors 1A, 1B, 2, Activin receptors 1(A), 1B, 2, and 2B. The result shows that all these antibodies are not specific to their corresponding antigens (part of the results shown in the Appendix 2). Thus, three commercial polyclonal antibodies specific for TGFβr-1, -2 and -3 (rabbit anti-TGFβ receptors 1, 2 and 3, Sigma) were obtained, two of which were validated by Western Blot technique. Using these antibodies, immunohistochemistry studies on mouse tissue sections were performed subsequently.

2.2.4.6 Immunohistochemistry for TGFβ receptors 1, 2 and 3

Adult mouse olfactory epithelium was prepared and sectioned as previously described. The tissue sections obtained from -80° freezer were left at room temperature for 2 h.
Sections were washed in PBS (23g Na₂HPO₄, 5.94 g NaH₂PO₄•2H₂O, and 90 g NaCl in 1 liter of water) for 3x5 min and incubated with PBS containing 0.3% H₂O₂ for 30 min, followed by another wash with PBS for 3 x 5min. Then sections were incubated with gentle agitation in PBS containing 2% BSA, 5% NFDM (nonfat dried milk), 0.1% Triton X-100 and 10% goat serum, for 1 h at room temperature and after that, in PBS containing 2% BSA, 5% NFDM (milk), 0.1% Triton X-100, 10% goat serum, primary antibodies (1/100 – 1/200 dilution of rabbit anti-TGFβ receptor 1, 2, or 3), for 1 h at room temperature. The blank control with no 1° antibody was prepared simultaneously. Following a wash in PBS for 3 x 5 min, sections were incubated in PBS containing 0.5% BSA, secondary antibodies (1/200, goat anti-rabbit IgG), for 1 h at room temperature, washed in PBS for 3 x 5 min, and incubated in avidin-biotin conjugate (ABC) solution (1/50 dilution, i.e., 20 μl A + 20 μl B + 980 μl PBS; prepared 1 h in advance), for 1 h at room temperature. Washed in PBS, 3 x 5 min, sections were color developed in DAB (PBS + 0.05% DAB + 0.004% H₂O₂) for less than 10 min and observed under microscope.

2.2.5 Competitive RT-PCR

2.2.5.1 IGF-1 treated human olfactory tissue

Human olfactory tissue was obtained with the donor’s permission from the donor undergoing routine nasal surgery after approval by Human Ethics Committee of Griffith University. Unlike the well established procedure for mouse olfactory tissue preparation, the preparation of human olfactory tissues is more sophisticated and the amount of the biopsies is limited, so histological confirmation of the tissue identity is not readily feasible considering the amount required for all processes of the competitive RT-PCR. The identification of the tissue was thus performed using two primers specifically for Olfactory Marker Proteins (OMPs; sequences of primers not shown).

The tissue was first sliced into 4 pieces of equal size. Two were incubated with 100 ng/ml IGF-1 in DMEM medium with 10% FCS at 37 °C for 4 hours and 48 hours, respectively
(T₄ and T₄₈) and the other two, serving as negative controls, were processed in parallel but without IGF-1 treatment (C₄ and C₄₈). The incubation times were determined from a published paper where IGF-1 has a greatest inducing effect on BMP-2 at the 4 hour incubation time point while for BMP-4 the optimal time point is 48 hour (Li et al., 1998).

### 2.2.5.2 Preparation of total RNAs

After IGF-1 treatment, total RNAs were extracted from the tissues and the concentrations were determined as previously described as previously described. The extracted RNAs were reverse transcribed into single-stranded cDNAs using 1st Strand cDNA Synthesis Kit (Boehringer). The purity/quality of the total RNAs were checked using GAPDH primers and no-RNA control (substitution of RNA with distilled water in the reverse transcription step).

### 2.2.5.3 Competitive PCR

Competitive PCR requires an internal reference DNA, known as competitive cDNA, to amplify along with target cDNA using the same primers. The competitive cDNAs against BMP 2 and BMP 4 cDNAs were previously constructed by digestion of a fragment of each target cDNA in pBluescript II KS- vector (Li, et al., 1998). The BMP 2 competitive cDNA was downsized to 618 bp from 818 bp. The BMP 4 competitive cDNA was downsized to 495 bp from 788 bp (Li et al., 1998).

For the competitive PCR, BMP 2 and BMP 4 target cDNAs were coamplified with dilutions of the competitor DNAs of known concentrations using the same oligonucleotide primers as below:

**Table 2-5 Primer sequences for both target cDNA and competitive cDNA** (Li et al., 1998)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence (5’Æ3’)</th>
<th>Product size with target cDNA (bp)</th>
<th>Product size with competitive cDNA (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMP2</td>
<td>Forward: 5’-GCCAGCCCGAGCCAAACACTGT-3’&lt;br&gt;Reverse: 5’-CGTCAAGGTACAGCATCGAG-3’</td>
<td>818</td>
<td>618</td>
</tr>
<tr>
<td>BMP4</td>
<td>Forward: 5’-ACTTCGAGGCGACACTTCTGC-3’&lt;br&gt;Reverse: 5’-CTGAAGTCCACATAGAGCGAGTG-3’</td>
<td>788</td>
<td>495</td>
</tr>
</tbody>
</table>

The primers and competitive cDNAs were kindly supplied by Ms Li, University of Queensland, St. Lucia, Queensland. The competitive cDNAs were validated by PCR.
using corresponding primers. The PCR reactions were carried out in a total volume of 20 μl, comprising 4 μl diluted competitive DNA (1/4 and 1/8 dilutions for BMP 2 assay; 1/1.6 and 1/3 dilutions for BMP 4 assay), 1x reaction buffer, dNTPs (0.4 mM), MgCl₂ (1.5 mM), 0.5 unit Taq DNA polymerase, and 0.2 mM of each primer. Amplification thermal cycle conditions were as follows: denaturation at 93°C for 3 min, followed by 35 cycles of denaturation at 93°C for 1 min, annealing at 60°C for 30 sec, and extension at 72°C for 1.5 min, followed by further extension at 72°C for 10 min.

For competitive PCR, the optimal dilutions were determined as 1 in 8 for BMP2 competitive cDNA and 1 in 3.2 for BMP 4 competitive cDNA because they were the points at which the unknown concentrations of BMP cDNAs are theoretically equal to the concentrations of their competitive cDNAs (Li et al., 1998). The PCR reactions were run simultaneously in a total volume of 20 μl, comprising target cDNA (4 μl, from 0.2 μg RNA), diluted competitive DNA (4 μl), 1x reaction buffer, dNTPs (0.4 mM), MgCl₂ (1.5 mM), 0.5 unit Taq DNA polymerase, and 0.2 mM of each primer. The amplification thermal cycle conditions were set as described above. After the competitive PCR reactions, products were electrophoresed in 1% agarose gel and photographed. The amounts of target DNA and competitive DNA present in each band were determined by densitometry. The level of induced BMP can be investigated by investigating the change in the ratio of target cDNA to competitive cDNA.
2.3 Results

2.3.1 RT-PCR detection of TGFβ receptors

2.3.1.1 Electrophoresis of total RNAs from different adult mouse tissues

Total RNAs were prepared from adult mouse tissues of OE, olfactory bulb, brain, heart, liver, lung, spleen, kidney, and muscle, and sent to spectrophotometry. The optic density (ODs) readings are listed below (Table 2-6):

Table 2-6. Spectrophotometry of total RNAs from different mouse tissues*

<table>
<thead>
<tr>
<th>Tissues</th>
<th>OD\textsubscript{260}</th>
<th>OD\textsubscript{280}</th>
<th>OD\textsubscript{260}/OD\textsubscript{280}</th>
<th>Sample Conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>OE</td>
<td>1.657</td>
<td>1.435</td>
<td>1.15</td>
<td>3.30 μg/μl</td>
</tr>
<tr>
<td>OLF. Bulb</td>
<td>0.624</td>
<td>0.349</td>
<td>1.78</td>
<td>1.25 μg/μl</td>
</tr>
<tr>
<td>Brain</td>
<td>0.604</td>
<td>0.367</td>
<td>1.65</td>
<td>1.21 μg/μl</td>
</tr>
<tr>
<td>Heart</td>
<td>0.480</td>
<td>0.313</td>
<td>1.54</td>
<td>0.96 μg/μl</td>
</tr>
<tr>
<td>Muscle</td>
<td>0.514</td>
<td>0.283</td>
<td>1.82</td>
<td>1.03 μg/μl</td>
</tr>
<tr>
<td>Liver</td>
<td>1.596</td>
<td>1.156</td>
<td>1.38</td>
<td>3.20 μg/μl</td>
</tr>
<tr>
<td>Lung</td>
<td>0.958</td>
<td>0.812</td>
<td>1.18</td>
<td>1.92 μg/μl</td>
</tr>
<tr>
<td>Kiney</td>
<td>1.325</td>
<td>0.973</td>
<td>1.36</td>
<td>2.65 μg/μl</td>
</tr>
<tr>
<td>Spleen</td>
<td>1.496</td>
<td>1.004</td>
<td>1.49</td>
<td>2.99 μg/μl</td>
</tr>
</tbody>
</table>

*Samples were read in 1/50 dilution with dH\textsubscript{2}O.

10 μg of each RNA extract was initially run in a formaldehyde denaturing gel (result not shown), where lung and liver samples did not demonstrate the typical rRNAs of 28S and 18S but a bright trace, which indicates the samples may be RNAse-contaminated. Because the purity of RNA preparations was very low according to the ratio of OD\textsubscript{260}/OD\textsubscript{280} in Table 2-6, the preparations were then re-purified with isopropanol. The readings after re-purification is listed Table 2-7.

Table 2-7. Spectrophotometry after re-purification of total RNAs from different mouse tissues*

<table>
<thead>
<tr>
<th>Tissues</th>
<th>OD\textsubscript{260}</th>
<th>OD\textsubscript{280}</th>
<th>OD\textsubscript{260}/OD\textsubscript{280}</th>
<th>Sample Conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>OE</td>
<td>0.671</td>
<td>0.363</td>
<td>1.85</td>
<td>1.34 ug/ul</td>
</tr>
<tr>
<td>Olf. bulb</td>
<td>0.473</td>
<td>0.253</td>
<td>1.87</td>
<td>0.94 ug/ul</td>
</tr>
<tr>
<td>Brain</td>
<td>0.558</td>
<td>0.313</td>
<td>1.78</td>
<td>1.12 ug/ul</td>
</tr>
<tr>
<td>Heart</td>
<td>0.161</td>
<td>0.097</td>
<td>1.67</td>
<td>0.32 ug/ul</td>
</tr>
<tr>
<td>Muscle</td>
<td>0.385</td>
<td>0.219</td>
<td>1.76</td>
<td>0.77 ug/ul</td>
</tr>
<tr>
<td>Kiney</td>
<td>0.461</td>
<td>0.243</td>
<td>1.90</td>
<td>0.92 ug/ul</td>
</tr>
<tr>
<td>Spleen</td>
<td>&gt; 3.0</td>
<td>2.717</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*Samples were read in 1/50 dilution with dH\textsubscript{2}O.
2.3.1.2 RT-PCR screening for TGFβ and BMP receptors

OLF442 cell line was cultured in DMEM with 1% BSA or 10% FCS (for differentiated OLF442 and undifferentiated OLF442 cells, respectively) as described previously in sections 2.2.2.2 and 2.2.2.3. Total RNAs of the cells were extracted by quanidinium thiocyanate, the RNA was quality checked and quantified as previously described in sections 2.2.3.2 – 2.2.3.4. Total RNAs of OLF442 cells and various mouse tissues were then reverse transcribed into single stranded cDNAs, which were later used for PCR and multiplex PCR reactions. The PCR or multiplex PCR conditions were as described in Table 2-4.

In Figure 2-1, the mouse brain (Br) and undifferentiated OLF442 cells were used to test the quality of the primers designed in the RT-PCR reactions. The top two gels in Figure 2-1 show that the primers designed for TGFβr-1, -2, -3, BMPr-1A, -1B, and -II are specific. Note that the electrophoretic bands of the foremost row, sized about 50 bp, are primer dimers.

In the RT-PCR assay, a serious problem occurred due to the low yield of total RNAs from olfactory epithelium and lamina propria, which made the experiments very time consuming and costly (RT-PCR results for individual TGF receptor investigation are not shown here). A multiplex RT-PCR was then introduced on the basis that primers for TGFβ and BMP receptors produce PCR products of different size but have similar GC contents and melting temperatures (see the bottom two gels in Figure 2-1).

In the multiplex RT-PCR result, all receptors are found to be expressed in various mouse tissues including the olfactory epithelium, lamina propria, olfactory bulb, brain, differentiated and undifferentiated OLF442.
Figure 2-1. Expression of TGFβ and BMP receptors. This figure includes RT-PCR (top two gels) and Multiplex RT-PCR (bottom two gels) detecting signals of mRNA expression of TGFβ and BMP receptors in mouse brain (Br), olfactory bulb (OB), olfactory epithelium (OE), lamina propria (LP), differentiated (D) and undifferentiated (U) OLF442 cells. 1= TGFβ receptor 2 (312 bp), 2=TGFβ receptor 1 (252 bp), 3= TGFβ receptor 3 (150 bp), 4=BMP receptor 2 (368 bp), 5= BMP receptor 1B (226 bp), 6= BMP receptor 1A (151 bp), M= 100 bp marker. All primers were validated and optimised using mouse brain tissue and undifferentiated OLF442 cells (top two gels). The bottom two gels have shown that TGFβ receptor type 1, 2, 3, BMP receptor type 1A, 1B and 2 are expressed in mouse olfactory epithelium, lamina propria, and olfactory bulb. The bottom bands of all gels are primer dimers.
Figure 2-2. Expression of TGFβ ligands and Activin receptors. RT-PCR signal of mRNA expression of TGFβ 1, 2, 3 (TGFβ-1, -2, and -3, respectively) and Activin receptors 1, 1B, 2, and 2B (ActR 1, 1B, 2, and 2B, respectively) in mouse olfactory epithelium (OE) and lamina propria (LP). Product sizes: TGFβ-1= 431 bp, TGFβ-2= 420 bp, TGFβ-3= 415 bp, ActR1= 150 bp, ActR-1B= 353 bp, ActR-2= 180 bp, ActR-2B= 321 bp. Water controls (W) were included in each batch of RT-PCR experiments where M is 100 bp marker. Bottom bands of the gels are primer dimers (not shown in the top right gel).
2.3.1.3 RT-PCR screening for ActR-1, -1B, -2, -2B, TGFβ-1, -2, and -3

To detect ActR-1, -1B, -2, -2B (receptors), TGFβ-1, -2, and -3 (ligands) in mouse olfactory epithelium and lamina propria, olfactory epithelium mucosa was removed from adult mice and the olfactory epithelium was separated from lamina propria as described in section 2.2.1. Total RNAs of the tissues were extracted, purified, quantified, reverse transcribed, and signal amplified using polymerase chain reaction as described. The primers used are listed in Table 2-2. Water controls were included to exclude non-specific amplification. In Figure 2-1, the RT-PCR result shows that all PCR products are of the right size on the agarose gels, indicating that all receptors of interest are expressed in both mouse olfactory epithelium and lamina propria.

2.3.2 Western Blotting

The concentrations of antisera for TGFβ receptors 1, 2, 3, BMP receptors 1A, 1B, 2, and Activin receptors 1(A), 1B, 2, and 2B were constructed in duplicate in a set of dilutions with 10% blocking solution as described previously: 1/1000, 1/300, 1/5000, and 1/7000. Since the result showed that all these antibodies were not specific to their corresponding target receptors (Some of the results are shown in the Appendix 2), three commercial polyclonal antibodies specific for TGFbr1, 2 and 3 (rabbit anti-TGFb receptors 1, 2 and 3; Sigma) were therefore introduced for the immunohistochemistry study. Anti-TGFbr 1 and 2 antibodies were validated by Western Blot technique (Figure 2-3). In figure 2-3, the TGFbr-1 and TGFbr-2 measure ~60 kD and 80 kD, which are at the predicted sizes for TGFβ receptor type 1 (~53 kD) and TGFβ receptor type 2 (70-85 kD) (Kingsley, 1994). Using these antibodies, immunohistochemistry studies on mouse tissue sections were performed subsequently (Figure 2-4). Because TGFβr-3 has a high molecular weight (280-330 kd; Wang et al., 1991) which is over the limit of the marker protein we used (It only measures up to 173 kd), the Western Blotting for TGFβr-3 was not performed.
Figure 2-3. Western blotting for TGFβ receptors. Western Blotting signals of TGFβ receptor type 1 and type 2 (TGFβr 1 & TGFβr 2) in mouse olfactory tissues. The sizes of TGFβr 1 and TGFβr 2 are approximately 60 kD and 80 kD, respectively. The optimal dilutions of primary antibodies towards the receptors are 1 in 3000 for TGFβr 1 assay and 1 in 5000 for TGFβr 2 assay.
2.3.3 Immunochemistry

The immunohistochemistry result shows that TGF β receptor 1 is expressed by a variety of cells in the olfactory system including neurons, globose basal cells (judging by the position of the cells), and cells in the lamina propria (Figure 2-4). Whether the labeled basal cells are globose basal cells needs to be confirmed by double labeling. An outstanding labeling is found in the neuronal layers in the olfactory epithelium, and axon bundles in lamina propria. The labeling of dendrites of olfactory receptor neurons is clearly seen. The control assay using the primary antibody previously incubated with blocking peptide shows a significantly reduced intensity of staining (data not shown). The blank control using water instead of the primary antibody remains clear. TGF β receptor 2 is abundantly expressed in olfactory epithelium and lamina propria. However, in contrast to TGFβr 1, labeling of the neurites of neurons and the axon bundles in lamina propria is not as strong. The ensheathing cells surrounding the axon bundles in lamina propria are more prominently labeled in TGFβr 1 study. Similarly, TGFβ receptor 3 is richly expressed in the olfactory mucosa. In some areas of the olfactory epithelium the receptor is diffusely distributed over the cell surface rather than the nuclear region (Figure not shown), which may be associated with its accessory function as a cell surface TGFβ–binding protein to modify the signaling pathways on the cell surface (Caestecker, 2004).
Figure 2-4. Expression of TGF β receptors. Expression of TGF βr 1 (A), TGF βr 2 (B), and TGF βr 3 (C) in adult mouse olfactory mucosa using immunohistochemistry. The result shows that these TGFβ receptors are abundantly expressed in adult mouse olfactory epithelium and lamina propria, especially in olfactory receptor neurons (ORN). TGF βr 1 is strongly expressed in ensheathing cells (white arrowheads) and nerve bundles (NB) in lamina propria where TGF βr 2 and TGF βr 3 have similar expression patterns. The supporting cells (white arrows) and horizontal basal cells of the basement membrane (black arrowheads) do not express any of TGF βr 1, TGF βr 2, and TGF βr 3. The three receptors are richly expressed in cilia of olfactory neuron (black arrows). Bars = 20 μm. D = Water Control (replacement of primary antibodies with water.
2.3.4 Competitive PCR for the effect of IGF-1 on BMP 2 and BMP 4

mRNAs can be semi-quantified using competitive PCR. The method of competitive PCR involves coamplification of two templates: the desired target sequence and a control template using the same primers where the control template must be distinguishable from the target sequence (Sambrook, et al., 1989). The control template is added in known amounts to a series of amplification reactions and should differ from the target sequence as little as possible to minimize systematic differences in the efficiency of amplification (Sambrook, et al., 1989). The relative amounts of the two amplified products therefore reflect the relative concentration of the control and target sequences in the original reaction mixture (Sambrook, et al., 1989).

In our assay, we used one competitive cDNA specific for each of BMP2 and BMP4 as the control templates which both were kindly supplied by Miss Li, H. (Li, et al., 1998). To obtain the target cDNAs, the human nasal biopsies were first processed as in section 2.2.5.1, then dissolved in solution D and homogenised by vigorous vortex. Total RNAs of IGF-1 treated test samples (samples treated with IGF-1 for 4 hours, $T_4$, and samples treated with IGF-1 for 48 hours, $T_{48}$) and control samples (samples with no IGF-1 treatment for 4 hours, $C_4$, and samples with no IGF-1 treatment for 48 hours, $C_{48}$) were extracted, quantified, and reverse transcribed as described in sections from 2.2.3.2 to 2.2.3.5.

Our initial preparations of the RNA samples were first decomposed by RNAse, which was detected by electrophoresis of total RNAs, and later suffered from genomic DNA contamination, which was identified, using various strategies including RNAse H digestion (Promega) and water control, as from Mini-Q water (these data are not shown here). Since the genomic contamination did not occur in our RNA extraction process, we checked the final preparation of test and control samples for the RNAse contamination using GAPDH primers (product size = 265 bp) in the PCR amplification reaction. as described in Table 2-4. To prevent the genomic DNA contamination from water, we used...
autoclaved water throughout the experiment and included a water control (substitution of total RNAs with autoclaved distilled water) in parallel with the sample batch. Our result shows that a consistent band sized 265bp appeared in each positive lane and no band was found in any water control, suggesting there was no RNAse contamination in the samples and no genomic DNA contamination in the water (see the top right gel of the Figure 2-5).

In a separate PCR reaction (see Chapter 2 for conditions), BMP2/4 competitive cDNAs and their specific primer pairs (see Table 2-5) were validated. The bottom left gel of the Figure 2-5C shows that BMP2 and BMP4 PCR products were both in their correct size (618 bp and 495 bp respectively), suggesting the specific primer pairs for BMP2 and BMP4 were working properly with their corresponding cDNAs. However, there was no reaction when target cDNAs were brought to interact with the same primer pairs on the same PCR conditions. To test the expression of BMP2/4/7 in the human olfactory mucosa, three different pairs of primers were specifically designed for each of the BMPs in an RT-PCR study but there was no signal detected (data not shown). In the RT-PCR identification of the human biopsies using two different pairs of primers against OMPs, we also failed to see the expression of OMPs in the tissues (data not shown). A possible explanation for these is that the dissection of the tissue was harder to perform in a living person than in a sacrificed mouse, so it is likely the tissues we used contained no olfactory components at all. Nevertheless, because of the limited amount of human tissues, we could not confirm the absence of OMPs in the tissues with proper controls.
Figure 2-5: Competitive PCR for BMP2/4. A: RNase detection using GAPDH primers on the RT-PCR conditions, where $C_4$ = BMP2 sample incubated without IGF-1 for 4 h, $T_4$ = BMP2 sample incubated with IGF-1 for 4 h, $C_{48}$ = BMP4 sample incubated without IGF-1 for 48 h, $T_{48}$ = BMP2 sample incubated with IGF-1 for 48 h. Product size = 265 bp. B: quality control for total RNAs, where $W$ = water control (using GAPDH primers but missing out the total RNAs in the RT-PCR). $C_{4g}$ = $C_4$ using GAPDH primers, $C_{48g}$ = $C_{48}$ using GAPDH primers, $T_{4g}$ = $T_4$ using GAPDH primers, $T_{48g}$ = $T_{48}$ using GAPDH primers. Product size = 265 bp. C: Quality check for BMP competitive cDNAs and primers. BMP2 competitive cDNA was diluted in 1/16 and 1/8 with distilled water. BMP4 competitive cDNA was diluted 1/6 and 1/3 with distilled water. Both competitive cDNAs were kindly supplied by Ms Li, University of Queensland, Brisbane, Australia. Left two bands are sized 618 bp (BMP2). Right two bands are sized 495 bp (BMP4). M = 100 bp ladder.
2.4 Discussion

This study showed that the mouse olfactory epithelium and lamina propria expressed the mRNAs for TGFβr-1, -2, and –3, BMPr1A, 1B and 2. These receptors were also expressed in both differentiated and undifferentiated OLF442 cells. The immunochemistry showed the proteins for TGFβr-1, -2, and –3 were expressed by olfactory receptor neurons and presumed globose basal cells in the olfactory epithelium and by ensheathing cells, nerve bundles in the lamina propria. The immunochemistry failed to identify the cells expressing BMPr1A, 1B, 2, and ActR 1(A), 1B, 2 and 2B due to the non-specific antisera used. However, mRNAs for ActR 1(A), 1B, 2 and 2B were found to be expressed in the olfactory epithelium and lamina propria by RT-PCR. This study also showed that the olfactory mucosa expresses mRNAs for growth factor ligands for TGFβ-1, -2, and -3. The competitive PCR failed to show the induction of BMP2 and BMP4 by IGF-1 in the human olfactory tissues.

2.4.1 TGFβ growth factor 1, 2 and 3

From our results, TGFβ growth factors 1, 2, and 3 are all expressed in olfactory epithelium. The immunochemistry has also shown that TGFβ receptors 1, 2 and 3 are also expressed in the area. Receptor-ligand studies have revealed that the TGFβ growth factors bind to the constitutively active TGFβ receptor 2, which then recruits the TGFβ receptor 1, resulting in trans-phosphorylation of the type 1 receptor and activation of downstream signals (Caestecker, 2004). The present report here has indicated paracrine and/or autocrine action for the growth factor signaling. The presence of the high molecular weight TGFβ receptor 3 has implicated its required existence as a co-receptor for supporting the TGFβ ligand (Blobe et al., 2001), or promoting the TGFβ ligand binding to the signaling receptors (Lopez-Casillas et al., 1993), or for inhibin promoting interactions between inhibin and the type 2 receptors, ActR 2, ActR 2B and BMPr 2, which are all expressed in the same areas as well (Caestecker, 2004).
2.4.2 Activin receptors

Since the Activin receptors, including AcR1A, AcR1B, AcR2(A) and AcR2B, bind to many different TGFβ ligands including TGFβ ligands, Activin A, MIS, BMP2, BMP6, BMP7, Nodal, GDF1, GDF5, GDF8, GDF11, and Inhibin A/B (see Table 2-1), the presence of all types of Activin receptors in the olfactory epithelium indicates the olfactory neurogenesis may involve those ligands. For example, one of many possible pathways is through Activin. The Activin signals through these serine-threonine kinase receptors 1 and 2. Binding of Activin induces the formation of heteromeric complexes of these receptors, and signaling is initiated when receptor 0 is phosphorylated and activated by receptor 2. The activated receptor 1 phosphorylates SMAD2. Activated SMAD2 associates with SMAD4 and mediates nuclear translocation of the heteromeric complex to activate specific gene transcription (Shimizu & Gurdon, 1999). Although the signaling mechanisms of Activin ligand/receptor are poorly understood in the olfactory system, there is evidence showing that GDF11, one of those ligands for Activin receptors, is expressed in nasal epithelium (Wu et al., 2003; Nakashima, et al., 1999).

2.4.3 The TGFβ receptor family

For the TGFβ receptor family, there are some issues here to be addressed. First, the expression of TGFβr-1, and -2 in the presumed globose basal cells has provided the site of action for TGFβ-2 in our working model to signal in the olfactory epithelium (Newman et al., 2000). That is, FGF2 stimulates proliferation of globose basal cells and TGFβ-2 binds the TGFβ receptor complex to in turn induce the differentiation of the FGF2-induced globose basal cells to promote neurogenesis. In addition, the expression of TGFβ-1, -2, and -3 in the olfactory epithelium implicates that TGFβ growth factors and receptors may follow an autocrine and/or paracrine action mode to signal and regulate neurogenesis.

Second, to study the TGFβ signaling pathways, it is inevitable to further study how the signals were generated and how they varied on different conditions. At our hands, the co-
expression of two types of TGFβ receptors in the olfactory epithelium and other tissues has supported the essential requirement of a receptor complex in the TGFβ signaling pathways. A depiction for the signaling pathways was the combinatorial theory by which different compositions of ligand-receptor complexes produce different responses and the type 1 receptor kinase plays a key role to determine the nature of the responses (Massague, 1996). As an example, TGFβr-1 and ActR-1B, both having a nearly identical kinase domain, can generate the same set of responses even if their respective ligands, type 1 receptors, and extracellular domains are quite different (Massague, 1996). However, the key role of receptor type 1 in generating specific signals was undermined by the evidence that the different responses could be generated by addition of a BMP ligand to preformed receptor complexes and by addition of the BMP ligand before the complexes form (Nohe et al., 2002).

The TGFβ signaling pathways may be far more complicated than imagined. The TGFβ family ligands have different affinities for different types 1 and 2 receptor combinations (See Table 2-1). TGFβ binds to the active TGFβ type 2 receptor, TGFβr-2, and then recruits the TGFβr-1 resulting in trans-phosphorylation of the type 1 receptor and activation of downstream signals (Casestecker, 2004). Activin, BMP6, and BMP7 have similar mechanisms (Attisano, et al., 1996; Ebisawa, et al., 1999). In contrast, BMP2 and BMP4 preferentially bind to the type 1 receptors BMPr-1A and BMPr-1B and recruit type 2 receptors into heteromeric signaling complexes (Reviewed by Caestecker, 2004).

In addition to the combinatorial model, the TGFβ signaling pathways were reported to be regulated by the concentrations of the receptors or the ligands. For example, it has been reported that the type 1 and 2 receptors that would normally not associate in the cell would spontaneously do so when the receptors are overexpressed (Shimizu & Gurdon, 1999). Another example is a finding for Activin that increasing occupancy of a single receptor type can cause cells to switch gene expression (Dyson & Gurdon, 1998).
The complexity of the combinatory system and/or the concentration effect is likely to explain the diversity of downstream TGFβ responses that can be generated by engagement of the receptors in different cell types (Caestecker, 2004). A future challenge for studying TGFβ signaling pathways in neurogenesis may be the studies for the functional significance of many different receptor-ligand combinations and for the quantification of expression of TGFβ superfamily in the olfactory system. In either case, the expression investigations in the olfactory system, particularly the in situ studies, are fundamentally important due to the insufficient information available to date.

2.4.4 Controls of RT-PCR
Controls are crucial in determining the quality of the samples (RNAs), water or procedures (RT or PCR reactions). Wrong interpretation on the controls may lead to wrong interpretation on the result thus leading to wrong hypothesis.

The positive control we used in RT-PCR was GAPDH primers against sample cDNAs, which is able to exclude RNAse contamination if no genomic DNA contamination is certain. The GAPDH primer pair was previously designed for the housekeeping gene, GAPDH, from human sequence, which was found to work across many other species and be able to endure a wide range of PCR conditions with the annealing temperature ranging from 45 to 68°C.

RNAse and water controls are negative controls for RT-PCR. In the RNAse control, the total RNAs are digested with RNAse and then run in parallel with the standard samples. This treatment allows detection of genomic DNA contamination in the samples if there is any. In comparison, the water control leaves out the addition of total RNAs in a standard batch but uses suspicious water source instead, serving to test if genomic DNA contamination exists in the water source. In these positive and negative controls, GAPDH primers instead of target primers are used. Listed in Table 2-8 is a suggested control scheme to test the RNA samples for RT-PCR studies, which might be particularly useful for investigating samples with limited availability.
Table 2-8 A scheme of quality controls used in RT-PCR reactions

<table>
<thead>
<tr>
<th>GAPDH</th>
<th>RNAse digestion</th>
<th>Water</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>-</td>
<td>-</td>
<td>RNA validated and no genomic DNA contamination in sample and water</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>-</td>
<td>RNAse contamination or RT failure</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Genomic DNA contamination in water</td>
</tr>
<tr>
<td>+</td>
<td>-</td>
<td>+</td>
<td>Technical errors</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>-</td>
<td>Genomic DNA contamination in sample</td>
</tr>
<tr>
<td>-</td>
<td>+</td>
<td>+</td>
<td>Technical errors</td>
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<tr>
<td>-</td>
<td>+</td>
<td>-</td>
<td>Technical errors</td>
</tr>
</tbody>
</table>

Note that all above three controls use GAPDH primers against the RNA samples in the PCR reactions, only leaving one condition different from the others. The ‘GAPDH’ control is a standard, while the ‘RNAse digestion’ control allows RNAse digestion before RT-PCR and the ‘water control’ uses water instead of RNA samples in the RT-PCR. + means appearance of expected band. – means no PCR product detected.
Chapter three

Identification and characterization of FGF growth factors and receptors in olfactory mucosa
3.1 Introduction

3.1.1 Fibroblast growth factors family

Fibroblast growth factors (FGFs) are a family including at least 23 peptide growth factors which interact with four tyrosine kinase receptors, FGFr 1-4 (Wiedlocha & Sorensen, 2004; Ohbayashi et al., 1998; Ornitz el al., 1996; Ozawa et al., 1996). Alternative splicing leads the four receptors to seven isoforms that bind FGF ligands with varying affinity (Ornitz et al., 1996). The seven isoforms differ from each other in the third extracellular immunoglobulin-like domain which changes the affinity of receptor for different FGFs (Hsu et al., 2001). FGF2 has highest specificity for FGFr1c, FGFr3c and FGFr4 (Ornitz et al., 1996).

Structural studies reveal that FGF2 is a single chain peptide composed of 146 amino acids that can also exist in a truncated form missing the first 15 amino acids. FGF1 is a 140-amino acid peptide that can also exist in a truncated form missing the first 6 amino acids (Gospodarowicz et al., 1987). The DNA sequences which code for FGF1 and FGF2 are more homologous than their protein counterparts, suggesting that the two proteins evolved from a common ancestral gene. Both lack a signal peptide consensus sequence that could account for their secretion from cells (Sporn and Roberts, 1991). FGF-1, FGF-2, FGF-3, and FGF-11-14 have been found intranuclearly as endogenous proteins. Exogenous FGF-1 and FGF-2 are internalized by receptor-mediated endocytosis, in a clathrin-dependent and -independent way (Reviewed by Wiedlocha & Sorensen, 2004). FGFs are reported to be synthesized in many cell types such as fibroblasts, vascular and capillary endothelial cells, smooth muscle cells, granulosa cells, adrenocortical cells, and astrocytes grown in cell culture. A large number of tumour cells, including those from glioma, rhabdomyosarcoma, leukemia, heptoma, and melanoma, are also known to synthesize and respond to FGFs. FGF2 is found nearly everywhere in adult body tissues but more abundantly distributed in pituitary tissues (Heath, 1993; Sporn and Roberts, 1991).
FGFs have been implicated in regulation of many key cellular responses involved in developmental and physiological processes, including proliferation, differentiation, migration, apoptosis, angiogenesis, and wound healing (Reviewed by Wiedlocha & Sorensen, 2004). Many reports suggest that FGF2 functionally acts as both a mitogen and a morphogen which stimulate proliferation and induce/delay the differentiation of neurons and their precursors in peripheral and central nervous systems or other tissues (Gensburger et al., 1987; Unsicker et al., 1987; Caday et al., 1990; Murphy et al., 1990; Kilpatrick et al., 1993; Dehamer et al., 1994; Calof, 1995; Murrell et al., 1996). The mitogenic role of FGF2 seems to be more predominant according to the evidence shown in many of these studies. However, there was evidence suggesting FGF2 suppressed neurogenesis in a cell line (Goldstein et al., 1997). It is evident that the effect of FGF2 is dose-dependent. With regard to differentiation, it is noteworthy that in presence of FGF2 in a low concentration, a cortical stem cell gives rise to neurons while in a higher concentration the stem cell gives rise to a non-neuronal glia cell (Qian et al., 1997). Moreover, the complexity of the action mechanism of FGF2 is also mirrored to a discovery that the expression of FGF ligands and their receptors varies significantly in different stages of a developing mouse brain (Ozawa et al., 1996).

FGFr 1 was found to be expressed in other tissues but not in the olfactory epithelium (Key et al., 1996). One study reported that FGF2 was expressed predominantly in the nuclei of supporting cells and olfactory receptor neurons (Goldstein et al., 1997) while another study showed that expression of FGF2 was regionally variable but when present there was extensive expression throughout the epithelium (Chuah and Teague, 1999). These two reports were in conflict perhaps because of differences in the age of the animals. But in either case, the identity of the cells producing FGF2 is unclear. Similarly, although FGFr-1 and FGFr-2 mRNA transcripts were identified in the olfactory extracts, the identity of the cells expressing receptors for FGF2 is not known (DeHamer et al., 1994).
3.1.2 Chapter aim

The objectives of this chapter are (1) to identify whether the FGFr-1 is present in the olfactory epithelium and to identify which cells express this receptor, (2) to identify cells expressing FGF2, and (3) to identify whether the olfactory cell line, OLF442, expresses FGF2. A paper based partly on the results of this chapter has been published (Hsu et al, 2001)
3.2 Methods and materials

3.2.1 In situ hybridisation

3.2.1.1 RNase-free management for in situ hybridisation

In situ hybridisation involving RNA requires strict RNase-free management to succeed. To deactivate RNAse, 0.5 – 0.1% diethyl pyrocarbonate (DEPC) was added in all solutions except for Tris-containing buffers. After overnight incubation at 37°C, the DEPC-treated solutions were autoclaved at 121°C for 45 min. Because DEPC can react with amines rapidly (Sambrook, et al., 1989), solutions containing Tris such as Buffer 1, 2, and 3 (see the following procedures) were prepared by DEPC-treated water and autoclaved. Glassware, plastic pipettes, tubes were autoclaved at 121°C for 45 min. Equipment which is not suitable for autoclaving was washed by 100% ethanol for 2 min and by 3% of H₂O₂ for 2 min. 2% SDS was then added into hybridization buffer to inhibit the RNAse.

3.2.1.2 Oligonucleotide probe

Oligoprobes have advantages of good tissue penetration, availability and single-stranded nature although published sequences are not always available and they may cause unstable hybrids. In the case of FGFr 1 mRNA detection in the olfactory epithelium, The ideal length of an oligoprobe is 24-45 nucleotides (Stahl et al., 1993). Based on the principle, two complementary oligonucleotide sequences (34-mer) we used were selected from one of the conserved regions in the mouse basic FGF receptor cDNA sequence (GenBank accession numbers: U22324, M28998, M33760, and M65053; see the following text for the probe sequences). This reserved region is considered as the potentially best target among the others for oligoprobe because it contains 33 nucleotides with a rather random distribution of nucleotides and has a high GC content (70%) which can be technically avoided. To reduce the GC content to the acceptable level of 50-60% (Stahl et al., 1993), four nucleotides (GGTG) were eliminated on the right hand side and 5 nucleotides (TGTATA), which were found identical among three isoforms of basic FGF receptor, were added on the left hand side to form a penetrative oligoprobe sequence.
(34-mer; Stahl et al., 1993) with a final GC content of ~53% but without any inclusion of polyG (more than GGG in a row) which favours the formation of probe tetramers (Sen and Gillett, 1988).

### 3.2.1.3 Oligonucleotide and RNA probes

To detect the FGFr-1, which has various binding affinity with FGFs (Ornitz et al, 1996), an anti-sense oligoprobe was specifically designed on published sequences (U22324, M28998, M65035, and M33760) and its sense sequence was used as a negative control. The oligonucleotide sequences were chemically synthesized by Pacific Oligos Pty. Ltd. and are listed below:

**Antisense:** 5′-GGACGTTCCTAGCAGCCAGGTCTCGGTGTATACA-3′

**Sense:** 5′-TGTATACACCGAGACCTGGCTGCTAGGAACGTCC-3′

For detection of FGF2 in the olfactory mucosa and the OLF442 cell line, an RNA probe, previously synthesized by vitro transcription of 184 bp of mouse FGF2 cDNA (M30644, Genbank; supplied by Kyra Sneesby, 1998) cloned downstream of T7 and SP6 RNA polymerase promotors in pBluescriptII SK (+/-), was used. Sense sequence of the RNA fragment was used as the negative control.

### 3.2.1.4 Labelling with DIG-ddUTP

Oligonucleotide sequences for FGF1 detection were enzymatically labeled at their 3’-end with terminal transferase by incorporation of a single digoxigenin-labeled dideoxyuridine-triphosphate (DIG-ddUTP; Boehringer Mannheim, DIG oligonucleotide 3’-end labeling kit).

Briefly, a mixture containing 5x reaction buffer (vial 1), 4 μl CoCl₂ solution (vial 2), 100 pmole oligonucleotide, 1 μl DIG-ddUTP solution (vial 3), 1 μl (50 units) terminal transferase (vial 4) was prepared in a tube with proper amount of distilled water to make up to the final volume of 20 μl. The mixture was incubated at 37°C for 15 min, and then placed on ice. The labeling reaction was stopped by 2 μl working glycogen solution [1 μl glycogen solution (vial 8) in 200 μl 0.2 mM EDTA solution, pH 8.0] for 5 minutes. Then
the labeled oligonucleotide was precipitated with 2.5 μl 4M LiCl and 75 μl prechilled (-20°C) absolute ethanol at –70°C for 2 hours. After a 30-min centrifugation at 14000 rpm, the pellet was washed with 50 μl cold ethanol (70% v/v), vacuum dried and stored at –20°C.

For the riboprobes, FGF2 antisense and sense sequences were labeled with digoxigenin (DIG) via random incorporation of DIG-UTP during transcription. The specificity of the probe was determined by Northern Hybridisation and sequencing (done by Kyra Sneesby, 1998). Probe labeling was confirmed by dot blot analysis (see Appendix 3 for the result).

3.2.1.5 Dot Blot test for RNA and oligonucleotide probes

To validate the DIG-labeled RNA and oligonucleotide probes, and to optimize the working concentration of the probes, a Dot Blot test (Boehringer Mannheim) was performed on the labeled probes in parallel with controls. For each riboprobe, a diluted concentration series of 100, 10, 1, and 0.1 pg/μl was established with DEPC-treated H₂O in different tubes. For each oligoprobe or control, a diluted concentration series of 0.5, 0.1, 0.02, and 0.004 pmoles/μl was prepared with dH₂O. The controls used were labeled and unlabeled oligonucleotides supplied by Boehringer Mannheim in the DIG oligonucleotide 3’-end labeling kit.

On a nylon membrane (Hybond™, Amersham), 1 μl of each diluted probe or control preparation was spotted in a row in a decreasing order of concentration from left to right. The membrane was air-dried for 30 min and then DNA or RNA was cross-linked to the membrane under UV light for 3 min. Following that, the membrane was washed with buffer 1 in a box. Removing the wash buffer, the membrane was incubated in 20 μl blocking solution at room temperature for 30 min. The blocking solution was decanted and the filter incubated in 20 ml antibody solution (1/5000 anti-DIG-phosphatase conjugate in blocking solution) at room temperature for another 30 min. After removing the antibody solution, the membrane was washed with buffer 1 for 15 min twice on a shaker and then incubated in 20 ml buffer 2 for 5 min. Finally, the membrane was covered by 25 ml of color substrate solution [200 μl NBT/BCIP (Boehringer Mannheim, Cat.
No. 1175041) in buffer 2) in the dark for 13 hours. To avoid excessive background resulting from over-coloration, the color reaction was checked every 1-2 hours. When the desired colour intensity was observed, the reaction was stopped with 20 ml buffer 3 for 2x 15 min. The quality and efficiency of labeling and the valid optimal concentrations were determined after the membrane was air dried completely (see Appendix 3 for the result).

3.2.1.6 In situ hybridisation

(A) FGFr-1 in olfactory mucosa

ISH using FGFr 1 oligoprobe on mouse sections followed the protocol below (Nonradioactive In Situ Hybridization Application Manual, 2nd edit., Boehringer Mannheim GmbH, Biocheica, 1996):

Cryostat sections were left at room temperature for 15 min and in a 40°C oven for 2 h. The sections were washed with RNAse-free PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na₂PO₄, pH 7.4) for 2 x 10 min and incubated in RNAse-free PBS containing 0.3% Triton X-100 for 15 min. Following a wash with RNAse-free PBS, 2 x 10 mins, the sections were postfixed at 4°C in 4% paraformaldehyde in RNAse-free PBS for 5 min and then washed with RNAse-free PBS for 2x10 min.

For prehybridisation and hybridisation, the sections were overlaid with prehybridization buffer (4xSSC, 25% deionised formamide (Sigma), 1 mg/ml sheared salmon sperm DNA) for 30 min at room temperature in a wet chamber. After removing the prehybridization buffer, sections were incubated in 10 – 15 µl hybridization buffer at room temperature overnight in the wet chamber. The hybridization buffer contains 4xSSC (1xSSC = 150 mM NaCl, 15 mM sodium citrate, pH 7.2), 25% formamide, 1mg/ml salmon sperm DNA, and probes (2 µl 3’ end-labeled probe in 30 µl prehybridization buffer). After that, sections were washed in 4xSSC for 2x15 min, in 2xSSC for 2x15 min, and in 0.5xSSC for 30 min at room temperature.
For immunohistochemistry reaction, sections were washed with Buffer 1 (100 mM Tris-HCl, pH 7.5, 150 mM NaCl) for 2 x 10 min and blocked in blocking solution (buffer 1 containing 0.1% Triton X-100, 1% normal sheep serum) for 30 min, and after decanting the blocking solution, incubated in anti-DIG solution (1 part of anti-DIG-alkaline phosphatase in 100-200 parts of buffer 1) for 2 h. The sections were then washed in buffer1 for 2x10 min, in buffer 2 (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 50 mM MgCl$_2$) for 10 min. After that sections were covered with a proper amount of color solution [25 μl of NBT/BCIP stock solution (Boehringer) in 1 ml of buffer 2, 1 μl 1 M levamisole (Sigma)] in a wet chamber for 2-24 hrs in the dark. When optimal color occurred, the color solution was discarded and the reaction was stopped with buffer 3 (10 mM Tris-HCl, pH 8.1, 1 mM EDTA) for 15 min. After the buffer was removed, sections were washed with tap water on a rocking platform for 15 min and mounted the sections with the aqueous mounting solution.

(C) FGF2 in olfactory mucosa:


Cryostat tissue sections at 8 um thick were left at room temperature for 15 min and in the 40°C oven for 2 hours before use. The sections were washed twice with RNase-free PBS (pH 7.4) for 5 min on a rocking plate and incubated in RNase-free PBS containing 0.3% TritonX-100 for 15 mins without shaking. Following an RNase-free PBS wash for 2x5min , the sections were properly labeled, transferred to a wet chamber and incubated with 1 μg/ml prewarmed (at 37°C) proteinase K (Sigma) in RNase-free TE buffer (100 mM Tris-HCl, 50 mM EDTA, pH 8.0) at the 40°C oven for 15 min. After removal of the solution, sections were flooded with RNase-free PBS containing 0.1 glycine solution (Sigma) to inhibit the activity of the proteinase K and then postfixed in 4% paraformaldehyde at 4°C for 5 min. The sections were washed in PBS for 2x5 min on the rocking plate, acetylated with 0.1 M triethanolamine (TEA) buffer, pH 8.0, containing 0.25% (v/v) acetic anhydride (Sigma) for 2 x10 min on the rocking plate, and then
incubated at 40°C with prehybridization buffer (5x SSC, 25% formamide) for 10 - 30 mins.

After discarding prehybridization buffer, the tissue sections were incubated with hybridization buffer overnight at 42°C. The contents of hybridization buffer used in the assay included 10% dextran sulfate, 2% SDS, 5x Denhardt’s solution, 100μg/ml sheared salmon sperm DNA, 10-30 ng of probes per 30 μl of hybridization buffer. Following the hybridisation step, the sections were washed in 4xSSC for 15 min, in 2xSSC for 15 min and in 0.5xSSC for 15 min at room temperature.

For immunohistochemistry reaction, sections were washed with Buffer 1 (100 mM Tris-HCl, pH 7.5, 150 mM NaCl) for 2 x 10 min and blocked in blocking solution (buffer 1 containing 0.1% Triton X-100, 1% normal sheep serum) for 30 min, and after decanting the blocking solution, incubated in anti-DIG solution for 2 h. The sections were then washed in buffer1 for 2x10 min, in buffer 2 (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 50 mM MgCl₂) for 10 min. After that sections were covered with color solution [25 µl of NBT/BCIP stock solution (Boehringer) in 1 ml of buffer 2, 1 µl 1 M levamisole (Sigma)] in a wet chamber for 2-24 hrs in the dark. When optimal color occurred, the color solution was discarded and the reaction was stopped with buffer 3 (10 mM Tris-HCl, pH 8.1, 1 mM EDTA) for 15 min. After the buffer was removed, sections were washed with tap water on a rocking platform for 15 min and mounted the sections with the aqueous mounting solution.

(A) FGF2 in OLF442:

ISH using FGF2 RNA probe on the olfactory cell line OLF442, a cell line derived from a mouse olfactory neuronal precursor (MacDonald et al., 1996), followed the protocol below:

Cells were cultured on round coverslips in a four well plate for 1-2 days. The wells were divided into two groups: one treated with DMEM medium containing 10 % FCS (for
undifferentiated cells) and the other, treated with FCS-free DMEM containing 1% BSA (for differentiated cells). Cells were cultivated with 1% BSA only when sufficient cells had grown semi-confluently and had been firmly attached to the coverslips. Cells reacting with sense riboprobe were negative controls.

Before fixation, cells were washed with TBS buffer (0.1 M Tris, 0.15 M NaCl, pH 7.4) at room temperature for 5 min. The intensity of aspiration was carefully controlled to prevent cell loss. Cells were fixed in freshly prepared 4% paraformaldehyde at 4°C for 30 min, washed with TBS for 5 min, and dehydrated in 70% ethanol for 30 min at -20°C to dissolve the lipids on the cell membrane so as to increase the cell permeability. Cells on coverslips were then washed in TBS for 10 min and immersed in 0.2 N HCl for 30 min, followed by wash in TBS wash for 10 min, in 2xSSC for 30 min, and in 4xSSC wash for 30 min.

For prehybridisation, cells were incubated in prehybridisation buffer [25% formamide, 4xSSC and 1xDenhardt’s solution (50 mg polyvinylpyrrolidone, 50 mg Ficoll, 50 mg BSA in 250 ml distilled water)] for 30 min at 37°C in a humid chamber. Following that the coverslips were left at room temperature for 90 min. For hybridisation, cells were incubated in 500 μl hybridisation buffer [(25% formamide, 4xSSC, 1xDenhardts solution and 5% dextran sulfate + sense/antisense 3’ end labelled oligoprobe (2 μl/1 ml hybridisation buffer)] at 40°C overnight. The posthybridisation conditions were: wash with solution A (25% formamide in 4xSSC) at 45°C for 45 min, with solution B (25% formamide in 2XSSC) at 45°C for 45 min, and with 2xSSC for 30 min at 45°C.

For immunohistochemistry, cells were washed in buffer 1 [100 mM Tris-HCl (pH 7.5), 150 mM NaCl] for 2 x 10 min on rocking plate, immersed in blocking solution [buffer 1 containing 0.1% Triton X-100 and 2% normal sheep serum for 5 min and after decanting the blocking solution, incubated for 2 hr in buffer 1 containing 0.1% Triton X-100, 1% normal sheep serum and 1 in 2000 dilution of sheep anti-DIG-alkaline phosphatase [Fab fragments]. Then cells were washed with buffer 1 for 2x10 min on the rocking plate and incubated in buffer 2 [100 mM Tris-HCl (pH9.5), 100 mM NaCl, 50 mM MgCl₂] for 10
min. In the coloration step, each coverslip were treated with 500 μl color solution [ 4 mL buffer2, 32 NBT/BCIP reagent, 4 μl levamisole stock solution (1M)] for 22 h in the dark. The reaction was stopped with buffer 3 [10 mM Tris-HCl (pH 8.1), 1 mM EDTA] and then the cells were washed with distilled water briefly. After counter-staining with 0.1% nuclear Fast Red in distilled water for 1 min, coverslips were washed with tap water for 2x10 min and mounted with Glycerol directly on glass slides.

### 3.2.2 Western blot for the specificity analysis of FGF2 antibodies

Olfactory mucosa was removed from mouse and proteins were extracted from the olfactory mucosa as previously described. Samples containing equal amounts of total protein (150 μg), with known amounts of standards were concentrated in 72% Trichloroacetic acid and electrophoresed using standard Tris-HCl SDS-PAGE as previously described. For the control, 0.01 μg human recombinant FGF2 was used. A 12% separation gel was used with a 4% stacking gel. The protein was transferred to a PVDF membrane (Millipore) in Transfer buffer (10 mM NaHCO3, 3mM Na2CO3, pH 9.9 in 20% (v/v) methanol). The blotted membranes were blocked with 10% non fat milk powder in TBST (25 mM Tris-HCl, pH 7.4, 137 mM NaCl, 3 mM KCl, 0.1% Tween 20 (v/v)). The primary antibody was applied at an appropriate dilution (Sigma, 0.4 μg/ml; Santa Cruz, 0.2 μg/ml) in the 10% blocking solution. The secondary antibodies were applied in a 5% blocking solution (goat anti-mouse IgG (H+L) horseradish peroxidase conjugate Bio-Rad Cat. No. 172-1011; rabbit anti-goat IgG IgG (H+L) horseradish peroxidase conjugate Bio-Rad Cat. No. 172-1034). The protein was visualised by the enhanced chemiluminescence system (ECL+Plus, Amersham Pharmacia Biotech, UK).

### 3.2.3 RT-PCR study for FGF 1 on olfactory mucosa

#### 3.2.3.1 Oligonucleotide sequences and tissue preparation

The primers used to detect FGF1 in mouse olfactory epithelium and lamina propria were a gift from Dr. James O. Pickles, University of Queensland, St. Lucia, Brisbane, Queensland:
Forward: 5′-CGTTGCTTCTTATATAGC-3′
Reverse: 5′-CTGGAGTTGGAGTTAGAATTG-3’

The mouse olfactory mucosa was removed from mouse and the olfactory epithelium was separated from lamina propria as previously described. Total RNAs of the tissues were extracted, quality checked, quantified and reverse transcribed as previously described in Chapter 2.

3.2.3.2 RT-PCR for FGF 1

RT-PCR for FGF 1 in mouse olfactory tissues followed the standard protocol (25 μl reaction) as listed in Table 2-4 with adjustments in template amounts (2 μl, 4 μl, 6 μl) and at an annealing temperature of 50.5°C.
3.3 Results

3.3.1 In situ hybridization

3.3.1.1 FGF receptor 1 expression in mouse olfactory mucosa

Figure 3-1A shows that the expression of FGF receptor type 1 (FGFr-1) was strongly distributed in the neuronal layer of olfactory mucosa and basal cell layers. Cells close the basement membrane were not labeled. Supporting cells close to the epithelial surface were also unlabeled (Figure 3-1A).

Fig 3-1. In situ hybridization of FGFr-1 mRNA in adult mouse olfactory mucosa. A: ISH specific for FGFr-1 using an antisense oligoprobe. B: sense control for the assay. S= cell body of supporting cell. Arrows indicate the position of basement membrane. Bars= 20 um.

3.3.1.2 FGF 2 expression in mouse olfactory mucosa and OLF442 cells

In Figure 3-2A, the result has shown that FGF2 was strongly expressed in the neuronal layer of olfactory mucosa and some basal cells. The basal cells, judging from their location, are presumed to be globose basal cells. Basal cells close to the basement membrane were not labeled. Thus, horizontal basal cells did not appear to express FGF2. FGF2 was also expressed in some gland-like tissues (see Hsu et al., 2001, for a clearer
view) and ensheathing cells in lamina propria. There was light, diffuse expression of FGF2 in supporting cell cytoplasm around the nucleus. The control (Figure 3-2B) hybridized with sense probe showed no labeling.


The cells on Figure 3-3A, B are undifferentiated OLF442 cells. The cells are flat, adherent epithelial-like, multipolar/bipolar with short processes and round refractile cells. The cells on Figure 3-3C, D are differentiated OLF442 cells that have rounded cell bodies and extended long bipolar processes (MacDonald et al., 1996). FGF2 mRNA, identical with the antisense probe, was found to be strongly expressed in cytoplasm of differentiated and undifferentiated OLF442 cells (Figure 3-3A, C). Control wells hybridized with the sense probe showed no labeling (Figure 3-3B, D).
Figure 3-3. In situ hybridisation of FGF2 mRNA in OLF442 cell line using a riboprobe. A: FGF2 ISH on undifferentiated OLF442 cells. B: sense probe control for the FGF2 ISH assay on undifferentiated OLF 442. C: FGF2 ISH on differentiated OLF442 cells. D: sense probe control for the FGF2 ISH assay on differentiated OLF442. The result shows that FGF2 was expressed in both undifferentiated and differentiated OLF 442 cells. Note that the images B, D were viewed with Nomarski optics to visualize the unstained cells. Bar = 20 um
3.3.2 Western blot study for anti-FGF2 antibodies

The specificity of two antibodies, polyclonal anti-human FGF2 antibody (Santa Cruz) and monoclonal anti-human antibody (Sigma), were studied using Western Blot technique. The antibodies were later used in our laboratory for FGF2 immunoreactivity in olfactory mucosa (Hsu et al., 2001). Each antibody detected a human recombinant FGF2 (Sigma, catalog number F0291) at 18 kD (Figure 3-4, C). In the mouse protein extracts from mouse olfactory mucosa, the polyclonal antibody identified a band at 18 kD as well as two higher molecular weight isoforms (Figure 3-4, SC). Using the same protein extract, the monoclonal antibody identified a band at 18 kD as well as other higher molecular weight isoforms (Figure 3-4, Sg).

![Western blot for FGF2 antibodies](image)

**Figure 3-4 Western blot specificity test for two commercial anti-FGF2 antibodies.**
Legends: C: positive control using FGF2 protein (human recombinant FGF2, Sigma, Catalog number F0291); SC: Western blot for FGF2 in adult mouse olfactory tissue using Santa Cruz polyclonal anti-human FGF2 antibody; Sg: Western blot for FGF2 in rat olfactory tissue using monoclonal anti-human FGF2 antibody from Sigma. Arrows indicate the protein of interest (~18kd). Non-specific bands generated in SC and Sg indicate higher molecular weight isoforms of FGF2 in both mouse and rat olfactory protein extracts.
Using the two anti-FGF2 antibodies, Hsu et al, 2001, has demonstrated that FGF2 immunoreactivity was more widely distributed in the olfactory mucosa than FGF2 mRNA, with the most intense FGF2-like immunoreactivity in the apically located cytoplasm of the supporting cells. Otherwise there was light staining distributed throughout all cell layers. Light staining was also found in gland-like and fibroblast-like cells. Cells associated with the olfactory nerve bundles deep in the lamina propria were strongly labeled (Data not shown here).

### 3.3.3 RT-PCR for FGF1 expression in olfactory epithelium

For expression of FGF1 mRNA in mouse olfactory mucosa, the RT-PCR result in Figure 3-5 has shown that FGF1 was expressed in both olfactory epithelium and lamina propria. All samples in different concentrations have each presented a band sized 243 bp.

This result is inconsistent with a previous finding that FGF1 is not present in the olfactory epithelium (Key et al., 1996). For further studies, an RT-PCR including a no-RNA control is suggested to exclude the possibility of genomic DNA involvement.

![Figure 3-5](image)

_Figure 3-5. FGF1 mRNA expression in mouse olfactory epithelium (OE) and lamina propria (LP). In the RT-PCR assay, the amounts of OE and LP templates were added in an increasing order from left to right (2 μl, 4 μl, 6 μl). PCR product = 243 bp (2109-2352 of BC037601, Genbank). M = 100 bp marker (Gibcol) with bands in 100 bp intervals._
3.4 Discussion

Our in situ hybridization studies have shown that FGF receptor type 1 (FGFr-1) and FGF2 were strongly expressed in the neuronal layer of olfactory mucosa and basal cell layers. Cells close the basement membrane, which are presumed to be horizontal cells, and supporting cells did not express FGFr-1 and FGF2 mRNAs. FGF2 was also expressed in some gland-like tissues and ensheathing cells in lamina propria. In the cell line OLF442, FGF2 mRNA was found to be strongly expressed in the cytoplasm of differentiated and undifferentiated OLF442 cells.

Two commercial anti-FGF2 antibodies were validated by Western Blot and were subsequently used in the immunochemistry studies (Hsu et al., 2001). In contrast to the FGF2 mRNA expression, FGF2 immunoreactivity was found to be more widely distributed in the olfactory mucosa than FGF2 mRNA with the most intense FGF2-like immunoreactivity in the apically located cytoplasm of the supporting cells. Light staining was also found in gland-like and fibroblast-like cells. Cells associated with the olfactory nerve bundles deep in the lamina propria were strongly labeled (The immunochemistry done by Dr Francois Feron is not shown here).

For the studies of FGFs, our RT-PCR has demonstrated that FGF1 was expressed in both olfactory epithelium and lamina propria which is inconsistent with a previous finding that FGF1 is not present in the olfactory epithelium (Key et al., 1996).

3.4.1 Actions of FGF2 and FGF receptor type 1 (FGFr-1)

This experiment has demonstrated that FGF2 and FGFr-1 (one of the four tyrosine kinase receptors which FGF2 binds with high affinity) are expressed mainly by the neuronal cells and by some of the basal cells in the olfactory mucosa. The basal cells, judging by their location, are presumed to be globose basal cells (Figure 3-1 and 3-2). In addition to FGFr-1, two other types of receptors, FGFr-2 and –3 were also expressed in the olfactory epithelium system (Hsu et al., 2001). The presence of FGF2, FGFr-1 and other receptors
in many adjacent cells provides a strong indication for the paracrine and /or autocrine actions of FGF growth factor signaling pathways (Hsu et al., 2001). Since FGFr-1 has high affinity with FGF2 (Ornitz, 1996), the presence of FGFr-1 in the presumed globose basal cells provides a basis for the proliferative role of FGF2 in globose basal cells (Newman et al., 2000).

3.4.2 The expression of FGF2 mRNA and FGF2 protein

The discrepancy between the expression of FGF2 mRNA (Figure 3-2) and the expression of FGF2 protein (Hsu et al, 2001) in the olfactory mucosa may be explained by that FGF2 protein content is not directly proportional to the steady-state FGF2 mRNA levels (Li & Murphy, 2000). The translation of FGF2 mRNA is regulated in part by an antisense FGF2 transcript that results from the bi-directional transcription of the gene. The expression of FGF2 protein is further complicated by that the antisense FGF2 transcript has alternative splice variants which are widely expressed in the central nervous system and these constructs were reported to disrupt the FGF2 autocrine pathway (Li & Murphy, 2000). In addition, the low molecular weight isoform of FGF2 is secreted or exported from cells, and may be taken up by other cells which do not normally produce it (Baird, 1994). The strong FGF2 immunoreactivity in the supporting cells may be the case, where FGF2 protein was taken up through the FGF2 secretion of glandular cells and accumulated in the cytoplasm of the supporting cells (Hsu et al., 2001).

3.4.3 Expression of FGF2 in OLF442

OLF442 is an immortalized neuronal progenitor from the basal cells. It was immortalized by incorporation of n-myc oncogene in retrovirus (MacDonald et al., 1996; refer to Chapter 5) and found to be analogous to an immature olfactory precursor at the intermediate stage of differentiation (Zehntner, 1998). Our results have shown that the presumed globose basal cells, immature and mature neuronal cells all express FGF2. Hence it is consistent because both differentiated and undifferentiated OLF442 express FGF2.
3.4.4 Role of FGF2 in neurogenesis

FGF2 can stimulate proliferation and induce or delay the differentiation in many neural or non-neural tissues in vitro (Gensburger et al., 1987; Gospodarowicz et al., 1987; Unsicker et al., 1987; Caday et al., 1990; Murphy et al., 1990; Kilpatrick et al., 1993; Dehamer et al., 1994; Calof, 1995; Murrell et al., 1996). It was also reported to be able to inhibit neurogenesis of a cell line derived from a rat OE (Goldstein., 1997). It is believed that FGF2 plays a role in regulating the number of cell divisions that precursor cells undergo (Calof., 1995). FGFs may effectively increase the number of precursor cells when they are present by early G₁ phase of the immediate neuronal precursor cell cycle (Dehamer et al., 1994). We have also reported that the increase in the number of neurons after FGF2 stimulation was due to the increase in the number of precursor cells (Newman et al., 2000).

Interestingly, in the G₁ phase, mitotic cells, under certain conditions, may leave G₁ and enter the G₀ phase in which cells are metabolically active and do not proceed through the cell cycle (Bradley et al., 1995). In this situation, there appear to be only two options for the cells that remain in this temporarily quiescent state: that is, either to return to G₁ for mitosis to continue or to remain in G₀ for a longer time as if they were suppressed by a growth inhibitor. This may explain that although FGF2 elicits typical proliferating responses in many cells, it has been reported as a growth inhibitor for some cultured tumour cells (Schweiger et al., 1987).

As mentioned above, it is possible FGF2 plays a role in determining which way the immediate neuronal precursors are to follow in G₁/G₀ phase. If this is the case, some questions may arise accordingly. What factors, if there is any, regulate FGF2 expression in the cells and how is this done? How does FGF2 exert its effects on precursor cells to determine the proliferating and differentiating potentials in the G₁/G₀ phase of the cell cycle so as to determine the fates of precursors? Or does FGF2 need some other factors to help regulate a neurodevelopmental mechanism? In consideration of the complicated roles the FGF2 plays in neurogenesis, most likely it can not accomplish all those reported...
tasks alone. The concentration of FGF2 (Qian et al., 1997), the various non-mitogenic responses of the cells expressing the FGF receptor to FGF2, isoforms of FGF2 and its receptors (Sporn and Roberts, 1991), other growth factors like IGF-1 (MacDonald et al., 1996) are possible factors involved in the action modes of FGF2 in regulating neurogenesis. Although the FGF2 was found active in vitro, there were only a limited number of reports providing in vivo evidence. Our studies now have provided evidence for the identity of the cells expressing FGF2 and FGFr-1 in the olfactory mucosa.
Chapter four

Identification and characterization of PDGF growth factors and receptors in olfactory mucosa
4.1 Introduction

4.1.1 Platelet-derived growth factor

Platelet-derived growth factor (PDGF) was originally identified in platelets and in serum as a mitogen for fibroblasts, smooth muscle cells and glia cells in culture. PDGF has since expanded to a family of dimers of at least four gene products, PDGF-A, PDGF-B, PDGF-C and PDGF-D, whose biological activities are mediated through two receptor types, PDGF receptor \( \alpha \) and PDGF receptor \( \beta \) (Betsholtz et al., 2001). The active PDGF molecule consists of 2 peptide chains linked together by disulfide chain (Heldin et al., 1993). Two PDGF receptor subtypes, PDGFr \( \alpha \) and PDGFr \( \beta \), have been identified; these are receptor tyrosine kinase subtypes encoded by different but related genes (Cooper & Kazlauskas, 1993). The PDGFr \( \alpha \) subunit binds PDGF-A, PDGF-B and PDGF-C chains, whereas the PDGFr \( \beta \) subunit binds the PDGF-B and PDGF-D chains (Betsholtz et al., 2001). A proposed model for PDGF ligand-receptor interaction postulated that PDGF dimers activate PDGF receptors through the generation of active PDGF receptor dimers in which each chain of the PDGF dimer binds one receptor molecule (Betsholtz, 1995). Since the number of \( \alpha \)-and \( \beta \)-receptor subunits varies in different cell types this determines the sensitivity of a cell to the different isoforms of PDGF (Sasahara et al., 1991). Within a cell line, PDGF receptor \( \alpha \) and PDGF receptor \( \beta \) dimers were reported to activate different responses (Eriksson, 1992).

It has been demonstrated in situ that PDGF-B and PDGFr \( \beta \) are extensively expressed in olfactory receptor neurons, supporting cells and probably horizontal basal cells in the olfactory epithelium, and ensheathing cells in lamina propria (unpublished data; Sneesby, 1998). In relation to previous identification of a secreted protein, SPARC (Secreted Protein Acidic and Rich in Cysteine) in olfactory epithelium, PDGF-B is postulated to regulate olfactory neurogenesis under the control of SPARC. The hypothesis is SPARC binds with PDGF-B to inhibit its binding to PDGFr \( \beta \) (Raines et al., 1992). PDGF-A and PDGF receptor \( \alpha \) were reported to be expressed in mouse embryo in that the in situ
hybridisation signal of PDGF-A was most strong in the epithelium while the signal of PDGF α was most strong in mesenchyme (Orr-urtreger and Lonai, 1992), although the cells expressing PDGF-A and receptor α were not identified in the studies. It is also known that the PDGF receptor α is expressed in neurons of central nervous system (Oumesmar, 1997). A recent study has reported that FGF-2 stimulates ERK1/2-dependent Sp1 phosphorylation, thereby repressing PDGF receptor α transcription via a cis-acting element in the PDGF receptor α promoter (Bonell & Khachigian, 2004), hence inhibiting the neurogenesis (see Chapter 6 for details).

4.1.2 Chapter Aim

The chapter aims to first screen mRNAs of PDGF-A, -B and PDGF receptors α and β in adult olfactory mucosa using RT-PCR. Any positive expression will be confirmed by in situ hybridisation. Since PDGF-B and PDGF receptor β have been well characterized in situ (Unpublished data from Sneesby, 1998), only PDGF A and receptor α are of our interest in the in situ hybridization study.
4.2 Methods and materials

4.2.1 RT-PCR

To investigate mRNAs of PDGF A, B, PDGF receptors α and β in adult mouse olfactory mucosa, mouse tissue was removed, olfactory epithelium and lamina propria were separated, and total RNAs were extracted as previously described in Chapter 2. The total RNAs were checked for quality on formaldehyde-containing gels, concentrations were determined by spectrophotometry, and then the RNAs were reverse-transcribed into single-stranded cDNAs using the methods as previously described (see Chapter 2). The reverse transcription reaction was controlled by GAPDH primers. The PCR reactions followed the standard protocol stated in Table 2-4 using an annealing temperature between 55 and 60 degrees. A No-RNA control using samples primers with omission of RNA template was included in each run of the experiment. The primers used for the sample tests are listed in Table 4-1 as below:

![Table 4-1. RT-PCR primers for PDGF A, B, PDGFr α, and β](#)

<table>
<thead>
<tr>
<th>Locus</th>
<th>Nucleotide sequence (5’→ 3’)</th>
<th>Product size (bp)</th>
<th>Genbank</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDGF A</td>
<td>Forward: CCATTGCAGGAAGAGAAGTA&lt;br&gt;Reverse: GGCAATGAAGCACCATACATA</td>
<td>436 *</td>
<td></td>
</tr>
<tr>
<td>PDGF B</td>
<td>Forward: CGACCAGCTCCATCGCCTCTT&lt;br&gt;Reverse: GCCAGGGGGTACTATTGTCT</td>
<td>461 *</td>
<td></td>
</tr>
<tr>
<td>PDGF rα</td>
<td>Forward: GTGAAATCAGAAGTGGAGGAC&lt;br&gt;Reverse: CCCATTTGCAAGACCTTTGAG</td>
<td>289 m84607</td>
<td></td>
</tr>
<tr>
<td>PDGF rβ</td>
<td>Forward: CATCAATACCGACATTTGACC&lt;br&gt;Reverse: CTTTTGGAGAATGTTGACTCC</td>
<td>303 x04367</td>
<td></td>
</tr>
</tbody>
</table>

* from Shaddy et al., 1996

4.2.2 In situ hybridisation (ISH)

For the in situ hybridisation, olfactory mucosa was prepared from adult Quackenbush mice as previously described in Chapter 2. Riboprobes were synthesized by Kyra Sneesby in our laboratory from pGEM-1 plasmids containing a 1.5 kb cDNA fragment of murine PDGF α-receptor cDNA and a 906 bp cDNA fragment of PDGF A chain (originally from Mercola, M., Harvard Medical School, Department of Cell Biology,
Boston, Massachusetts). They were labeled, checked for quality and quantified by Sneesby before use for in situ hybridization assay.

4.2.2.1 Detection of PDGF A expression by ISH using a riboprobe

For PDGF-A in situ hybridisation assay, cryostat sections were left in 38°C oven for 2 h, washed with PBS for 2x5 min, 0.3% Triton X-100 in PBS for 15 min, PBS for 2x5 min, incubated in 1 μg/ml proteinase kinase for 15 min at 38°C, washed with 0.1M Glycine solution for 5 min, and fixed in 4% paraformaldehyde for 5 min, followed by a few washes including PBS for 2x5 min and 0.25% Acetic anhydride in TEA buffer for 2x10 min. Sections were then placed in pre-hybridization buffer (5xSSC+25% formamide) for 30 min and hybridized in hybridization buffer (5xSSC, 25% formamide, 5x Denhardt solution, 2% SDS, 100 μg/ml sheared salmon sperm DNA, 30 ng/30μg probe) overnight at 38°C. After hybridization, the sections were washed in 2xSSC for 15 min and 1xSSC for 15 min and 0.1xSSC for another 15 min. Following incubation with buffer1 for 2x5 min, sections were blocked in blocking solution for 30 min and treated with anti-DIG solution (1/200) for 2 h. Tissue sections were then washed in buffer1 for 2x10 min, buffer 2 for 10 min, and color-developed in chromogen with 0.5xLevimisole solution for 3 hours.

4.2.2.2 Detection of PDGF receptor α expression by ISH using a riboprobe

For PDGFα in situ hybridisation assay, cryostat sections were left in 38°C oven for 2 h, washed with PBS for 2x5 min, 0.3% Triton X-100 in PBS for 15 min, PBS for 2x5 min, incubated in 1 μg/ml proteinase kinase for 15 min at 38°C, washed with 0.1M Glycine solution for 5 min, and fixed in 4% paraformaldehyde for 5 min, followed by a few washes including PBS for 2x5 min and 0.25% Acetic anhydride in TEA buffer for 2x10 min. Sections were then placed in pre-hybridization buffer (5xSSC+25% formamide) for 30 min and hybridized in hybridization buffer (5xSSC, 25% formamide, 5x Denhardt solution, 2% SDS, 100 μg/ml sheared salmon sperm DNA, 30 ng/30μg probe) overnight at room temperature. After hybridization, the sections were washed in 2xSSC for 5 min and 1xSSC for another 5 min. Following incubation with buffer1 for 2x5 min, sections were blocked in blocking solution for 30 min and treated with anti-DIG solution (1/100)
for 2 hours. Tissue sections were then washed in buffer 1 for 2x5 min, buffer 2 for 10 min, and color-developed in chromogen with 0.5xLevimisole solution for 2 hours.
4.3 Results

4.3.1 Expression of PDGF A, B, PDGF receptors α and β detected by RT-PCR

In Figure 4-1, the result has shown four unique bands specific for PDGF A, B, and PDGF receptors α and β at their correct sizes. The PDGF A, B, PDGF receptors α and β have been demonstrated to be expressed in both olfactory epithelium and lamina propria. The no-RNA controls all remained clear, which has excluded the possibility of genomic DNA incorporation in the samples.

4.3.2 Expression of PDGF A and PDGF receptor α in adult mouse olfactory mucosa detected by ISH using riboprobes

Figure 4-2 shows the expression of PDGF A and PDGF receptor α in adult mouse olfactory mucosa. In Figure 4-2A, PDGF A was strongly expressed in the neuronal layer of olfactory epithelium including mature and immature neurons and found to be expressed moderately in some basal cells, supporting cells, and some tissues in lamina propria. Some tissues in lamina propria were labeled in the sense control (Figure 4-2B) perhaps because of insufficient posthybridisation wash. In Figure 4-2C, PDGFr α was moderately expressed in the neuronal layer and was weakly expressed in the supporting cell. The sense control was not labeled (Figure 4-2D).
Figure 4-1. RT-PCR detection of PDGF A, B and PDGF receptors α and β in mouse olfactory epithelium (OE) and lamina propria (LP). Image A shows PDGF A and PDGF B in OE and LP. Image B shows PDGF receptors α and β in OE and LP. C= no-RNA control. M= 100 bp marker. The product size for PDGF A is 436bp, for PDGF B, 461bp, for PDGFr α, 289bp and for PDGFr β, 303bp.
Fig 4-2. Expression of PDGF A and PDGF receptor α mRNAs in adult mouse olfactory mucosa detected by in situ hybridisation (ISH) using riboprobes. PDGF A was strongly expressed in the neuronal layer of olfactory epithelium including mature and immature neurons and expressed moderately in some basal cells, some supporting cells, some tissue in lamina propria (A) in comparison with the sense control (B). The sense control took some stain in lamina propria, which might be caused by insufficient wash in ISH. PDGFr α was also expressed in the neuronal layer (C). Some supporting cells were also weakly labeled (arrows). The sense control (D) was not labeled. Bars = 20 um.
4.4 Discussion

The RT-PCR, PDGF-A, -B and PDGF receptors α and β were expressed in the olfactory epithelium and lamina propria. The in situ hybridization has provided evidence for the expression of PDGF A and PDGF receptor α in the areas, supporting the RT-PCR result.

4.4.1 Expression of PDGF B and PDGFβ

Using in situ hybridization, Kyra Sneesby in our laboratory has previously demonstrated that PDGF-B and PDGFβ are extensively expressed in olfactory receptor neurons, supporting cells and probably horizontal basal cells in the olfactory epithelium, and ensheathing cells in lamina propria (Data not shown). The expression patterns were similar to the results we obtained for PDGF A and PDGF receptor α in the studies.

4.4.2 Actions of PDGF in olfactory epithelium

The function of PDGF receptor α in neurogenesis is poorly understood but PDGF was initially described for its potent mitogenic activity on smooth muscle cells, fibroblasts, glial cells, and mediates other crucial functions during embryonic development such as tissue repair, chemotactism and cytoskeleton rehandling (Reviewed by Oumesmar et al., 1997). In our laboratory, Newman et al., 2000, observed that PDGF-AB promotes the survival of immature neurons in a cell culture (Newman et al., 2000). The expression of PDGF-A and PDGF receptor α in the olfactory epithelium and lamina propria may be associated with these functions. In combination with Sneesby’s findings for PDGF B and PDGFβ, it is possible that PDGF-AB signals directly through PDGF receptors α and β on the immature neurons to enhance olfactory neurogenesis.
Chapter five

Identification and characterization of an unknown gene 16b5 in olfactory mucosa and OLF442 cells
5.1 Introduction

5.1.1 OLF442 and 16b5

OLF442 is an immortalized olfactory cell line which was originally established by MacDonald et al., 1996. OLF442 was immortalized by retroviral infection of the dividing cells in regenerating olfactory epithelium. Practically, the retrovirus infected the cells with \( n-myc \) oncogene that was linked to an antibiotic resistance gene \( zen-neo \). Cells with the antibiotic resistance were then selected, cultured, cloned and characterized (MacDonald et al., 1996). Since the recombinant murine retrovirus only infects dividing cells, the cells that can be immortalized are limited to the olfactory neuronal progenitors, precursor cells, and the slowly dividing supporting cells of the epithelium (Zehntner, 1998). OLF442 is the designation of one of the clonal cell lines generated (Zehntner, 1998).

OLF442 cells were described as flat adherent epithelial like cells multipolar/bipolar cells with short processes and round refractile cells (MacDonald et al., 1996). To differentiate OLF442, Simone Zehntner in our laboratory exposed the OLF442 cells to a number of differentiating agents and found cells cultured in serum-depleted conditions appeared significantly more differentiated than in other conditions (Zehntner, 1998). Differentiated OLF442 cells have rounded cell bodies and extended long bipolar processes, which make the cells more neuron-like. According to the altered expression of a number of neuronal and olfactory markers, the differentiated OLF442 cells were suggested to have a more neuronal-like phenotype (Zehntner, 1998).

Differential display technique is a reverse-transcriptase polymerase chain reaction (RT-PCR) based technique, which allows a side-by-side comparison of mRNA from different cell types or stages of differentiation, and isolation and identification of differentially expressed genes. As development and regeneration are characterized by changes in cell and tissue morphology, the identification of novel cell- or tissue-specific genes will help
understand the complex developmental processes (Zehntner, 1998). For example, the undifferentiated and differentiated OLF442 cells mimic two stages of developing olfactory epithelium. In Zehntner’s previous work, the gene sets of the two types of cells were differentially displayed, the expression of cell-specific genes were observed, and novel fragments were identified, isolated and characterized.

16b5 is a novel fragment previously isolated from OLF442 cells by the differential display technique. The 16b5 was observed to be upregulated by differentiation and the observation was confirmed by RNAase protection (Zehntner, 1998). The 16b5 was further characterised by Zehntner using Marathon 5’ Race protocol and its sequence was studied against the existing blast machine, GENBANK and EMBL (Figure 5-1). There was no in vivo evidence to support these findings.

The 16b5 poly A\(^+\) mRNA is a 1593 sequence showing no homology to identified genes within the GENBANK or EMBL although the sequence has 100% homology to numerous mouse expressed sequence tags (EST’s) as well as up to 80% homology with a number of human EST’s (Zehntner, 1998). The 16b5 contains a complete open reading frame (ORF) beginning after the stop codon at the 157 bp position and codes for 289 amino acids, with a terminal stop codon at 1024 bp (Figure 5-1). The start codon (ATG-methionine) begins 60 bp downstream at the 217 bp position. The predicted ORF beginning at the methionine encodes a portion of 268 amino acids and the predicted protein folding indicates 5 \(\alpha\)-helixes and 7 \(\beta\)-pleated sheet structures. Amino acid sequence alignment showed the sequence has homology to a motif of a putative bipartite nuclear-localization signal at 261-278 amino acids (Zehntner, 1998; data not shown).

5.1.2 Chapter aim

This chapter aims to confirm differential regulation in OLF442 cells, to investigate expression of the novel fragment in vivo, and to obtain more information by blasting the sequence of 16b5 against existing gene databases. In situ hybridisation using an oligonucleotide probe will be used to detect 16b5 in both the differentiated OLF442 cells and in the olfactory mucosa.
5.2 Methods and materials

5.2.1 Tissue and cell preparations

Mouse olfactory tissue was dissected from adult mouse and sectioned as described Chapter 2. Other tissues were removed from the mouse, sliced into a proper size and sections prepared in the same way. For RT-PCR, the olfactory epithelium was separated from lamina propria as described in Chapter 2. For RT-PCR assays, differentiated and undifferentiated cells were prepared and, tissues were RNA-extracted as described in Chapter 2. Total RNAs from cells and tissues were checked for quality, concentration-determined and then reverse-transcribed as described in Chapter 2. For in situ hybridization, OLF442 cells were grown on round coverslips as described in the following text.

5.2.2 RT-PCR screening

5.2.2.1 Primer selection

Based on the full sequence of 16b5 fragment isolated by Marathon 5’ RACE (Figure 5-1, from Zehntner S.P., 1998, unpublished), a pair of primers were selected to screen the 16b5 poly A⁺ mRNA over a range of mouse tissues using RT-PCR. The oligonucleotide sequences were synthesized by Pacific Oligo Pty. Ltd. and their concentrations were re-measured as described in Chapter 2. The sequences of the primers and the predicted product size are listed in Table 5-1 below:

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Primer sequence (5’ → 3’)</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>16b5</td>
<td>Forward: GAGTCCACAGTCTTCCAGTCCAGAGACGTT</td>
<td>799</td>
</tr>
<tr>
<td></td>
<td>Reverse: CACACTCGGTACCAACAGCGTGAGAT</td>
<td>799</td>
</tr>
</tbody>
</table>

Table 5-1 Primer sequences for 16b5
5.2.2.2 RT-PCR

A 25 μl reaction mix (Boehringer Mannheim) was prepared as follows: 2 μl 10x reaction buffer, 1.5 μl 25 mM MgCl₂, 0.1 10 mM dNTP, 0.3 μl 10 pmole/μl primer, 0.25 μl Taq DNA polymerase, 16.55 μl distilled water and 4 μl 1st strand cDNA previously prepared using 1st Strand cDNA Synthesis Kit (Boehringer Mannheim). The PCR conditions were: 1 cycle of initial denaturation- 95°C for 5 min; 35 amplification cycles- 95°C for 1 min, 68°C for 1 min, 72°C for 2 min; 1 cycle of final extension-72°C for 7 min, in a thermal cycler (Perkin-Elmer). Reactions were analysed on 2% agarose gel and visualised with ethidium bromide.

5.2.3 In situ hybridisation using oligonucleotide probe

5.2.3.1 Probe selection

The oligonucleotide sequences (Table 5-2) were synthesized by Pacific Oligo Pty. Ltd., concentration-determined, and 3’ end labeled as described in Chapter 2.

Table 5-2. 16b5-specific oligonucleotide sequences selected for in situ hybridisation

<table>
<thead>
<tr>
<th>Probes</th>
<th>Primer sequence (5’Æ3’)</th>
<th>Position</th>
<th>ISH result</th>
</tr>
</thead>
<tbody>
<tr>
<td>probe1</td>
<td>GAGTCCACAGTCTTCCAGTTCAGAGACGTT</td>
<td>506-535</td>
<td>x</td>
</tr>
<tr>
<td>probe2</td>
<td>ATCTCACGCTGTGGTACCGAGTG</td>
<td>1284-1309</td>
<td>✓</td>
</tr>
</tbody>
</table>
5.2.3.2 Detection of 16b5 in OLF442 by in situ hybridization

Cells were cultured with DMEM containing 10% FCS and 1/200 streptomycin (S/P) on coverslips in a four well plate for 1-2 days until a sufficient amount of cells were grown and evenly distributed on coverslips. The wells containing cells were divided into two groups and labeled. A group labeled as ‘Serum-containing’ was treated with DMEM containing 10% FCS and 1/200 S/P. The other, labeled with ‘Serum-depleted’, was treated with FCS-free DMEM containing 1% BSA and 1/200 S/P. Because the serum-
depleted cells were only weakly attached to the base, care was taken of to ensure a sufficient amount of cells had grown before the serum-depletion step initiated.

The cells on the coverslips were fixed in 4% freshly prepared and filtered paraformaldehyde at 4°C for 15 min. Cells were washed with PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na₂PO₄, pH 7.4) for 5 min, dehydrated in 70% ethanol for 30 min at -20°C, washed in TBS (0.1 M Tris, 0.15 M NaCl, pH 7.4) for 10 min and incubated in 0.2 N HCl for 30 min. After another PBS wash for 10 min, cells were incubated in 250 μl prehybridisation buffer (10% formamide, 5xSSC) for 30 min at 37 °C in a humid chamber. Cells were then incubated in 60 μl hybridisation buffer [10% formamide, 5x SSC, 5x Denhardt's solution (250 mg polyvinylpyrrolidone, 250 mg Ficoll, 250 mg BSA in 250 ml distilled water), 10% dextran sulfate, 100 μg/ml sheared salmon sperm DNA and sense/antisense 3’ end labelled oligoprobe (2 μl per 30 μl hybridisation buffer)] at 37 – 40 °C overnight. Following that, cells were washed in 2x SSC for 15 min and 1xSSC for 15 min at room temperature.

For immunohistochemistry to detect the DIG, cells were washed in buffer 1 [100 mM Tris-HCl (pH 7.5), 150 mM NaCl] for 2 x 5 min on a rocking plate, blocked in blocking solution [buffer 1: 0.1% Triton X-100 and 2% normal sheep serum] for 30 min and after decanting blocking solution, incubated for 2 hr with 1/500 dilution of sheep anti-DIG-alkaline phosphatase [Fab fragments] in buffer 1, followed by two washes with buffer 1 for 5 min and an incubation with buffer 2 [100 mM Tris-HCl (pH9.5), 100 mM NaCl, 50 mM MgCl₂] for 10 min. After that, coverslips were color developed in a proper amount of color solution [ 80 μl NBT/BCIP reagent and 4 μl levamisole stock solution (1M) in 4 ml buffer 2 ] for a few hours to overnight in the dark and the coloration was stopped in buffer 3 [10 mM Tris-HCl (pH 8.1), 1 mM EDTA] for 2x15 min.. Cells were then washed with tap water for 5-15 min until blue precipitates in cells were observed under microscope.
5.2.3.3 Detection of 16b5 mRNA in mouse tissues using in situ hybridisation

In practice, probe1 (table 5-2) did not work with ISH using a variety of ISH conditions. Therefore probe2 was selected and attempted on mouse sections using the following protocol (Nonradioactive In Situ Hybridization Application Manual, 2nd edit., Boehringer Mannheim GmbH, Biochemica, 1996):

For prehybridisation, cryostat sections were left at room temperature for 15 min and then in a 40°C oven for 2 hrs. The sections were washed for 2 x 10 min with RNAse-free PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na₂PO₄, pH 7.4), followed by incubation with with RNAse-free PBS containing 0.3% Triton X-100 for 15 min. After that sections were washed with RNAse-free PBS for 2x10 min, postfixed with 4% paraformaldehyde in RNAse-free PBS at 4°C for 5 min, and washed for 2 x 10 mins with RNAse-free PBS. Then the tissue sections were overlaid with prehybridization buffer (4xSSC, 25% deionised formamide (Sigma), 1 mg/ml sheared salmon sperm DNA) for 30 min at room temperature.

After removing the prehybridization buffer, sections were incubated in 10 - 15 μl hybridization buffer [4xSSC (1xSSC = 150 mM NaCl, 15 mM sodium citrate, pH 7.2), 25% formamide, 1mg/ml salmon sperm DNA, and probes (2 μl 3’ end-labeled probe in 30 μl prehybridization buffer)] at room temperature overnight in the wet chamber. After the hybridisation, the sections were washed in 4xSSC for 2x15 min, 2xSSC for 2x15 min, and 0.5xSSC for 30 min at room temperature.

For immunohistochemistry, sections were washed with Buffer 1 (100 mM Tris-HCl, pH 7.5, 150 mM NaCl) for 2 x 10 min, tissue blocked in blocking solution (buffer 1 containing 0.1% Triton X-100, 1% normal sheep serum) for 30 min, and then incubated with anti-DIG solution (1 part of anti-DIG-alkaline phosphatase in 100-200 parts of buffer 1) for 2 hrs after the blocking solution was decanted. The tissues were washed with buffer1 for 2x10 min and buffer 2 (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 50 mM MgCl₂) for 10 min. After that, the sections were covered with proper amount of color
solution [25 µl of NBT/BCIP stock solution (Boehringer) in 1 ml of buffer 2, 1 µl 1 M levamisole (Sigma)] in a wet chamber for 2-24 hrs in the dark. When an optimal color intensity was obtained, the color solution was discarded and the coloration was stopped in buffer 3 (10 mM Tris-HCl, pH 8.1, 1 mM EDTA) for 15 min. The sections were then washed with tap water on a rocking platform for 15 min and mounted with an aqueous mounting solution.
5.3 Results

5.3.1 RT-PCR

Differentiated and undifferentiated OLF442 cells and several mouse tissues were screened for 16b5 mRNA using RT-PCR. The tissues of interest include mouse brain (Br), olfactory epithelium (OE), lamina propria (LP), olfactory bulb (OB), liver (Lv), kidney (K), lung (Lu), heart (H) and muscle (Mu). In Figure 5-2, bands in OLF442 cells and bands across all tissues but muscle were sized at 799 bp whilst the negative controls using water instead of cDNA template remained clear, which indicates that 16b5 mRNA was expressed in differentiate and undifferentiated OLF442 cells and in nearly all tissues investigated except for the muscle tissue. Because of the presence of 16b5 in olfactory epithelium, we moved further to studying the cells expressing 16b5 in the OLF442 cells and the olfactory epithelium using in situ hybridization.

Figure 5-2. RT-PCR detection of 16b5 mRNA over a range of adult mouse tissues. The RT-PCR shows that the adult mouse olfactory epithelium and other tissues expressed 16b5, where OE= olfactory epithelium, LP= lamina propria, OB= olfactory bulb, Br= brain, H= heart, Lv= liver, Lg= lung, and K= kidney, U= undifferentiated OLF442, and D = differentiated OLF442. Nothing was detected in muscle tissue (Mu) and in water controls (W). Product size = 799 bp. M= 100 bp marker.
5.3.2 In situ hybridisation

5.3.2.1 Expression of 16b5 mRNA in OLF442 cells

In the in situ hybridisation investigation, 16b5 was found to be expressed in differentiated (serum-depleted) OLF442 cells, but not detectable in undifferentiated (serum containing) cells (Figure 5-3C). This result supports the previous finding that 16b5 was upregulated by differentiation (Zehntner, 1998). The sense control for the undifferentiated cells was slightly labeled by non-specific stain on the foreground, which might be caused by insufficient posthybridisation wash.

Figure 5-3. Detection of 16b5 mRNA in OLF442 cell line using in situ hybridization. The result shows that 16b5 was expressed in differentiated OLF442 cells (C) but not in undifferentiated cells (A). Sense control for the undifferentiated (B) was labeled slightly by some non-specific stain. Sense control for the differentiated OLF442 cells (D) were not labeled.
5.3.2.2 Expression of 16b5 mRNA in mouse olfactory mucosa

Figure 5-4 shows that the neuronal layer of olfactory epithelium, including mature and immature olfactory receptor neurons, expressed 16b5 to a weak degree. 16b5 mRNA was also expressed slightly in supporting cells in a sporadic fashion and, some superficial basal cells which, judging by their location, is presumed to be globose basal cells. Horizontal basal cells closest to the basement membrane appeared not to be stained. No significant labeling was observed in sense probe controls.

Fig 5-4. Detection of 16b5 mRNA in adult mouse olfactory mucosa using in situ hybridization. The in situ hybridization result shows that 16b5 mRNA is mainly expressed in the neuronal layer of olfactory epithelium including mature and immature neurons (A). Some superficial basal cells were stained. The weak labeling suggests that the copy number of the unknown gene in olfactory neurons is low. No labeling was observed in ISH using sense oligoprobe (B). Arrows indicate the basement membrane. Bars = 20 um.
5.3.3 Sequence blast

The entire sequence of 1593 nucleotides of the novel fragment was blasted against the gene databases maintained in NCBI (National Center of Biotechnology Information; http://www.ncbi.nlm.nih.gov), using the BLASTN 2.2.10 blasting machine. The result showed that the 16b5 sequence had 98% homology (1201 out of 1221 nucleotides) to the mouse RIKEN cDNA 5930416I19 gene (GeneID = 72440). The gene contains a conserved domain of Presenilin which is involved in Alzheimer’s disease (Moretti et al., 2004). The 16b5 sequence also had about 80% homology to the cloned sequences from human hepatoblastoma and neuroblastoma, and about 80% homology to the ESTs from adult mouse retina and liver.
5.4 Discussion

Our in situ hybridization result has shown that the differentiated OLF442 cells expressed 16b5 mRNA while the undifferentiated OLF442 cells did not express the 16b5 to a detectable degree. However, the more sensitive RT-PCR shows that the undifferentiated OLF442 cells indeed express 16b5 (Figure 5-2). In the mouse section, 16b5 mRNA was expressed in neuronal cells including immature and mature neurons and, appeared to be expressed in globose basal cells rather than horizontal basal cells. RT-PCR has demonstrated that 16b5 mRNA were expressed in a wide range of other mouse tissues.

5.4.1 The unknown gene, 16b5

The unknown gene, 16b5, previously identified by differential display, isolated by RNAse protection assay, and charaterised by Marathon 5’ RACE, was found to be upregulated by differentiation in the OLF442 cells which is an immortalized olfactory cell line (Zehntner, 1998). The present study observation using in situ hybridization has confirmed this in OLF442 cells. In the in situ hybridization study on the OLF442 cells, 16b5 was strongly expressed in differentiated cells rather than in undifferentiated cells suggesting the unknown gene was upregulated by differentiation. To study the significance of the unknown gene, 1655 was blasted and found to have no homology to identified genes within the GENBANK or EMBL although the sequence had 100% homology to numerous mouse expressed sequence tags (EST’s) as well as up to 80% homology with a number of human EST’s (Zehntner, 1998). A recent research had similar results (see section 5.3.3). Further studies can not move on until there is more information available.
5.4.2 RT-PCR screening

In the RT-PCR studies, undifferentiated and serum-depleted OLF442 cells, olfactory mucosa and a variety of other mouse tissues were found to express 16b5, including mouse brain, olfactory epithelium, lamina propria, olfactory bulb, liver, kidney, lung, and heart. Muscle did not express 16b5. Since the expression in mouse tissues was comprehensive, studies on the 16b5 were discontinued in the middle of the research.

5.4.3 Findings for OLF442

When undifferentiated, OLF442 was found to express high level of the neurofilament protein NF-M in a Western study and the neurofilament protein NF-H was not detected by immunochemistry. Following serum depletion, the expression of NF-M was decreased while the expression of NF-H became immunochemically detectable (Zehntner, 1998). The more sensitive RT-PCR detected low level of NF-L mRNA in both differentiated and undifferentiated cells (Reviewed by Zehntner, 1998). Olfactory marker protein and β−tubulin were immunochemically detected in serum-depleted OLF442 although their expression could not be detected by Western or Northern blot (MacDonald et al., 1996; Zehntner, 1998). However, the neural cell adhesion molecule (N-CAM) normally found in globose basal cells could not be detected in OLF442. In the studies of MacDonald et al., 1996, neurofilament and glial fibrillary acidic protein were found to be co-expressed in bipolar cells of a particular morphological class. According to the expression of these neuronal markers, OLF442 has been characterized to be similar to an immature olfactory precursor, at an intermediate stage of differentiation, being potentially able to provide insight into how undifferentiated, immature neuronal precursors give rise to mature olfactory receptor neurons (Zehntner, 1998; Heras, 2003).

In theory, the above findings can only show that OLF442 cells are in the neuronal populations of the olfactory epithelium but can not clearly define the stage of maturity of the cells. For example, the expression of OMP but no expression of N-CAM suggest separate stages of maturity of a neuronal cell, in the serum-depleted OLF442 cells. One explanation for this is OLF442 cells, either undifferentiated or differentiated by serum
depletion, were under the influences of the *n-myc* oncogene that may have altered the genetic phenotype of the dividing olfactory cells when immortalized, eventually leading to change of expression of some neuronal markers but not others.

In our result, the differentiated OLF442 cells expressed 16b5 mRNA while the undifferentiated cells did not express 16b5 mRNA to a detectable level (see Figure 5-3). However, the more sensitive RT-PCR showed that 16b5 was also expressed in undifferentiated OLF442 cells. In the mouse section, 16b5 mRNA was expressed in neuronal cells including immature and mature neurons and, appeared to be expressed in globose basal cells rather than horizontal basal cells although the identity of globose basal cells needs to be verified by a double labeling method (see Figure 5-4).
Chapter Six

General discussion
The work of the thesis demonstrates the expression of multiple growth factors and growth factor receptors in the olfactory epithelium and lamina propria. TGFβ superfamily ligands and receptors were expressed in both olfactory epithelium and lamina propria (Table 6-1) except for BMP2/4/7 that were not detected by RT-PCR using three different pairs of primers (data not shown). To work out the problem of detection of BMP2/4/7, incorporation of a positive control tissue may be helpful for further studies. In the Table 6-1, the immunocytochemistry has shown that TGFβ receptors 1, 2 and 3 were expressed in presumed globose basal cells, immature and mature neurons where the expression of TGFβ receptor 2 in these areas has been confirmed by a recent in vivo study (Getchell et al, 2002). Horizontal basal cells and supporting cells did not express the mRNAs of these receptors. Other members of the TGFβ superfamily were found to be expressed in olfactory epithelium and lamina propria using RT-PCR but the identity of the cells expressing them remains to be investigated.

In the studies of FGF2, FGF2 mRNA was strongly expressed in presumed globose basal cells, immature and mature neurons, glandular cells in lamina propria, and weakly expressed in the apically located cytoplasm of the supporting cells. Using the FGF2 antibodies validated by Western blotting in this project, the FGF2 protein was subsequently demonstrated to be expressed most intensely in the apically located cytoplasm of the supporting cells (Hsu et al., 2002). The expression of the detected FGF2 protein was cytosolic and is different from that of high molecular weight FGF2 proteins (22, 23, and 24 kd) which are generally nuclear and are limited to mature neurons and supporting cells of the olfactory epithelium (Goldstein et al., 1997). The discrepancy of FGF2 identification between in situ hybridization and immunocytochemistry studies may be due in part to the fact that FGF2 protein content does not correlate directly with the steady-state FGF2 mRNA levels because its expression is complex and is regulated transcriptionally and post-transcriptionally in a cell type specific manner (Hsu et al., 2002, and references therein). FGF2 protein may also be secreted and taken up by neighbouring cells, so that mRNA and protein expressions do not match.
FGFr-1 mRNA was found to be present in the presumed globose basal cells, neurons, and probably also in supporting cells. FGFr-1 immunoreactivity was identified throughout the epithelium and significantly in the ensheathing cells around the nerve bundles in lamina propria (Hsu et al., 2002). In another study of the FGF receptor isoforms, FGFr-1b, FGFr-1c and FGFr-2c were expressed in the olfactory epithelium while FGFr-1b, FGFr-1c, FGFr-2b, FGFr-2c, FGFr-3b, and FGFr-3c were expressed in lamina propria. These findings as well as the presence of FGF2 in olfactory epithelium suggest that FGF2 is an autocrine or paracrine signals for the proliferation of neuronal precursors in vivo (Hsu et al., 2002). Because only three receptor isoforms are present in the olfactory epithelium and because FGF receptors have different specificities for FGF ligands (Ornitz et al., 1996), the FGF signaling pathway for neurogenesis has been limited to only several possibilities.

**Table 6-1.** Expression of known growth factor ligands and receptors and 16b5 in the olfactory epithelium

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<tr>
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**Notes:** HBC= horizontal basal cell; GBC= globose basal cell; IN= immature neuron; MN= mature neuron; SC= supporting cell; OE= olfactory epithelium; LP= lamina propria; U= undifferentiated OLF442 cells; D= differentiated OLF442 cells. ICC = immunocytochemistry; ISH = in situ hybridisation.; + = positive expression; - = not detected; +/- = low level of expression.
6.1 TGFβ growth factor signaling

TGFβ-2 has been reported to stimulate terminal differentiation of olfactory neurons in vitro and promote the survival of N-CAM positive cells hence facilitating neurogenesis (Mahanthappa and Schwarting, 1993). In cultures containing only basal and supporting cells of olfactory epithelium, TGFβ-2 induced differentiation of keratin-positive basal cells which were postulated to be globose basal cells (Newman et al, 2000). TGFβ-2 mRNA has been localized in the olfactory epithelium of 14.5 – 16.5 day mouse embryos (Millan et al., 1991). Both TGFβr-1 and TGFβr-2 have been detected in the olfactory of E11-E16 mouse embryos (Mariano et al., 1998). In this project we have demonstrated that the immature and mature olfactory receptor neurons, and the presumed globose basal cells in adult mouse mucosa expressed TGFβ receptors 1, 2 and 3, suggesting these cells are targets for TGFβ signaling. The co-localization of TGFβ ligands 1, 2 and 3 in the same areas suggests the TGFβ signaling follow an autocrine and/or paracrine fashion.

The presence of TGFβr-2 in mature neurons implies that TGFβ may play a role in maintaining the mature neurons. The presence of TGFβr-2 in immature neurons has provided supportive evidence that TGFβ promotes terminal differentiation (Getchell et al., 2002). The expression of receptors for TGFβ–2 in the presumed globose basal cells has supported our working model for signaling control in olfactory epithelium (Newman et al., 2000). That is, FGF2 stimulates proliferation of globose basal cells and TGFβ-2 binds the TGFβ receptor complex to in turn induce the differentiation of the FGF2-induced globose basal cells. TGFβ signaling through TGFβr-2 on the globose basal cells may be a mechanism by which proliferation is regulated to maintain the balance between postmitotic immature neurons and their progenitors (Getchell et al., 2002). However, the identity of the presumed globose basal cells should be further verified by double labeling.

Previous receptor-ligand studies have revealed that the TGFβ growth factors bind the constitutively active TGFβ receptor 2, which then recruits the TGFβ receptor 1, resulting in trans-phosphorylation of the type 1 receptor and activation of downstream signals
through Smad proteins that associate common mediator Smads to eventually trigger the context-specific regulation of gene expression in the nucleus (Caestecker, 2004; Massague, 1998). The presence of the high molecular weight TGFβ receptor 3 has implicated its required existence as a co-receptor for supporting the TGFβ ligand (Blobe et al., 2001), or promoting the TGFβ ligand binding to the signaling receptors (Lopez-Casillas et al., 1993), or for inhibin promoting interactions between inhibin and the type 2 receptors, ActR 2, ActR 2B and BMPr 2, which are all expressed in the same areas as well (Caestecker, 2004).

Members of TGFβ superfamily do not always follow the same pathway to transduce signals. In contrast to the signaling of TGFβ subfamily, BMP2 and BMP4 preferentially bind to the type 1 receptors BMPR-1A and BMPR-1B and recruit type 2 receptors into heteromeric signaling complexes (Reviewed by Caestecker, 2004; refer to Table 2-1). BMPs may follow a concentration-specific, ligand-specific pathway for the signaling. For example, low concentrations of BMP4, but not BMP7, promote neurogenesis by supporting survival of N-CAM+ olfactory receptor neurons (Shou et al., 2000), and some BMPs, including BMP4 at high concentrations, inhibit neurogenesis by decreasing progenitor cell proliferation (Shou et al., 1999). Another example is a finding for Activin that increasing occupancy of a single receptor type can cause cells to switch gene expression (Dyson and Gurdon, 1998). The presence of all studied BMP receptor types in olfactory mucosa suggests BMPs play a role in regulating olfactory neurogenesis. The failure of detecting BMP2/4/7 in our RT-PCR may be because there was no human olfactory mucosa in the biopsies at all. Another possibility is the effect of BMPs in vitro (Caestecker, 2004, and references therein; refer to Table 2-1) was due to the effect of the receptors such as BMPR-1A, BMPR-1B, BMPR-2, ActR-2A or ActR-2B, and the growth factors that normally act on those receptors in vitro are different in vivo.

Inhibition of growth factor signaling is an important part of regulation of neurogenesis. The inhibition of TGFβ signaling pathway takes place in different ways. The inhibitor could be growth factor ligands themselves. For example, functional Activins are dimeric combinations of monomeric Activin β subunits (Pangas and Woodruff, 2000); but
Activin β and Inhibin α ligand subunits can heteromerically combine to form Inhibins A and B (Pangas and Woodruff, 2000), which competitively interacts with Activin type 2 receptors, but can not recruit the ActR-1B/ActR2 signaling complex, thereby inhibiting the signaling pathway of Activins (Lewis et al, 2000). Because TGFβr-3 not only promotes TGFβ ligand binding to the signaling receptors (Lopez-Casillas et al., 1993) but also acts as a co-receptor for Inhibin promoting interactions between Inhibin and the type 2 receptors ActR2, ActR2B and BMPr-2 (Caestecker, 2004), the co-expression of TGFβr-3 with these receptors in the olfactory epithelium suggests TGFβr-3 may have a role of functionally inhibiting Activin and BMP-dependent signaling. Another example is a recent finding that the overexpression of TGFα, a member of epidermal growth factors, downregulates the expression of TGFβ–2 and hence blocks the terminal differentiation of neurons induced by TGFβ–2 (Getchell et al., 2002).

It is possible that the inhibition of TGFβ growth factor signaling involves exogenous agents or plays out by a way of autoregulation. For example, the anti-neurogenetic effect of BMP4 in progenitor cells can be blocked by noggin, a specific BMP antagonist (Shou et al., 1999). An example of autoregulation is demonstrated in a recent study of the Growth and Differentiation Factor 11 (GDF11 is also known as BMP11; see Table 2-1) where the GDF11, expressed in neurons and progenitors, inhibited olfactory neurogenesis in vitro by inducing p27Kip1, a cyclin-dependent kinase inhibitor, and reversible cell cycle arrest in progenitor cells (Wu et al., 2003; Nakashima, 1999).
6.2 Fibroblast growth factor signaling

Fibroblast growth factors (FGFs) are potent growth factors controlling or influencing growth of epithelial, mesodermal, and neuroectodermal cells and their functions in the nervous system range from development to adult plasticity (Reviewed by Plendl et al., 1999). The best known and the most abundant FGFs in normal tissues are FGF1 and FGF2, previously known as acidic and basic FGF (Plendl et al., 1999). They perform action on cellular proliferation and differentiation, and have neurotrophic and angiogenic properties, by triggering specific receptors present at the surface of target cells (Plendl et al., 1999). FGF1 binds to four FGF receptors with high affinity while FGF2 binds to FGFr-1 with high specificity (Ornitz et al., 1996).

FGF1 was found to be expressed by olfactory ensheathing cells which are believed to modulate olfactory axon growth between the neuroepithelium and olfactory bulb (Key et al., 1996). In cultures of olfactory tissue, FGF1 stimulated morphological differentiation of olfactory ensheathing cells and primary sensory neurons (Key et al., 1996). In our RT-PCR experiment, we detected FGF1 signal in both olfactory epithelium and lamina propria, which is in conflict with previous studies that did not detect FGF1 in these areas (Key et al., 1996). Considering its receptors present in the olfactory mucosa (Hsu et al., 2001), FGF1 signal may have a role in neurogenesis but this is yet to be discovered.

FGF2 was demonstrated in mature olfactory neurons and supporting cells in vivo (Goldstein et al., 1997). In the present project we have demonstrated that FGF2 mRNA and its receptor FGFr-1 are expressed by superficially located basal cells, immature and mature cells, suggesting FGF2 is an autocrine and/or paracrine signal for the proliferation of neuronal precursor in vivo (Hsu et al., 2001). FGF2 promotes proliferation of neuronal precursors, globose basal cells, and a cell line derived from globose basal cell (Newman et al., 2000; Reviewed by Hsu et al., 2001). These findings altogether strongly suggest the proliferative role of FGF2 in olfactory neurogenesis in vivo.
FGF2 lacks a conventional signal sequence, which makes the export of the protein from the cell a puzzle to scientists. A possible mechanism is that the high molecular weight FGF2 proteins (the low molecular weight FGF2 may be included) are secreted via apoptosis (Goldstein et al., 1997; Newman et al., 2000) by which mature cells such as supporting cells and receptor neurons release FGF2 after they die, thus stimulating proliferation of globose basal cells, which would lead to neuronal replacement. Alternatively, the low molecular weight FGF2 protein possibly follows a transmembrane-associated, energy-dependent pathway (Florkiewicz et al., 1995) by which the protein is transported to any cells that are unable to produce FGF2 but close to the source of the protein. For example, the 18 kD FGF2 protein may be secreted by the alternative pathway from the glandular cells of Bowman’s gland (as identified in the present study) to the apical region of the olfactory epithelium (as observed in the immunochemistry experiment in Hsu et al., 2001) and accumulated in the supporting cells (Hsu et al., 2001).

6.3 Platelet derived growth factor signaling

PDGF A and PDGF receptor α were found in globose basal cells, neurons and possibly supporting cells. In separate studies, PDGF B and PDGF receptor β were expressed in the similar manner (Unpublished data from Sneesby, 1998). This is the case that the target cells for PDGF were the cells producing them, suggesting an autocrine and/or paracrine signaling pathway for PDGFs.

Functionally, PDGF AB promotes the survival of immature cells in cell cultures (Newman et al., 2000). It is proposed that PDGF dimers activate PDGF receptors through the generation of active PDGF receptor dimers in which each chain of the PDGF dimer binds to one receptor molecule (Betsholtz, 1995). The PDGFα-receptor subunit is able to bind to PDGF-A, and -B ligand subunit with high affinity, whereas the PDGFβ-receptor subunit binds only to the PDGF-B ligand subunit with high affinity (Betsholtz, 1995). So the differential expression of receptors α and β on a cell may affect the PDGF signaling on the cell. For example, if the expression of PDGF α is reduced in the cells of the
epithelium, the formation of αα and αβ receptor dimers may be decreased and the ββ receptor relatively increased. Because PDGF AB, in theory, is bound to the receptor dimers, αβ and αα, but not to the receptor dimer, ββ (Betsholtz, 1995). Thus, the relative decrease in expression of the receptor dimers for PDGF AB may weaken PDGF AB signal to promote cell survival.

PDGF signaling pathway may be regulated by FGF2 at the transcriptional level. It was reported that FGF-2 stimulates ERK1/2-dependent Sp1 phosphorylation, thereby repressing PDGF receptor α transcription via a cis-acting element in the PDGF receptor α promoter (Bonell and Khachigian, 2004). Because PDGFα-receptor subunit is able to bind PDGF-AA, -BB and –AB dimers with high affinity and because FGF2 is also expressed in the epithelium olfactory epithelium (see Chapter 3), the presence of PDGF receptor α in the immature neurons provides a possibility for FGF2 controlling PDGF cell survival function.

6.5 Molecular regulation of olfactory epithelium

In summary, FGF2 stimulated proliferation of a basal cell in cell cultures, TGFβ−2 induced differentiation of the ‘basal cell’ into an immature neuron and PDGF-AB promoted the survival of the immature neurons to enhance the survival of immature neurons (Newman et al., 2000). For TGFβ-2, our expression studies suggest that the basal cell is likely to be the globose basal cell because of its superficial position to the olfactory basement membrane. Once the identity of the cell verified by double labeling, the target cells for TGFβ−2 will be ascertained to exist in the globose basal cell populations. In combination with Sneesby’s work on PDGF B and PDGF receptor β (1998), here we have provided a possibility that PDGF-AB signals through PDGF receptors α and β at the surface of the immature neurons. TGFβ−2 and PDGF receptor α may be regulated by other growth factors. In addition to the proliferative role in neuronal progenitors, FGF2 can repress the transcription of PDGF receptor α (Bonell and Khachigian, 2004) at the transcription level. Recent evidence also shows that overexpression of TGFα
downregulates the expression of TGFβ-2, which consequently promotes proliferation of horizontal basal cells on the one hand and inhibits the terminal differentiation of mature neurons on the other (Getchell et al, 2002; Farbman & Ezeh, 2000; Farbman et al. 1994).

Of other important growth factors, BMP2/4 at a concentration above a threshold inhibits olfactory neurogenesis via a transcription factor, MASH1 (Shou et al., 2000; Shou et al., 1999). GDF11 is expressed in olfactory epithelium by neurons and progenitors (Wu et al., 2003; Nakashima, 1999). Released from mature neurons or progenitors, GDF11 inhibits the proliferation of progenitor cells (Wu et al., 2003). Other than the growth factors, leukemia inhibitory factor (LIF) is an important cytokine that has been found to enhance proliferation of neuronal proliferation in injury-induced neurogenesis (Bauer et al., 2003). In vivo evidence shows that the injured olfactory receptor neurons release LIF as a stimulus to initiate their own replacement (Bauer et al., 2003).

Based upon the information available, our previous working model for molecular regulation of olfactory neurogenesis (see Figure 1-4) can be modified as in Figure 6-1. The working model shows the growth factors regulating olfactory neurogenesis and their sites of action on identified cell types. Of course, these are probably not the only growth factors acting and these growth factors may have actions other than those illustrated. Some other growth factors such as IGF-1, or other members of FGF and BMP families may also play a role in the regulation of neurogenesis.
Figure 6-1. A schematic model of molecular regulation of olfactory neurogenesis. HBC = horizontal basal cell; GBC = globose basal cell; IN = immature neuron; MN = Mature neuron; The effect of FGF2 and TGFβ-2 on neurons is unclear. Broken lines indicate inhibitory effects. Whether FGF2 is released through apoptosis remains to be investigated.
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Appendix 1: Quality control of total RNAs

Figure A-1. Electrophoresis of total RNAs from different adult mouse tissues on a formaldehyde denaturing gel. 1 = olfactory epithelium; 2 = olfactory bulb; 3 = brain; 4 = heart; 5 = muscle; 6 = liver; 7 = lung; 8 = kidney; 9 = spleen. Bottom left: RNA in lane 7 (i.e., lung) shows no band indicating the RNA extraction was not effective. A clear trace is shown in lane 9 without typical 28S and 18S rRNAs indicating the RNAs have degraded. The 28S and 18S rRNAs are seen in the other 7 lanes showing the total RNA contents remain intact. Top two pictures were obtained from the 7 valid samples detected from the bottom left after repurification by isopropanol. The migration distances of 28S and 18S in the top left picture were measured and their log values were compared with a reference curve constructed from the marker lane (M) to obtain their sizes (data not shown).
Appendix 2: Specificity test for the antibodies of TGFβ family of receptors

Figure A-2. Specificity test for antibodies towards TGFβ family of receptors using Western blotting techniques. The film (5 sec exposure) above only show a part of the results where polyclonal antibodies were applied in a dilution gradient of 1/1000 (=1), 1/3000 (=3), 1/5000 (=5), and 1/7000 (=7). Higher dilutions of 1/10000 and 1/12000 were tried but the results are not shown here. The concentration of secondary antibody was once lowered to various degrees in several attempts in order to eliminate the dirty background but these attempts were of no avail, indicating the contamination came from the antisera. The left film was for TGFβr-1 and TGFβr-2, and the right film was for BMPr-1A. Both failed to show the bands of expected size (molecular weight for type 1 receptor ~ 53 kD, for type 2 receptor = 70 - 75 kD, and for type 3 receptor = 200 - 400 kD; Massague, 1990; Kingsley, 1994). C is negative control where the primary antibody was omitted.
Appendix 3: Dot Blot test

Figure A-3. Dot Blot test for DIG-labeled probes. 1, 5 = FGFr-1 oligoprobe; 2, 4 = 16b5 unknown gene oligoprobe; 3: Labeled control oligoprobe (in antisense area) and unlabeled control oligoprobe (in sense area); 6 = FGF2 riboprobe; 7 = PDGF A riboprobe; 8 = PDGF α riboprobe; 9 = PDGF B riboprobe; 10 = PDGF β riboprobe. Except for the row 3 of controls, the left four dots were spotted with antisense probes while right four dots were spotted with sense probes. Concentrations for oligoprobe dots including controls were 0.5 pmole/μl, 0.1 pmole/μl, 0.02 pmole/μl, and 0.004 pmole/μl, respectively. Concentrations for riboprobes were 100 pg/μl, 10 pg/μl, 1 pg/μl, and 0.1 pg/μl, respectively. Color was developed for 13 hours.