Chapter One: General Introduction

Introduction
Many examples exist of simple mendelian inheritance in a variety of organisms particularly in those whose genetics have been most thoroughly dissected. The compound eye of Drosophila and the vulva of C. elegans have contributed greatly to an understanding of the genetic basis for phenotypic variation in multicellular animals. As a result of their relative ease of genetic manipulation and short gestation period, an understanding of the genetic interaction between alleles at multiple loci is also becoming evident from studies of these two species. Often, a result of this genetic analysis has been the identification of biochemically interacting molecules which are components of conserved signalling pathways.

A large number of documented mouse mutations also exhibit a simple mendelian inheritance although the underlying molecular basis of the mutation is in most cases unknown (Jackson Laboratory Web Page). These mutations may provide a similarly powerful tool for dissecting the complex genetic interactions regulating mammalian development. Many of these mutations disrupt the normal development of the hair follicle suggesting a functional role for these gene products in regulating its growth and development (Sundberg, 1994). In this study, an examination of several hair mutations resulting from a disruption of function of members of the EGF and FGF families of peptide growth factors has been undertaken. By first reviewing the experimental data as to whether these molecules interact at a biochemical level a hypothesis is presented that it is likely that they will also interact genetically. Therefore in this study, in contrast to others, evidence for a biochemical interaction was first established prior to conducting an analysis of a genetic interaction. Assessing the phenotypic consequences of combining these biochemically interacting mutations could therefore increase our understanding of genetically disrupting a biochemically conserved mammalian signalling pathway.

The Hair Follicle

Embryogenesis of the Hair Follicle
The hair follicle has evolved as an epidermal structure with a wide range of functions. These include: protection, temperature regulation, excretion of metabolic by-products and sexual signalling. Hair, and other epidermal appendages such as feathers, scales, claws, horns and wool, are formed from embryonic tissues of both mesodermal and ectodermal origin. A series of inductive interactions between the embryonic mesoderm and ectoderm determine the site and type of epidermal appendage formed (Hardy,
The nature of this induction is unknown although an initial competence signal from the dermis (mesoderm) is thought to induce the generalised formation of skin appendages in vertebrates. Tissue recombination experiments have revealed that mouse dermal tissue is capable of inducing the formation of feathers or scales when combined with chicken or reptile epidermis respectively (Sengel, 1958). The inductive capacity of dermal tissue has also been demonstrated by the ectopic induction of hair follicles with transplanted rat vibrissae dermal papilla (see Jahoda, 1992 for review). The inductive interactions of dermal and epidermal tissues are therefore likely to play an important role in regulating hair follicle growth not only during embryogenesis but also in the mature cycling follicle.

The mouse has at least eight hair types which are produced in different body regions (Sundberg, 1994). These include the four types of pelage hairs, vibrissae or sensory hairs, clia (eyelashes), tail hairs, ear hairs and hair around the feet, nipples and perianal/genital regions. The first hair follicles initiated during mouse embryogenesis are the vibrissae or whisker follicles which form primordia at approximately 12 days of gestation while pelage hair follicle primordia form at 14 days gestation. Follicle initiation continues until birth and possibly several days after this period, although independent observations vary regarding this time frame (Raphael, 1982). The development of the hair follicle is first visible as a thickening in the embryonic epidermis overlying the presumptive mesenchymal condensate. Epidermal placode formation is then followed by an involution of this region of the epidermis into the dermis with the concomitant formation of the mesenchymal condensate, the pre-papilla, which may be the result of an inductive signal from the epidermis. A second inductive dermal signal from the pre-papilla may then stimulate epidermal proliferation inducing the downgrowth of epithelial cells to form the bulbous hair peg of the hair follicle anlagen (Hardy, 1992). As the follicle matures the epidermally derived cells differentiate to form the mature hair follicle (Figure 1.1) which consists of several discrete cylindrical tissue layers surrounded by a dermal sheath. These include the Outer Root Sheath (ORS), Inner Root Sheath (IRS) and hair follicle matrix cells enclosing the dermal papilla. A comprehensive analysis of eight stages of embryogenesis of the hair follicle in the mouse has been carried out (Hardy, 1949).

**The Mature Hair Follicle**

The hair follicle is a relatively simple structure with several subdomains of cellular activity definable in the mature follicle. The most mitotically active regions are the bulb consisting of hair matrix cells which, during the actively growing stage, are continuously dividing and differentiating to form the mature fibre. The mature anagen follicle (Figure 1.1) consists of a series of concentric cellular layers organised around a central axis which arises from the rapidly proliferating epithelial cells of the follicle bulb. The three innermost layers, the medulla, cortex and hair cuticle comprise the mature hair fibre and
are characterised by the expression of hair follicle specific keratins. The medulla is comprised of regularly arranged cells separated by air-filled spaces while the cortex is a hollow keratinised cylinder. The hair cuticle is a tube of thin overlapping scales which encloses the cortex. These three layers constitute the visible hair fibre which emerges through the epidermal surface via the pilary canal. The next three layers are collectively referred to as the Inner Root Sheath (IRS) and consist of cuticle, Huxley's layer and Henle's layer.

The final concentric layer is the Outer Root Sheath (ORS) which is continuous with the basal layer of the interfollicular epidermis and is the epidermal layer of the follicle in closest contact with the dermal sheath. The sebaceous glands and sweat glands are formed concomitantly with the ORS. The hair fibre develops as the rapidly dividing cells of the hair matrix are displaced or migrate distally toward the skin surface and undergo a poorly understood differentiative process to form the discrete layers of the hair follicle. The assignment of differentiated cell fates to the proliferating bulb cells has been proposed to occur via a process involving morphogenic gradients and cell-cell interaction (Kopan and Weintraub, 1993).

The mature follicle is divisible into several zones of cellular differentiation. The first is the proliferative or mitotically active zone of the follicle bulb, where the rapidly dividing cells of the hair matrix are located. The next region is the keratogenous zone in the lower third of the follicle consisting of bulb cells which have differentiated to form the distinctive cell layers of the mature follicle. The expression of hair follicle specific keratins is also initiated in this region. This is followed by the zone of hardening where the program of keratinisation is completed followed by the zone of sloughing where the IRS cells separate from the emerging hair fibre and slough into the pilary canal formed by the ORS cells. This is also the area into which secretions from the sebaceous and sweat glands are dispersed.

The adult hair coat of mice consists of four distinct hair types (Dry, 1926). These are: the guard hairs; the longest fibre type which grows with no constrictions and comprise a small portion of the total pelage hairs, the aochene and awl hair types which are shorter than guard hairs with aochenes containing one constriction; and the zig-zag fibres which comprise the majority of the pelage and possess several constrictions. The relative proportions of each fibre type including number, length and gross morphology are affected in several mouse hair mutations (Sundberg, 1994).

The existence of a potential stem cell population in the hair follicle is uncertain. A putative localisation has been proposed in the 'bulge' region of the ORS located in the nonregressing portion of the hair follicle situated below the arrector pili muscle (Cotsarelis et al., 1990). Epidermal ORS cells in this region are relatively
undifferentiated and slow cycling but can be induced to proliferate upon exposure to TPA(12-tetradecanoylphorbol-13-acetate), a potent tumor inducing agent. Highly proliferative keratinocytes have also been isolated from the putative bulge region of rat vibrissae (Kobayashi et al., 1993).

The bulge region in rodents is a prominent feature during embryogenesis but this regresses to become almost indistinguishable in the mature follicle (Pinkus, 1958). In other species, such as the Merino sheep, a bulge region has not been observed (Auber, 1950). An analysis of the proliferative capacity of the human hair follicle has found regions of the ORS which are highly proliferative in vitro but are not associated with the putative bulge region (Rochat et al., 1994). An alternative localisation of a stem cell population has also been suggested to reside in the proliferating cells of the hair matrix in the follicle bulb (Reynolds and Jahoda, 1991).

The Hair Cycle

In most mammals the hair follicle undergoes a cycle of active growth followed by a period of regression and quiescence (Figure 1.2). This recurrent change in cellular
activity makes the hair follicle a unique structure which regularly regenerates in the adult. Changes in the growth rate of the hair follicle can be influenced by a number of factors including photoperiod, seasonal changes, sexual maturity as well as nutritional, environmental and physiological stress. In the mouse, development of the first hair coat occurs in a synchronous wave with the initiation of follicle formation occurring around 14 days of gestation. Three main phases of hair follicle growth have been identified in the mouse (Dry, 1926).

![Diagram of hair follicle growth phases](image)

**Figure 1.2** Schematic representation of the stages of the murine hair cycle (reprinted with permission from Lauer et al., 1995). The period from mid anagen of an actively growing hair follicle is shown progressing through catagen, telogen and then the resumption of anagen growth.

The anagen phase of active growth is followed by the catagen phase, when an abrupt cessation of mitotic activity in the bulb occurs and the transient portion of the hair follicle regresses, resulting in the formation of a hair club loosely anchored in the epidermis. The hair club is not in contact with the dermal papilla but is surrounded by an ORS structure, the epidermal sac, and is connected to the dermal papilla via the hair germ. The degeneration of hair matrix cells which occurs during catagen is largely due to programmed cell death and terminal differentiation. Changes also occur in the dermal papilla during this period including the degradation of the ECM, a decrease in intracellular space and an aggregation of the dermal papilla cells (Lavker et al., 1994). This degenerative phase is followed by a period of mitotic inactivity, the telogen phase, which can vary in length before the reinitiation of anagen growth. Once anagen is reinitiated and a new hair follicle is formed the original fibre is dislodged from the epidermis and shed. The anagen phase has been subdivided into six substages, anagen i-VI (Chase et al., 1951) and generally progresses over a 15 day period in the
mouse, while the catagen phase has been subdivided into eight substages, catagen I-VIII (Straie et al., 1961) and rapidly progresses to the telogen phase which usually lasts from 7 to 10 days.

The cessation of the first hair cycle in the mouse is synchronous and progresses from the head to the posterior regions with subsequent hair cycles becoming more asynchronous. The first hair cycle may also be unique due to the embryonic nature of the first hair coat and distinct from the subsequent cycling of the mature hair follicle (Sundberg and King, 1996). Other species, such as humans, and Merino sheep, have a mosaic or random cycling of hair follicles, and do not exhibit synchronous growth regulation except in the neonate. They also have longer periods of anagen growth. Changes in the thickness of the skin also occur during the various stages of the hair cycle (Chase et al., 1959). Skin thickness is at its maximum during the anagen phase with the onset of catagen resulting in a decrease in thickness of the dermal and adipose layers and a slight thickening of the epidermis. The skin is at its thinnest during the resting or telogen stage of the hair cycle.

The hair cycle is thought to be under the control of both systemic and local factors with a number of hypotheses having been proposed to explain their influence on hair growth control. The accumulation of an inhibitor of hair follicle growth during the anagen phase of the hair cycle which reaches a threshold level and activates the transition to catagen, the Chalone hypothesis, has been suggested as a potential mechanism of hair growth inhibition (Chase and Eaton, 1959). Others have proposed that the proliferative cells of the hair follicle bulb have a finite capacity for cell division which, once exhausted, allows the follicle to regress and enter the catagen state (Cotsarelis et al., 1990). However, these intrinsic control mechanisms of follicle growth regulation do not explain how exogenous agents such as Cyclosporin A (Urabe et al., 1995) can increase the period of time spent in anagen if threshold levels of an inhibitor have been reached or the transit amplifying cell population has been exhausted.

Peptide Growth Factors and Hair Development

Peptide growth factors are small polypeptides involved in the regulation of a wide variety of cellular activity. Activation of a cellular response is dependent on the presence of an appropriate receptor on the responding cell-type initiating a cascade of intracellular signalling events. This can result in a spectrum of cellular responses ranging from proliferation to differentiation depending on the cellular context in which they are activated. A number of these molecules have been shown to affect the development and activity of the embryonic and mature hair follicle although the exact nature of the cellular events perturbed remains unclear.
The genetic basis of several spontaneous mouse mutations affecting growth and development of the hair follicle has indicated that members of this broadly definable group of molecules are involved in the regulation of hair growth (Sundberg and King, 1996). Several of these genes encode members of the Epidermal Growth Factor (EGF) and Fibroblast Growth Factor (FGF) families of polypeptide growth factors. Comparative developmental biology is revealing that evolutionarily conserved cellular signalling pathways are involved in the seemingly disparate morphological development of the nematode vulva, the Drosophila eye, the chicken limb bud, and, potentially in the formation of epidermal appendages such as the hair follicle. These conserved pathways involve peptide growth factors, their transmembrane receptors, and intracellular signalling molecules in a complex biochemical network regulating a number of decision points determining cell fate.

Members of the Receptor Protein Tyrosine Kinase (RPTK) family of transmembrane signalling molecules and their associated ligands are components of these networks and, when mutated result in anomalous hair follicle development and growth regulation in the mouse (Sundberg and King, 1996). By combining information gathered from molecular developmental biology with a knowledge of the genetic basis for several mouse hair mutations, a novel experimental approach may be possible which could increase our understanding of these biochemical networks and their role in mammalian development.

The Receptor Protein Tyrosine Kinase (RPTK) Family

The RPTK family of transmembrane molecules are members of the superfamily of protein kinases which are involved in a wide range of intracellular signalling events utilising a conserved catalytic protein kinase domain (Van der Geer and Hunter, 1994). The RPTKs are Type I transmembrane spanning molecules containing an extracellular ligand binding domain, a single transmembrane region and an intracellular region consisting of a conserved tyrosine kinase autocatalytic domain and several tyrosine residues which are autophosphorylated upon receptor activation. The current RPTK family consists of 14 subfamily groupings (Van de Geer et al., 1994), several of which have been shown to be present in the embryonic and mature hair follicle (Stenn, 1994). The Epidermal Growth Factor Receptor (EGFR) and Fibroblast Growth Factor Receptor (FGFR) RPTK subfamilies and their associated ligands will be considered with regard to their role in epidermal development and regulation of hair follicle growth. A potential interrelationship between these two RPTK subfamilies is also examined.

The EGFR subfamily

EGFR ligands

The members of the EGF ligand family which are known to activate the EGFR currently consists of five peptides; EGF, TGF-α, amphiregulin, betacellulin and Heparin Binding-
EGF (HB-EGF). These molecules belong to a larger grouping of transmembrane glycoproteins which contain varying repeats of an EGF-like domain, a protein sequence consisting of a characteristic spacing of six cysteine residues which can pair to form three disulfide bonds (Massagué, 1990). The mature glycosylated peptide is translocated to the cell-surface with the EGF domain presented extracellularly. This region may interact as a membrane-bound molecule with the EGFR (Brachmann et al., 1989; Wong et al., 1989) or it can be proteolytically cleaved, possibly by an autocrine mechanism, to generate a diffusible ligand (Baselga et al., 1996). Both membrane bound and soluble forms of the ligand are biologically active and although their different functions are unclear they may mediate intercellular communication in a juxtacrine or paracrine manner. The intracellular carboxy terminus of membrane bound TGF-α can also associate with intracellular proteins containing tyrosine/serine/threonine kinase activity suggestive of a form of reverse cellular signalling by the membrane bound ligand as well as the activation of signal transduction in cells presenting the EGFR (Shum et al., 1994). A functional requirement for heparin sulphate proteoglycans by two members of this ligand family, HB-EGF and amphiregulin, has been reported (Piepkorn et al., 1994; Hashimoto et al., 1994) while a high molecular weight pro-EGF form also binds to heparin (Parries et al., 1995).

Members of this family of growth factor ligands were among the first shown to have an effect on the development of the hair follicle when preparations of EGF from murine submaxillary gland administered to newborn mice inhibited the normal growth and development of the first hair coat (Levi-Montalcini and Cohen, 1960; Cohen, 1962). Dorsal skin from newborn mice treated with EGF exhibited hyperplasia, increased keratinisation and an inhibition of hair growth. Accelerated eyelid opening and tooth eruption as well as a decrease in body weight were also observed. A more comprehensive study of daily injections for a 14 day period post-partum resulted in a retardation of hair growth with a decrease in hair diameter and length of the follicle bulb (Moore et al., 1983). Purified TGF-α also induces similar changes in mouse epidermal development and hair growth (Tam, 1985).

The generation of transgenic mice containing either null alleles or an overexpression of TGF-α have revealed a functional role in vivo for this member of the EGF ligand family during epidermal development. Mice homozygous for null alleles of the TGF-α locus present a phenotype that has been shown by complementation analysis to be allelic with the waved-1 mutation (Luetteke et al., 1993; Mann et al., 1993). These mice exhibit premature eyelid opening and tooth eruption, and the regular orientation of hair follicles is disrupted resulting in the production of waved-hair fibres. A potential role for TGF-α in maintaining the normal differentiation of the hair fibre and as an epidermal orientation signal during follicle development has been suggested to account for the observed phenotype of mice lacking this growth factor. Conversely, mice
overexpressing a TGF-α transgene in epidermally derived tissue developed fewer hair follicles and displayed epidermal hyperplasia, hyperkeratosis and a thinner dermis in the neonate. Spontaneous papillomas also developed in adult animals by 3-4 months of age (Dominey et al., 1993).

Members of this ligand family are expressed in both the proliferative and differentiating regions of the hair follicle and the interfollicular epidermis (Table 1.1). Localisation of TGF-α transcript in the mature wild-type mouse hair follicle was observed in the region of the IRS defined by the zone of keratinisation and in a limited region of the ORS (Lustteke et al., 1993). In the sheep, immunohistochemical analysis has detected TGF-α protein in the ORS, sebocytes and epidermis. These sites all show a decrease in immunoreactivity at the onset of a photoperiod induced catagen state which is re-established in late proanagen (Nixon et al., 1996). Human skin displays weak immunoreactivity for TGF-α in the IRS, parts of the ORS, sebocytes and both the basal keratinocyte layer and intermediate layers of the epidermis. Strong TGF-α immunoreactivity was also noted in the matrix cells of the bulb (Aklyama et al., 1996). The immunolocalisation of a putative sheep EGF has been noted in the periderm of embryonic epidermis, and the ORS, sebocytes and pilary canal of the mature follicle (du Cros et al., 1993). In human epidermis weak EGF immunoreactivity was noted in the periderm as well as IRS, parts of the ORS, the basal and intermediate layers of the epidermis and the hair matrix. Increased immunoreactivity was also observed in the sebocytes where EGF may be secreted (Aklyama et al., 1996). Other EGFR ligand family members have not been examined in situ and their localisations in the hair follicle are unknown.

Several EGFR ligands stimulate an autocrine mitogenic response in keratinocytes in vitro including EGF, TGF-α (Coffey et al., 1987), amphiregulin (Cock et al., 1991) and HB-EGF (Hashimoto et al., 1994). The autocrine stimulation of human keratinocytes has revealed that a coordinated transcriptional crossregulation of members of this ligand family may occur in vitro (Barnard et al., 1994). When cultured cells were stimulated with each of the EGF ligand family members, the activation of transcripts for all members was induced, with two members, HB-EGF and amphiregulin, exhibiting an immediate-early transcriptional response. A degree of functional redundancy within this gene family has been indicated by utilising keratinocytes from both wild-type and Tgfα-/- mice (Dlugosz et al., 1995). The autocrine response of keratinocytes derived from either animal was examined in vitro and in vivo for transforming ability when transfected with the v-rasH oncogene. All known EGFR ligands, except EGF, were upregulated in transformed keratinocytes in vitro. Keratinocytes grafted onto Nude mice from both TGF-α deficient and wild-type cell lines induced squamous cell tumors in vivo suggesting that TGF-α was not required for cellular transformation. The authors have
postulated that other members of the EGFR ligand family are able to compensate for
the absence of a functional TGF-α gene product.

Other EGF domain-containing proteins have recently been shown to interact with the
EGFR and may indicate that a more complex regulation of ligand-induced activation of
the EGFR may be occurring. A potential negative trans regulatory function of the EGF
domain-containing molecule neuregulin (NDF) has been postulated and NDF may inhibit
EGF binding to the EGFR in vitro (Karunageran et al., 1995, Chen et al., 1996). A
similar negative trans regulatory mechanism has been proposed for the argos gene in
Drosophila and its EGFR homologue, DER (Schweitzer et al., 1995b). Several isoforms
of neuregulin exist which vary in their EGF-like domain, receptor binding affinity and
effect on cell proliferation. The neuregulin β isoform is able to interact with the EGFR via
erbB3 heterodimerisation (Pinkas-Kramarski et al., 1998). The effect of neuregulin
isoforms on mouse keratinocyte proliferation revealed a decreased mitogenic effect on
cell growth compared to EGF suggestive of a differential regulation of EGFR activity
(Markovsky et al., 1995).

The potential functional role of these EGFR ligands has been complicated by the
degree of heterodimerisation that can occur between members of the EGFR subfamily.
A broader functional activity may exist within this ligand family involving differential
interactions between various erbB heteromers and specific ligands (Beerli and Hynes
et al., 1996). The number and combination of EGFR subfamily members present on the
cell surface, as well as the particular ligand bound, may determine the intracellular
signalling pathways that can be stimulated in a specific cellular context.

**EGFR**

The EGFR (erbB-1) is one of 4 currently identified members of this RPTK subfamily
which includes erbB-2(neu, HER-2,p185), erbB-3(HER-3) and erbB-4(HER-4). These
molecules are widely expressed during development and at maturity in a range of
tissues, an observation consistent with a pleiotropic effect of mutation in members of
this RPTK subfamily. Aberrant expression of the EGFR subfamily and their associated
ligands have also been implicated in a variety of carcinomas (Khazale et al.,., 1993).
The classical model of EGFR activation is the formation of a homodimeric signalling
complex upon ligand binding. This results in the autophosphorylation of the intracellular
C-terminus by the autocatalytic tyrosine kinase domain at a number of tyrosine
residues. These may then act as docking sites for SH-2 containing intracellular
signalling molecules (Schlessinger and Ullrich, 1992).

Several examples of the intracellular signalling molecules activated by the EGFR
include the well known sos/Grb2 activation of the MAPK cascade via ras/raf activation
(Eagan et al., 1993), other adaptor molecules such as Shc (Bonfini et al., 1995), PLCγ
activation of phospholipid turnover (Van der Geer and Hunter, 1994), phosphorylation of various PKC isoforms (Denning et al., 1996), the activation of a variety of intracellular kinases containing SH-2, SH-3 and pleckstrin homology domains (Pawson, 1995; Marengere and Pawson, 1994) and a number of potential transcription factors that may translocate to the nucleus after EGFR activation. These include members of the STAT family (Fu and Zhang, 1993) and a novel zinc finger-containing molecule (Galchevargova et al., 1996). A comprehensive review of intracellular signalling pathways activated by the EGFR subfamily has been published (Van der Geer and Hunter, 1994).

*ErbB-2* is considered an orphan receptor as no specific ligand has been found which binds to this subfamily member. It does, however, play an integral role in the activation of downstream signalling events due to its ability to heterodimerise with all other members of the EGFR subfamily and may be a shared subunit (Karunagaran et al., 1996; Chen et al., 1996). *ErbB-3* is a catalytically inactive member of this subfamily but its activity is restored when its ligand, neuregulin, is bound and heterodimerised with the subfamily members EGFR and erbB-2. This association permits the autophosphorylation of erbB-3 and the activation of specific intracellular signalling molecules associated with this receptor (Pinkas-Kramarski et al., 1996).

*ErbB-4* is also bound by isoforms of neuregulin and can heterodimerise with the EGFR (Cohen et al., 1996). The EGFR-binding ligand betacellulin, may also activate the erbB-4 receptor and potentiate a distinct intracellular signalling cascade (Riese et al., 1996a). A complex hierarchical network of ligand-receptor interactions and the activation of multiple downstream signalling events is an emerging paradigm for signal transduction within this RPTK subfamily (Tzahar et al., 1996; Riese et al., 1996b). The degree of heterodimerisation and the intracellular signalling events initiated may be determined by the number of receptors on the cell surface and the activating ligand. The possible transmodulation of EGFR activity by other RPTK family members (Davis and Czech, 1987; Chantr, 1995) and G-protein-coupled receptors (Daub et al., 1996) is another area of interaction which complicates the signalling paradigm further.

The only naturally occurring mutation in the EGFR subfamily currently known to affect hair growth is the *Egfr*-a mutant which results in a phenotype of waved-hair fibres and disorganised hair follicles (Luetteke et al., 1994). Several other EGFR subfamily members are expressed in the epidermis or in keratinocytes *in vitro* although their roles in the regulation of epidermal development are unclear (Table 1.1). The targeted disruption of the EGFR generating a null allele generally results in embryonic lethality although some animals do survive postnatally depending on the genetic background of the mouse strain utilised (Miettinen et al., 1995; Threadgill et al., 1995; Sibilia and Wagner, 1995). Mice surviving postnatally exhibit an immature epithelial phenotype,
generalised organ hypoplasia and are significantly smaller than their healthy littermates. A more defined epidermal function for the EGFR has been revealed with the construction of an epidermally expressed dominant negative EGFR mutation (Murillas et al., 1995). Mice homozygous for this mutation developed a wavy-hair phenotype consistent with the Egfr<sup>−/−</sup> mutation with hair follicles failing to enter the catagen stage of the normal hair cycle and eventually undergoing necrotic cell death. This suggests a functional role for the wild-type EGFR in the regulation of the anagen to catagen transition of the murine hair cycle. Homozygous transgenic null alleles of erbB-2 and erbB-4 result in mid-gestational lethality with mice exhibiting neural and cardiac developmental defects (Lee et al., 1995; Gassman et al., 1995).

Epidermal localisations of EGFR have revealed that this protein is expressed during the early stages of follicle morphogenesis and in the mature follicle (Table 1.1). Generally EGFR localisations have been determined using immunohistochemistry or the binding of radiolabelled [125]<sub>I</sub>EGF to putative EGFR sites. During embryogenesis of the hair follicle detectable levels of EGFR are present in the epidermis of rat and human skin with a localised decrease in [125]<sub>I</sub>EGF binding or EGFR immunoreactivity observed in the epidermal area of the developing hair germ (Green and Couchman, 1984, Nanney et al., 1990). In embryonic sheep epidermis a more generalised decrease in epidermal [125]<sub>I</sub>EGF binding is observed coincident with follicle initiation (Wynn et al., 1995). In the mature wool follicle, the EGFR is associated with proliferative populations of epidermal cells in the basal keratinocyte layer, sebocytes, the follicle bulb, and the IRS/ORS layers of the wool follicle (Wynn et al., 1989). [125]<sub>I</sub>EGF binding sites in adult rat epidermis are detected in the ORS, follicle bulb and sebocytes.

An analysis of [125]<sub>I</sub>EGF binding during the rat hair cycle suggests that an appreciable change in EGFR levels may occur during the late anagen and early catagen stages. A decrease in the number of [125]<sub>I</sub>EGF binding sites is observed in the hair matrix cells adjacent to the upper regions of the dermal papilla during this period (Green and Couchman, 1984). In human epidermis, EGFR immunoreactivity is detectable in the basal keratinocyte layer, sebocytes, the hair matrix and the ORS while weaker levels are observed in the intermediate layers of the epidermis and the IRS of the hair follicle (Akiyama et al., 1986, Nanney et al., 1990). The transcript for erbB-2 has been detected in a number of tissues including the epidermis where it is expressed in the proliferative cells of the basal keratinocyte layer in the rat (Kokai et al., 1987) while immunoreactive erbB-2 protein product has been detected in the ORS of human hair follicles (Maguire et al., 1989). Other members of the EGFR subfamily have not been examined in situ although mouse keratinocytes do express erbB-3 in vitro. (Marikovsky et al., 1995).
The FGFR sub-family

FGFR ligands

The FGF ligand family currently consists of ten members, FGF-1(aFGF), FGF-2(bFGF), FGF-3(int-2), FGF-4(hst/kFGF), FGF-5, FGF-6, FGF-7(KGF), FGF-8(AIGF), FGF-9(GAF) and FGF-10 (Yamasaki et al., 1996). These proteins are expressed in a variety of cellular contexts during embryogenesis and at maturity and have been implicated in a range of processes including organogenesis, angiogenesis and oncogenesis. Several members are characterised by a requirement for heparin sulphate proteoglycans which may mediate receptor binding and growth factor activity (Schlessinger et al., 1995). Most members have an amino terminal secretory domain with the exception of FGF-1 and FGF-2 which lack this region and are presented on the cell surface. One member, FGF-5, has an alternative splice variant (Haitor et al., 1996), the only member of the FGF ligand family currently known to produce a truncated transcription product. Another family member, FGF-8, can generate several isoforms varying in their N-terminus coding region (Crossley and Martin, 1995) which may bind splice variants of the FGFR sub-family with varying affinity (MacArthur et al., 1995).

Several FGF family members also affect the growth and development of the hair follicle. A targeted disruption of the murine FGF-5 gene results in a knockout mouse which has been shown, by complementation analysis, to be allelic with the mouse angora mutation, Fgflf, suggesting that members of the FGF family are involved in the regulation of the hair cycle (Hébert et al., 1994). The extension of the anagen growth phase in angora mice supports a potential inhibitory role for FGF-5 in the regulation of the murine hair cycle. FGF-1 and FGF-2 have also been shown to have an inhibitory effect on hair follicle growth in mice when recombinant protein is administered subcutaneously (du Cros, 1993). Both growth factors delayed the normal process of hair development decreasing mitotic activity in the adult skin and hair follicle density in the neonate.

Conversely, recombinant FGF-7(KGF) has a stimulatory effect on hair growth. Intraperitoneal administration of FGF-7 protein to Nude (nu/nu) mice results in a generalised recovery of hair growth whereas subcutaneous injection induces a localised regrowth at the sites of administration (Danilenko et al., 1995). Histological analysis has revealed that FGF-7 promoted a hypertrophy of the hair follicle and sebaceous gland in treated mice. Topical application of FGF-7 to mice homozygous for the hairless mutation also results in the recovery of hair growth (Danilenko et al., 1994). Transgenic mice homozygous for a null mutation in the FGF-7 gene have been generated and exhibit a hair phenotype similar to the now extinct rough mutation (Guo et al., 1996). Over-expression of FGF-7 in the basal keratinocyte layer results in a hyperthickened, hyperproliferating epidermal phenotype with a decrease in the number
of hair follicles (Guo et al., 1993). This observation is inconsistent with the stimulatory effects of the recombinant protein on hair growth and may be related to its ectopic expression in the epidermis. The transcript for FGF-7 is also markedly upregulated after epidermal wounding and may be involved in epithelial regeneration (Werner et al., 1992). However, the work of Guo et al. (1993) tends to disagree with this.

Localisations of several FGF family members in the hair follicle have revealed distinct expression domains which, in some instances, are dependent on the stage of the hair cycle (Rosenquist and Martin, 1998; Hébert et al., 1994). The transcript for FGF-5 is present only during the anagen to catagen transition period and was detectable in the ORS of the follicle bulb during anagen VI. FGF-7 is detectable in the dermal papilla from early anagen to mid anagen and decreases by anagen VI to undetectable levels during the catagen and telogen stages of the hair cycle. In the Merino sheep, FGF-1 protein has been localised in the IRS possibly coincident with its high affinity receptor, FGFR4, while FGF-2 protein has been detected in the basement membrane of the ORS and hair matrix of the wool follicle bulb (du Cros et al., 1993).

Of the FGF ligand family members examined, only the dermally expressed FGF-7 has a stimulatory effect on hair growth while the epidermally expressed FGF-1, FGF-2 and FGF-5 exhibit an inhibitory effect. The possible involvement of FGF-2 in the initiation of epidermal appendage formation has been recently described in the chicken epidermis (Song et al., 1996). The spatially restricted transcription of FGF-2 in the epidermal placode and FGFR-1 in the dermal condensate of normal chicken epidermis but not in the skin of the scaleless mutation (a skin mutant which fails to form feather placodes) indicates that a paracrine interaction between the dermis and the epidermis could be occurring. Ectopically applied FGF-2 rescued the mutant scaleless phenotype suggesting that the expression of FGF-2 in the epidermis may be involved in the inductive events of epidermal appendage formation. A more comprehensive analysis of the inducing effects of FGF-1, FGF-2 and FGF-4 on feather formation has revealed that the ectopic application of these recombinant proteins results in the induction of feather follicle primordia with a concomitant increase in the expression of components of the ras signalling pathway. This effect was abrogated by the use of the tyrosine kinase inhibitor, Genistein, suggesting that the activation of a tyrosine kinase signalling cascade is involved in the formation of feather buds after treatment of the skin with recombinant FGF’s (Widelitz et al., 1998).

FGFR

The FGFR subfamily of RPTK’s currently consists of four members FGFR1(igf), FGFR2(bek), FGFR3 and FGFR4 which are characterised by an extracellular region consisting of three immunoglobulin-like domains, a single transmembrane domain and an intracellular tyrosine kinase autocatalytic domain. Alternative splice variants are known
to occur which utilise deletions or alternative exons of the extracellular immunoglobulin-like region. Splice variants result in receptors with differing ligand affinities. For example, the IIIb(KGFR) and IIIc(bek) isoforms of FGFR2 bind either FGF-7(KGF) or FGF-2 with higher affinity (Miki et al., 1992). Binding of an appropriate ligand to its receptor is also dependent on the formation of a multimeric ligand interaction with cell surface heparin sulphate proteoglycans. This increases receptor binding affinity resulting in the dimerisation and autophosphorylation of the receptor and the activation of downstream signalling events (Schlessinger et al., 1995). A translocation of the receptor bound ligand to the nucleus may also occur depending on the receptor isoform and whether the ligand contains a nucleolar localisation sequence (Prudovsky et al., 1996). Mutations in members of this RPTK subfamily have been associated with a number of genetic syndromes in humans resulting in craniofacial abnormalities. These include allelic variants of FGFR2 which result in the clinically definable Crouzon, Apert, Pfeiffer and Jackson Weiss syndromes. Pfeiffer syndrome has also been shown to be the result of mutations in FGFR1 while mutations in FGFR3 result in achondroplasia and thanatophoric dwarfism (Mulvihill, 1995; Muenke and Schell, 1995).

Transgenic mice have revealed that null mutations in members of the FGFR subfamily result in embryonic lethality when mice are homozygous for FGFR1 or FGFR2 null alleles, while homozygous FGFR3 null mutants survive to birth but exhibit skeletal deformities (reviewed by Muenke and Schell, 1995). The epidermal function of FGFR1 has been examined by utilising an epidermally expressed dominant negative mutation lacking the intracellular tyrosine kinase domain (Warner et al., 1993). A phenotype of varied thickening of the epidermis and an increase in nucleated cells of the suprabasal epidermal layers occurs. This has suggested that epidermally expressed FGFR1 may act as a differentiation factor rather than in promoting epidermal proliferation. This conclusion may need to be reconsidered as FGFR1 is usually dermally expressed (Rosenquist and Martin 1996) and the resulting phenotype could be due to its ectopic expression in the epidermis.

Epidermal localisations of the FGFR subfamily reveal a spatial and temporal regulation in the hair follicle and during the hair cycle (Rosenquist and Martin, 1996) with distinct expression domains observed in the mid-anagen hair follicle. FGFR1 is expressed in the dermal papilla while FGFR2 is expressed in the hair matrix cells of the follicle bulb. The expression of FGFR3 is observed one cell layer removed from those in contact with the dermal papilla in the hair bulb. FGFR4 exhibits a comparatively widespread distribution in the follicle present in the IRS/ORS and also in the hair matrix region. FGFR1 and FGFR2 expression ceases during the anagen to catagen transition while FGFR3 and FGFR4 remain detectable until mid-catagen. During telogen, no expression is detectable for all four FGF receptors (Rosenquist and Martin, 1996). The binding affinities of the various receptors and their isoforms are suggestive of a potential
dermal-epidermal paracrine relationship between ligand and receptor during the regulation of hair growth. The high affinity receptor for the dermally expressed FGF-7, FGFR2, is expressed in the hair matrix while a high affinity receptor for the epidermally expressed FGF-5, FGFR1 is expressed in the dermal papilla. Interestingly, FGF-5 can also bind FGFR2 (Clements et al., 1993) raising the possibility that epidermally expressed FGF-5 may compete with dermally expressed FGF-7 for FGFR2 binding during the anagen to catagen transition.

A Potential Interaction between the EGFR and FGFR sub-families

A functional association may exist between the ligands and receptors of the EGFR and FGFR subfamilies, with a number of studies suggesting a potential cross-regulatory relationship. An in vitro study of mouse and human keratinocytes has been undertaken to determine whether ligands of the FGFR subfamily can activate the EGFR pathway by analysing simple epithelial keratin regulation indicative of EGFR activation (Dlugosz et al., 1994). Of the FGFR ligands assayed, FGF-5 and FGF-7 induced changes associated with EGFR activation. The authors have postulated that since FGF-2 and FGF-7 both bind to FGFR-2(KGFR), the activation of the EGFR pathway may be occurring distally to an FGFR-2 mediated signalling cascade. Human and mouse keratinocytes stimulated with FGF-2 and FGF-7 also increase TGF-α production, down-regulate the activated EGFR and increase keratinocyte proliferation. This effect on proliferation was abrogated by the administration of neutralising antibodies to both FGF-7 and TGF-α but not to either growth factor alone, suggesting that the mitogenic effects of KGF in vitro may be partly mediated by TGF-α. The potential cross induction of TGF-α and subsequent activation of the EGFR by these two FGFR ligands is suggestive of an interaction between these two RPTK subfamilies. Further analysis of this potential interaction involved the use of mouse keratinocytes from an EGFR null mutant (Threadgill et al., 1995). These keratinocytes, which lack a functional EGFR, were unable to respond mitogenically to TGF-α administration but were still able to exhibit a proliferative response to FGF-7 and FGF-2. This suggests that EGFR activation is only partly responsible for the mitogenic effects of these two FGFR ligands and other proliferative signalling pathways are also activated.

A more direct functional relationship between the FGFR and EGFR subfamilies is apparent from the observation that the transcription of Fgt5 mRNA is induced as an immediate-early response gene in vitro when human foreskin fibroblasts are treated with the EGFR ligands, EGF and TGF-α. (Werner et al., 1991a, Werner et al., 1991b). The induction of FGF-5 transcription was cycloheximide insensitive indicating that de novo protein synthesis was not required. This transcriptional induction of FGF-5 may be mediated by the phosphorylation and translocation of cytoplasmic transcription factors to the nucleus as a result of EGFR activation.
Table 1.1: Protein and transcript localisations for the EGFR and FGFR subfamilies and associated ligands in the mouse, rat, sheep and human epidermis. Hair cycle specific regulation is indicated where known.

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Molecule</th>
<th>Localisation</th>
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Nanney et al. (1990)  
Green and Couchman (1984)  
Nanney et al. (1984a, 1984b)  
Wynn et al. (1995)  
Nanney et al. (1990)  
Wynn et al. (1999)  
Akiyama et al. (1996) |
| erbB-2       | erbB2                  | Basal Keratinocyte layer (rat)  
ORS (human) | Kokai et al. (1987)  
Maguire et al. (1989) |
| erbB-3(HER3) | erbB3                  | Mouse keratinocytes in vitro| Marikovsky et al. (1995) |
| EGF          | Epidermal Growth Factor | Periderm and intermediate layers of foetal epidermis. ORS, sebaceous gland and pilary canal (sheep) IRS/ORS, sebaceous gland, matrix cells, basal keratinocyte layer and intermediate layers of the epidermis (human)| du Cros et al. (1992)  
Akiyama et al. (1996) |
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Table 1.1 (continued)
Genetic Universality of the EGFR Signal Transduction Cascade in Eukaryotes

A consistent model of EGFR signal transduction is emerging from the study of a number of mutations affecting the development of multicellular organisms. The molecular basis of several mutations has revealed that a variety of developmental decision points affecting seemingly unrelated tissues in C. elegans, Drosophila and the mouse are the result of mutations in the EGFR signal transduction pathway (Figure 1.3). This may be an example of a highly conserved intercellular signalling mechanism regulating cell growth and differentiation during development.

The C. elegans EGFR homolog, let-23, has a number of allelic variants with varying degrees of phenotypic penetrance dependent on the location of the mutation relative to the conserved functional domains of the EGFR (Aorian et al., 1994). The observed phenotype is dependent on whether the mutation results in a receptor with activity ranging from partial to complete loss-of-function or, in some cases, constitutive activation (Sternberg et al., 1995). A larger number of alleles of the Drosophila EGFR homologue DER, have been characterised including the mutants; torpedo, faint little ball and ellipse. These mutations express a variety of phenotypes depending on their effect on the functional domains of the DER (Clifford and Schupbach, 1994; Perrimon, 1994). Several loss-of-function phenotypes have been characterised in the faint little ball and torpedo categories while ellipse mutants exhibit a semi-dominant gain-of-function phenotype (Shilo and Raz, 1991).

A number of potential upstream ligands and downstream effectors of both let-23 and DER have been revealed by combining the genetic analysis of epistatic relationships between mutations with the molecular biology of signal transduction pathways. In C. elegans vulval development a TGF-α like ligand, lin-3, activates let-23 and subsequently let-60 (ras), resulting in the induction of vulval cell fates (Hill and Sternberg, 1992). Genes negatively modifying let-23 activity have been identified from genetic screens of mutations suppressing an overexpressed lin-3 or restoring inactive or partially functional let-23 mutants (Sternberg et al., 1994; Sternberg et al., 1995). These include mutations such as unc-101, sli-1, lin-15A, lin-15B and rok-1. A gene product for several of these mutations has been identified. lin-15A and lin-15B encode novel proteins while sli-1 appears to encode a C. elegans homologue of the c-cbl proto-oncogene, a molecule which may interact indirectly with the EGFR via its SH-3 domain and adaptor proteins such as Grb-2. Others, such as lin-7 and lin-2, have been shown to facilitate let-23 signalling by positioning the receptor at cell junctions (Simske et al., 1996).

Similarly, in Drosophila two TGF-α-like ligands, gurken and spitz, have been implicated in a variety of morphogenetic events (Perrimon, 1994), including the establishment of the
anterior-posterior and dorsal-ventral embryonic axis during oogenesis (González-Reynes et al., 1995). Both 
\textit{gurken} and \textit{spitz} have been shown to activate the vertebrate EGFR (Perrimon, 1994) while secreted \textit{spitz} (but not membrane bound) activates DER \textit{in vitro} (Schweitzer et al., 1995a). Other \textit{spitz} class molecules such as \textit{painted}, \textit{rhomboid} and \textit{star} may also play a role in regulation of the DER signalling complex (Schweitzer et al., 1995a). The transmembrane proteins \textit{rhomboid} and \textit{star} have been implicated in the processing of the \textit{spitz} ligand, possibly determining a graded activation of the DER in embryonic ventral ectoderm and the subsequent determination of several cell fates (Golembio et al., 1996b). The \textit{painted} gene, a transcription factor, acts downstream of the activated DER in an antagonistic relationship with the \textit{yan} transcription factor and is involved in the regulation of the expression of a number of genes including \textit{orthodonticle}, \textit{tartan} and \textit{argos} (Gabay et al., 1996).

A negative regulator of DER signalling, \textit{argos}, acts as an extracellular inhibitor of DER activation by \textit{spitz} (Schweitzer et al., 1995b). Argos is a secreted molecule containing a single EGF motif similar to that present in \textit{spitz} and is induced following \textit{spitz} activation of DER. Competition for binding of DER with \textit{spitz} by \textit{argos} may prevent DER dimerisation modifying the degree of DER activation via an inhibitory feedback mechanism and possibly mediates the lateral inhibition of EGFR activation in neighbouring cells (Golembio, 1998a). This combination of a graded DER activation by secreted \textit{spitz} and the induction of negative regulators of DER activity has been suggested as a mechanism which may result in the induction of discrete cellular fates in the embryonic ventral ectoderm (Golembio et al., 1996b).

An intracellular signalling cascade involving the \textit{ras}, \textit{raf} and MAPK components is also apparent from the genetic study of the \textit{Drosophila} EGFR (Doyle and Bishop, 1993; Rogga et al, 1991). A recent study has also confirmed that the DER is used reiteratively during \textit{Drosophila} compound eye development and is required in concert with the \textit{sevenless} receptor for the differentiation of all eye cell types (Freeman, 1996). This is suggestive of a conserved intracellular signalling cassette mediating RPTK signalling from the cell surface to the nucleus (Diaz-Benjumea et al., 1994; Brunner et al., 1994).

In contrast to the relatively well understood genetics of EGFR signalling in simpler multicellular organisms, the information available in vertebrates is limited. Several mutational variants of the EGFR in mice have recently been characterised with phenotypic effects varying from the relatively mild to embryonic lethality. Identification of the locus responsible for the recessive \textit{waved -1} mutation (Crew, 1933) in mice was the first indication that mutations in the EGFR signalling pathway were involved in determining qualitative phenotypic differences in vertebrates. Mice with a targeted disruption in the \textit{TGF-α} locus exhibit an easily discernible phenotype of wavy
vibrissae and coat hairs characteristic of the waved-1 mutation (Luettkes et al., 1993; Mann et al., 1993). Complementation analysis has revealed that the disrupted TGF-α locus is allelic for the waved-1 mutation.

A second recessive wavy haired mutation, waved-2 (Keeler, 1935) which is phenotypically similar to Tgfa<sup>wa</sup>, had been genetically mapped close to the EGFR locus on mouse chromosome 11. Analysis of waved-2 EGFR coding sequence revealed that a point mutation was present in subdomain III of the tyrosine kinase catalytic domain of the EGFR, generating an amino acid substitution and a partial loss of ligand-dependent autophosphorylation activity (Luettkes et al., 1994). The Tgfa<sup>wa</sup> and Egfr<sup>wa</sup> mutations are the only known spontaneous mutants involved in the mammalian EGFR signalling pathway. Several other waved-hair mutants have been genetically mapped close to other members of the EGFR subfamily of receptors and ligands. Amphiregulin and betacellulin map to mouse chromosome 5 within the linkage group containing the now extinct wavy haired recessive mutation marcel (Pathak et al., 1995), while a known heteromer of the EGFR, erbB-2, maps to distal chromosome 11 in the vicinity of several dominant Rex alleles (Sundberg, 1994).

The conservation of the ras, raf and MAPK signalling cascade in the mammalian EGFR pathway is also highly conserved with many components of this pathway encoding potential proto-oncogenes (Hunter, 1997). Although signalling by the mammalian EGFR pathway is considerably more complex, with the existence of several ligands and receptors that may mediate various heteromeric associations, the intracellular activation of the conserved MAPK pathway and other intracellular signalling cascades is suggestive of a conserved signalling network mediating RPTK activation (Schlessinger and Bar-Sagi, 1994; Wilson, 1994; Hunter, 1997).

A number of other spontaneous mutations affecting epidermal development may be implicated in the EGFR signalling cascade. These include the fsn (flaky skin) and Ta (Tabby) mutations which exhibit an elevation and a depression of EGFR levels respectively (Nanney et al., 1998; Vargas et al., 1996). The genetic basis of both mouse mutations is currently unknown although a potential human homolog of the X-linked Tabby locus may be involved in anhidrotic ectodermal dysplasia (EDA), a clinical syndrome in humans exhibiting similar abnormalities in epidermal development (Kere et al., 1996). The mutated gene resulting in EDA is a novel transmembrane protein and its relationship with the EGFR signalling pathway is unclear. Topically applied EGF suppresses the phenotypic expressivity of the fsn mutation (Nanney et al., 1998), while systemic administration of EGF to Ta mice has a similar normalising effect on this mutant phenotype (Kapalanga et al., 1990). This modifying effect of administered EGF may indicate that the mutations involved could be regulated by the EGFR signalling pathway. As the genetic maps for the mouse genome are refined and further genes are
knocked out, a more complete picture will emerge of the EGFR signalling pathway in the mouse of at least similar complexity to that currently being uncovered in *C. elegans* and *Drosophila*.

Utilising the mouse to genetically dissect the EGFR pathway in mammals in a similar manner to that achieved in *C. elegans* and *Drosophila* has been proposed (Della et al., 1995). An understanding of the developmental events influenced by this highly conserved and pleiotropic RPTK signalling cascade may be better understood by producing allelic combinations of mouse mutations encoding putative signalling pathway components or downstream effectors, and observing their phenotypic effect. An attempt to genetically disrupt components of the EGFR signalling cascade has recently been reported by combining homozygous *Egfr*<sup>++</sup> mutants and *Sos1* heterozygous null alleles (Wang et al., 1997). A relatively mild enhancement of the sporadic *Egfr*<sup>++</sup> eye phenotype was observed, suggesting that the genetic dissection of the EGFR signalling pathway in mammals may be possible.

Figure 1.3 An evolutionarily conserved signalling cascade (modified from Stemberg et al., 1994). A simplified comparison of the EGFR signalling cascade in *C. elegans*, *Drosophila* and the mouse. Only the intracellular ras pathway components of EGFR activation are shown. Potential negative regulators have been omitted.
The Hair Follicle as a Developmental Paradigm

The development of epidermal appendages, the mouse hair follicle in particular, has been proposed as a model system which could provide an understanding of the inductive events associated with tissue growth and differentiation (Hardy, 1992). Current vertebrate models including the Zebrafish, Xenopus and the chicken limb bud, have contributed to an awareness of the conserved molecular events regulating organogenesis. Mouse hair mutations and their associated effects on hair follicle growth may provide an opportunity for a similar developmental study in mammals.

A number of proteins which have been implicated in the initiation and morphogenesis of a variety of tissues are also expressed in the hair follicle (Messenger, 1993; Panaretto, 1993; Stenn et al., 1994). Several of these growth factor molecules have also been shown to act as potential morphogens, molecules which can influence cell fates in a juxtacrine and/or paracrine manner. The relative mesodermal and ectodermal localisations of these polypeptides in the developing and mature hair follicle have similarities to those described in other tissues, particularly the chicken limb bud, and imply a conserved role for these growth factor families in mediating organogenesis. A representative sample of several of the more widely studied families of developmentally significant proteins, their location in the embryonic epidermis and in the mature cycling hair follicle, are presented in Table 1.3.

Other groups comprising the structural elements of the epidermal cell such as the keratins, cell adhesion molecules and the ECM (Extra Cellular Matrix) are also of central importance in epidermal morphogenesis and have recently been reviewed (Stenn, 1994). Several classes of ECM molecules, notably the heparin sulphate proteoglycans, may modulate peptide growth factor localisation and activity. These are also differentially expressed during the hair cycle being present to a greater or lesser degree during the various growth stages (Couchman, 1983).

A potential diffusible morphogen, Sonic hedgehog (SHH), is expressed in the embryonic and adult epidermis (Bitgood and McMahon, 1995). Expression occurs in the epidermal placode just prior to, or coincident with, the early stages of hair follicle formation and is continued in the anterior epithelium of the mature hair follicle and in the IRS of vibrissae. SHH is a member of a unique family of secreted signalling molecules related to the Drosophila segment polarity gene hedgehog and is expressed in regions associated with organising activity. These inductive regions may regulate cellular patterning during organogenesis and include areas such as the embryonic node, the notochord, the ventral floor plate of the neural tube and the ZPA (Zone of Polarising Activity) (Bueno et al., 1996, Echelard et al., 1993, Riddle et al., 1993, reviewed by Smith, 1994). Secreted SHH is cleaved into two fragments, a cholesterol modified amino
SHH/patched interaction during tissue development is unclear although SHH is known to relieve the inhibitory effects of patched expression, resulting in the induction of genes from the TGF-β and Wnt growth factor families (Goodrich et al., 1996). One of the most widely studied TGF-β family members known to be induced by SHH is decapentaplegic (dpp) the Drosophila homologue of the vertebrate BMP-2 and BMP-4 genes (Hogan, 1996). Repression of TGF-β and Wnt family gene products by patched and a negative autoregulation of its own expression, are necessary in maintaining epidermal homeostasis. The aberrant deactivation of patched results in an uncontrolled epidermal proliferation which progresses to basal cell carcinoma (Johnson et al., 1996).

Other aspects of the SHH signalling pathway may also be of relevance during hair follicle development. These include the activation of PKA (cAMP-dependent Protein Kinase A), a negative regulator of SHH signalling (Hammerschmidt et al., 1996). Differential expression of PKA and PKC (Protein Kinase C) activity in the developing chicken epidermis suggests that these intracellular signalling molecules may be involved in regulating epidermal appendage formation. During feather formation, a widespread dermal and epidermal expression of PKA activity becomes confined to the epidermis of developing feather placode and is absent from the interplacode region. A transient expression of PKA activity is also detected in the mesenchymal condensate between stages 33-36 (Nouveen et al., 1995a), a period that coincides with the expression of SHH in the epidermal placode at approximately stage 34 (Nohno et al., 1995). In contrast, PKC activity is initially detected throughout the dermis and exhibits a localised decrease in the dermal regions underlying the presumptive feather placode. The application of Protein Kinase A agonists results in an increased formation of feather bud primordia while Protein Kinase C agonists enhance the interbud domain inhibiting feather bud formation (Nouveen et al., 1995a).

The use of chicken skin explant culture has also helped to reveal a temporal relationship between several of these peptide growth factors and the formation of the feather bud (Chuong et al., 1996). By recombining chicken skin epithelium with dermis, the induction of new feather bud primordia occurs. The temporal appearance of several molecules was studied during this reinitiation of feather bud primordia by in situ hybridisation and revealed that FGF-4, BMP-2 and BMP-4 were initially induced in the developing placode followed by SHH and Wnt7a. The homeobox genes Msx1 and Msx2 were next induced with the cell adhesion molecule NCAM and the homeobox gene Hox-C6 last in the series examined. The homeobox genes, Msx1 and Msx2, are repressed by activation of the PKA pathway and both genes exhibit a polarised expression in the epidermal placode of the developing feather bud (Nouveen et al., 1995). They could also be involved in the activation of the transcription factor LEF-1, possibly via BMP-4 induction (Kratochvil et al., 1998).
The observation of a localised decrease in EGFR immunoreactive material in regions of the epidermis overlying the mesenchymal condensate in rat and human skin (Table 1.1) may be related to the activity of PKA and PKC in the mammalian epidermis. The activation of PKC is regulated by membrane phospholipid turnover and the subsequent increase of diacylglycerol and Ca\(^{2+}\) levels, both of which are induced by a number of RPTK family members including the EGFR. The PLC\(\gamma\) sub-family of phospholipases (Van der Geer and Hunter, 1994) as well as phosphatidylinositol-3-kinase activation (Soltoff et al., 1994) are examples of gene products that can be regulated by the EGFR. Interestingly, PKA activation may inhibit activity of the MAPK kinase pathway via a cAMP-dependent inhibition of ras activation of ras and MAPK (Wu et al., 1993; Cook and McCormick, 1993). This is suggestive of an antagonistic relationship between the PKA pathway and the RPTK signal transduction mechanism and may explain the restricted and non-overlapping activity of PKA and PKC in the developing chicken epidermis.

Several members of the TGF-\(\beta\) superfamily are expressed in the developing epidermis and the hair follicle, with some exhibiting hair cycle-dependent regulation (Table 1.3). Dermal expression of BMP-4 adjacent to epithelial tissue expressing SHH (Biltgood and McMahon, 1995) is consistent with an inductive interaction between the epidermis and dermis of the developing hair follicle. Transgenic studies indicate that BMP-4 may play a role in hair follicle regulation. Mice carrying a bovine cytokeratin IV/BMP-4 construct expressing in the ORS develop an initial hair coat but are unable to regrow a subsequent coat after the first hair cycle (Blessing et al., 1993). The ectopic expression of BMP-4 in the epidermis may be misleading considering the normal mesodermal expression of BMP-4 in the hair follicle. Two other BMP molecules, BMP-3 and BMP-7, have also recently been localised to the developing and mature hair follicle in the rat (Takahashi and Ikeda, 1996). Bmp-3 mRNA was present in the IRS/ORS, dermal sheath and the mesenchymal condensate of the developing follicle with expression becoming weaker as the follicle matured. Bmp-7 mRNA was detected in the IRS, ORS and the dermal sheath of the developing follicle while in the mature follicle it was expressed in the dermal papilla and lower half of the ORS.

Other members of the TGF-\(\beta\) superfamily may be differentially regulated during the hair cycle. TGF-\(\beta\)1 is expressed during early anagen in the IRS while its receptor, TGF-\(\beta\)RI, increases in the ORS during the anagen to catagen transition (Wilton et al., 1998). The growth inhibitory effects of several of the TGF-\(\beta\) growth factors and the appearance of the appropriate receptor during the anagen to catagen transition may be related to the subsequent inhibition of hair growth. Transgenic mice overexpressing TGF-\(\beta\)1 in the epidermis exhibit a marked decrease in the proliferative capacity of the basal epidermal cells and a decreased follicle density. The hair follicles that did form were short and stunted, a phenotype which is consistent with a growth inhibitory effect.
Expression of TGF-β superfamily members may also be correlated with the hyperproliferative growth state of some skin carcinomas. TGF-β1 and the receptor TGF-βRII, exhibit increased expression in basal cell carcinomas (Schmidt et al., 1998), an observation consistent with the loss of repression of this growth factor superfamily by patched. Recombinant TGF-β2 has been shown to induce mesenchymal condensation in chicken skin which has had the epidermal layer removed, suggesting a potential involvement in epidermal induction during appendage formation (Ting-Berreh and Choung, 1996). The authors have also shown that ectopic expression of SHH in chicken epidermis results in the induction of TGF-β2 and larger mesenchymal condensates, suggesting that TGF-β2 is downstream of SHH which is also expressed in the epidermal placode.

A number of transcription factor proteins also participate in the development of the epidermis, two of which, LEF-1 and AP-2, have been localised to the developing hair follicle. The appearance of an AP-2 transcript in the epidermis occurs at an early stage of follicle formation prior to the morphological changes associated with the appearance of the epidermal placode (Byrne et al., 1994). LEF-1 exhibits a similar epidermal expression pattern but mRNA is also detected in the mesenchymal condensate and during the formation of the dermal papilla, an epidermal-dermal expression pattern that is also observed with BMP-2 and Msx-2 (Zhou et al., 1995). A number of keratin promoter regions contain consensus binding sequence for both of these transcription factors, while the EGFR has an AP-2 responsive promoter in vitro (Johnson, 1996). LEF-1 seems to be essential for epidermal appendage development with transgenic LEF-1 null mutants lacking teeth, whiskers and hair (van Genderen et al., 1994).

Subsequent experimentation using mice overexpressing an epidermal LEF-1 transcript indicate that an epidermal induction of the underlying dermis by LEF-1 results in a disorientated follicle patterning and the ectopic formation of hair follicles (Zhou et al., 1995). Tissue recombination experiments with epidermis from LEF-1 null mutants suggest that LEF-1 expression is first required in the mesenchyme followed by its epidermal expression (Kratochwil et al., 1996). This is consistent with the classical hypothesis that the dermis is the initiator of an inductive signal resulting in the formation of an epidermal appendage (Hardy, 1992). Induction of LEF-1 expression by BMP-4 also defines this transcription factor as a potential downstream effector (Kratochwil et al., 1996). The involvement of LEF-1 downstream of the Wnt signalling pathway has also been suggested and may involve the cytoplasmic protein β-catenin, a member of the armadillo family of repeat proteins (Behrens et al., 1996). An interaction between β-catenin and the EGFR has also been shown to occur (Hoschuetzky et al., 1994) suggesting that this cytoplasmic anchorage protein may be involved in the signalling events of a number of peptide growth factor receptors. The Wnt signalling pathway also includes a putative receptor frizzled and the cytoplasmic proteins dishevelled and
zeste white -3. The dishevelled protein interacts antagonistically with the Notch signalling pathway during Drosophila wing formation (Axelrod et al., 1996). It is interesting to note that the smoothened receptor has homology to frizzled suggesting a potential link between the regulation of SHH and Wnt signalling pathways (Stone et al., 1996; Hunter, 1997).

The involvement of Notch during wing morphogenesis and its antagonistic involvement in the Wnt signalling cascade may indicate a potential role for this transmembrane protein in the hair follicle. The expression of Notch protein in the hair follicle may also be a link between the activation of the EGFR signalling cascade and the Notch signalling pathway. A murine homologue of the Drosophila Notch protein, mNotch, has been localised to the cells of the hair matrix (Kopan and Weintraub, 1993). mNotch expression is restricted to follicle bulb cells one layer removed from the adjacent dermal papilla in the anagen follicle and in the differentiating cells of the IRS and cuticle. At the end of the first hair cycle mNotch levels decrease but are restored when the second hair cycle is initiated. A potential role for mNotch in maintaining the differentiated cellular states of the developing and actively growing follicle has been suggested (Kopan and Weintraub, 1993).

During C. elegans vulval development a homolog of Notch, lin-12, may determine the differentiation of various cellular states in concert with the EGFR signalling pathway (Kenyon, 1995). Varying levels of diffusible lin-3, the nematode TGF-α homologue, disrupts the differentiation of vulval precursor cells. Higher concentrations of lin-3 override the normal differentiative process which is thought to involve lin-12 in a signal transduction mechanism which has evolved to ensure the correct differentiation of a tissue. An interaction between the EGFR signalling pathway and Notch may determine the differentiated cellular states of the proliferating hair follicle bulb in a similar manner to that occurring in the nematode vulva. Considering the coincident localisations of the EGFR and mNotch in the hair follicle bulb, a lateral induction of cell fates may be possible.

Although the peptide, Bcl-2, is not usually considered a growth factor, it is of interest in the development of the hair follicle considering the spectrum of cellular states present during follicle initiation and in the mature cycling hair follicle. Regulation of cell death, or apoptosis, plays a central role in tissue development and the factors influencing this cellular state are being widely studied (White, 1996). The bcl-2 gene is broadly expressed during embryogenesis and becomes restricted to populations with stem cell potential at later stages of development. Its role seems to be that of a ‘death repressor’, the presence of which inhibits a cell from progressing to an apoptotic state.
A study of the Bcl-2 protein product during the formation of the hair follicle and its subsequent regulation during the hair cycle, has revealed that discrete populations of cells seem to be coordinately regulated to express Bcl-2 (Stenn et al., 1994). During anagen, Bcl-2 is detected in the proliferating regions of the hair follicle; the hair matrix, ORS and the bulge region of the ORS, an area hypothesised to contain a hair follicle stem cell population (Cotsarelis et al., 1990). Entry into catagen resulted in a decreased expression of Bcl-2 with a further downregulation occurring during telogen, when the epidermal cells in contact with the dermal papilla regressed and underwent apoptosis. Notably, Bcl-2 expression was not maintained in the bulge region of the follicle at this stage although the expression of Bcl-2 in the dermal papilla was detected throughout all stages of the hair cycle. The expression of Bcl-2 protein in other epithelial stem cell populations examined by the authors did not decrease, an observation inconsistent with the localisation of a putative stem cell population in the bulge region of the hair follicle.

The genetic study of a wide variety of developmental processes in a number of multicellular organisms has revealed that groups of molecules that are highly conserved across species boundaries are involved. These molecules, of which only a few have been considered here, may constitute a molecular ‘tool kit’ (Palopoli and Patel, 1996) that can be applied to determine the processes of cellular differentiation and organogenesis during evolution. Not only have these developmentally significant molecules been conserved across species barriers but their reiterative use during embryogenesis suggests that these molecules are fundamentally involved in the developmental process. The expression of members of these conserved gene families in the mammalian hair follicle may be an indication that the regulation of its growth and development involves molecular mechanisms similar to those described in other systems.
Table 1.2: Developmentally significant molecules in epidermal development of vertebrates. Hair cycle specific regulation is indicated where known.

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Molecule</th>
<th>Localisation</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>SHH</td>
<td>Sonic Hedgehog</td>
<td>Epidermal placode (mouse, chicken)</td>
<td>Bitgood and McMahon (1995)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IRS (mouse vibrissae)</td>
<td>Nohno et al. (1995)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Iseki et al. (1998)</td>
</tr>
<tr>
<td>Wnt-12</td>
<td>Wnt-12</td>
<td>Follicle primordia (mouse vibrissae)</td>
<td></td>
</tr>
<tr>
<td>Ptc</td>
<td>Patched</td>
<td>Epidermis (human)</td>
<td>Christiansen et al. (1994)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Vibrissa primordia (mouse)</td>
<td></td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
<td>Activity localised to epidermal placode. Transient activity in the mesenchymal condensate (chicken)</td>
<td>Johnson et al. (1996)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Goodrich et al. (1996)</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
<td>Localised dermal decrease in activity beneath epidermal placode (chicken)</td>
<td>Noveen et al. (1995a)</td>
</tr>
<tr>
<td>Bmp-2</td>
<td>Bone morphogenetic protein 2</td>
<td>Epidermal placode and precortical cells (mouse vibrissae)</td>
<td>Lyons et al. (1990)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Bitgood and McMahon (1995)</td>
</tr>
<tr>
<td>Bmp-3</td>
<td>Bone morphogenetic protein 3</td>
<td>IRS/ORS and dermal sheath of developing follicle (rat)</td>
<td>Takahashi and Ikeda (1995)</td>
</tr>
<tr>
<td>Bmp-4</td>
<td>Bone morphogenetic protein 4</td>
<td>Mesenchymal condensate, dermal papilla, precortical cells and IRS (mouse hair and vibrissae)</td>
<td>Jones et al. (1991)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Bitgood and McMahon (1995)</td>
</tr>
<tr>
<td>Bmp-7</td>
<td>Bone morphogenetic protein 7</td>
<td>IRS/ORS and dermal sheath of developing follicle. Dermal papilla and lower half of ORS in mature follicle.</td>
<td>Takahashi and Ikeda (1996)</td>
</tr>
<tr>
<td>Gene</td>
<td>Description</td>
<td>References</td>
<td></td>
</tr>
<tr>
<td>--------------</td>
<td>------------------------------------------------------------------------------</td>
<td>-----------------------------------</td>
<td></td>
</tr>
<tr>
<td>TGF-β2</td>
<td>Transforming Growth Factor -beta 2 ORS (human)</td>
<td>Schmid et al. (1996)</td>
<td></td>
</tr>
<tr>
<td>TGF-βRI</td>
<td>TGF-beta Receptor I ORS during anagen to catagen transition and in sebaceous gland (mouse).</td>
<td>Wollina et al. (1996)</td>
<td></td>
</tr>
<tr>
<td>TGF-βRII</td>
<td>TGF-beta Receptor II Epidermis and low levels in dermis(human) Sebaceous gland (mouse)</td>
<td>Schmid et al. (1996)</td>
<td></td>
</tr>
<tr>
<td>Notch(lin-12)</td>
<td>Notch Hair matrix (mouse)</td>
<td>Kopan and Weintraub (1993)</td>
<td></td>
</tr>
<tr>
<td>Bcl-2</td>
<td>Bcl-2 Epidermal expression in hair matrix and ORS during anagen, decreases during catagen and absent in telogen. Dermal papilla expression constant.</td>
<td>Stenn et al. (1994)</td>
<td></td>
</tr>
<tr>
<td>AP-2</td>
<td>Activator Protein 2 Restricted epidermal expression prior to formation of the epidermal placode.</td>
<td>Byrne et al. (1994)</td>
<td></td>
</tr>
<tr>
<td>Msx-1(Hox-7)</td>
<td>Msx-1 epidermal placode (chicken)</td>
<td>Noveen et al. (1995b)</td>
<td></td>
</tr>
<tr>
<td>Msx-2(Hox-8)</td>
<td>Msx-2 epidermal placode (chicken)</td>
<td>Noveen et al. (1995b)</td>
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</table>
Aims of this work

The role of peptide growth factors during mammalian development is an area of active study due to their involvement in the progression of many cancers. An understanding of their normal physiological role is incomplete, with a complex relationship existing between the effects of these molecules on cellular proliferation and the spatial and temporal regulation of their appearance. To develop a better understanding of the functional role of these molecules, I have combined information from molecular biology, developmental genetics and studies of hair growth regulation to examine an in vivo relationship between members of the EGFR and FGFR subfamilies. A potential functional relationship is based on several experimental observations which have revealed that members of these growth factor families can influence the period of time that a hair follicle remains in anagen growth. These are:

- The targeted disruption of the murine FGF-5 locus resulting in an FGF-5 null mutation exhibiting the angora phenotype, Fgf5<sup>−/−</sup> (Hébert et al., 1994). This suggests that the absence of a functional FGF-5 gene product delays the normal transition from the actively growing anagen phase to the regressing catagen phase of the hair cycle. A potential inhibitory role for this growth factor on cellular proliferation of the hair follicle has been proposed.

- A transcriptional immediate-early response for FGF-5 is induced in vitro when cells are treated with a number of peptide growth factors, notably EGF and TGFα (Werner et al., 1991). This observation suggests that the transcriptional 'turn on' switch for FGF-5 may be the binding of a peptide growth factor ligand, such as EGF or TGF-α, to the EGF receptor, triggering the activation of an intracellular kinase cascade and the transcriptional induction of FGF-5.

- Disruption of the hair cycle in mice expressing a defective EGFR in the epidermis (Murillas et al., 1995). These animals also exhibit a failure to enter the catagen state of the normal hair cycle suggesting that a functional EGFR is required for the transition from anagen to catagen, a function related to that of FGF-5.

- The inhibitory effects that EGF and TGF-α have on hair follicle growth when administered to newborn mice (Moore et al., 1981; Tam, 1985). This effect may be mediated by the EGFR signalling cascade, which in the hair follicle appears to have an inhibitory effect on cell growth.

Considered collectively, these experimental observations suggest a relationship between epidermally expressed EGFR ligands, activation of the EGFR, the transcriptional induction of FGF-5 and the inhibition of cell proliferation in the hair follicle during the anagen to catagen transition of the murine hair cycle. This postulated biochemical interaction between a ligand-stimulated activation of one receptor tyrosine
kinase and the resulting induction of a ligand for another receptor tyrosine kinase is illustrated below in Figure 1.2.

\[
\begin{align*}
\text{TGF-\alpha / EGF} \\
\downarrow \\
\text{EGFR} \\
\downarrow \\
\text{Immediate-early Response} \\
\downarrow \\
\text{FGF-5} \\
\downarrow \\
\text{Anagen to Catagen Transition of the Hair Cycle}
\end{align*}
\]

Figure 1.2 A depiction of the potential biochemical interrelationship between ligands for the EGFR, the resulting receptor activation and the downstream immediate-early transcriptional response of FGF-5.

To test this hypothesis I have utilised two experimental models. The first approach was to examine the effects of these growth factors in vivo by analysing the epidermal phenotype of animals carrying combinations of mutations in this potentially conserved signal transduction pathway. To achieve this, I have used three molecularly defined mouse mutations; the Tgfa\textsuperscript{enu} mutation carrying a disrupted null allele of the Tgfa gene (Mann et al., 1993), the Egfr\textsuperscript{vma-2} mutation (Luetteke et al., 1994), a partially functional mutation in the EGFR, and Fgf5\textsuperscript{enu}, a deletion mutation in the Fgf5 gene (Hébert et al., 1994). A characterisation of the genotype and resulting epidermal phenotypes of progeny with various allelic combinations of these three mutations may help to define their physiological roles in vivo and their effects on mammalian epidermal development.

It is useful at this point to state clearly the null and alternative hypotheses of this genetic study. The null hypothesis is that there is no interaction between these mutations and the phenotypes observed will be a summation of the phenotypes of each locus. The compound mutants will display an additive combination of the individual mutant phenotypes and no novel phenotypes will be observed. Furthermore, all genotypes will be present at their expected mendelian frequency. The alternative hypothesis is that these loci may interact and extreme phenotypes, which may not represent a summation of the individual mutant phenotypes, will be observed for particular allelic combinations. The observation of novel phenotypes and a
statistically significant divergence of genotype frequency from those expected would be considered as evidence for rejecting the null hypothesis in favor of this alternative.

A second experimental approach taken will be to examine the epidermis of EGF treated Merino sheep at various times after EGF administration. This treatment, results in a dramatic inhibition of wool follicle growth which is morphologically similar to the early stages of catagen in the mouse hair cycle (Hollis et al., 1983). TGF-α and FGF-5 will be examined by in situ hybridisation to determine whether their transcriptional regulation is altered following EGF treatment and the induction of a catagen-like state in the wool follicle. Taken together, these two approaches, one genetic, the other physiological, may give an indication as to the importance the EGFR and FGFR dependent signalling pathways during mammalian epithelial development.
Chapter Two: Materials and Methods

Materials

Major Chemicals
[α-32P]dCTP was purchased from Bresatec. Size markers Spp-1(EcoR1) and PUC-19 (HpalI) were purchased from Progen. DNA grade agarose was purchased from Progen. Major chemicals were purchased from BDH, Sigma and Oxoid. All other chemicals and solvents were Analytical Reagent grade.

Enzymes
Proteinase K was purchased from AMRESCO. Restriction endonucleases and appropriate 10X digestion buffers were purchased from Promega, Progen or Boehringer Mannheim. RED HOT Taq DNA polymerase was purchased from Advanced Biotechnologies. T7 and Sp6 RNA polymerases were purchased from Promega or Boehringer Mannheim.

Kits
Wizard plasmid purification kits were purchased from Promega. Message Maker transcription kit and Gigaprive radiolabelling kits were purchased from Bresatec.

Bacterial strains
HB101 supE, hsdR20(r5′m5), recA13, ara-14, proA2, lacY1, galK2, rpsL20, xyl-5, mtl-1

XL-1 supE44, hsdR17, recA1, endA1, gyrA46, thi, relA1, lac

Plasmids
pGEM-3Zf+ (Promega)
pLIIT-28 (New England Biolabs)
pCR II (Invitrogen)
Plasmids were purchased from the appropriate supplier, transformed into HB-101 cells and maxiprep isolations carried out in the laboratory.

Media, Common Solutions and Buffers
L-Broth

1.0% bactotryptone
0.5% yeast extract,
0.5% NaCl
LB- Agar

Add 15g agar per 1 litre L-Broth

Antibiotics (working concentration)

- Ampicillin 50µg/ml
- Tetracycline 12.5µg/ml

1 X TBE

- 90mM Tris-HCl pH 7.5
- 90mM boric acid
- 2mM EDTA pH 8.0

TE pH 8.0

- 10mM Tris-HCl pH 8.0
- 1mM EDTA pH 8.0

1 X SSPE

- 150mM NaCl
- 10mM sodium phosphate pH 7.4
- 1mM EDTA

1 X SSC

- 150mM NaCl
- 15mM sodium citrate pH 7.0

DNA loading dye (neutral dye mix)

- 0.25% (w/v) xylene cyanol
- 0.25% (w/v) bromophenol blue
- 50% (w/v) glycerol (sterile) and 50% H₂O sterile.
Dissolve in glycerol and then H₂O, stored a 4°C

RNase A (10mg/ml)

- 10mM Tris-HCl pH 7.5
- 15mM NaCl
Heat treat for 15 minutes at 100°C, stored at -20°C

Ampicillin (50mg/ml)
Made up 5mls in dH₂O and filter sterilized through 0.2µM filters and stored as 1ml aliquots at -20°C. Final selection was carried out using a 50µg/ml concentration.
Tetracycline (5mg/ml)
Made up in absolute ethanol and stored as 1ml aliquots at -20°C. Final selection was
carried out using 12.5μg/ml in plates

Ethidium Bromide (10mg/ml)
Made up in ddH₂O and stored in an aluminium foil wrapped bottle (1ml aliquots) at room
temperature. Added 10μl per 200mls 1 X TBE agarose gel to give a final concentration
of 0.5μg/ml.

Sevage
A CHCl₃/isoamyl alcohol mix (24:1), stored in an aluminium foil wrapped bottle at room
temperature.

Phenol
Phenol was equilibrated with 1M Tris-HCl (pH 8.0) until a pH greater than 7.8 was
obtained. 1%(w/v) 8-hydroxyquinolone was then added and stored in an aluminium foil
wrapped bottle at 4°C.

Miscellaneous Materials
X-ray film was from Fuji Photo Film Company. Photographic film for in situ hybridization
and histology was from Kodak. Polaroid film was from Kodak. Hybond N+ hybridization
transfer membrane was purchased from Amersham.

Equipment

Centrifuges
Bench top Eppendorf 5415C centrifuge
Beckman J2-21M Induction Drive centrifuge
Clements GS200 swinging bucket rotor

PCR machines
A Stratagene ROBOCYCLER Gradient 96 PCR machine was used for
optimisation and genotyping of all samples.

Electrophoresis Systems
Large gel apparatus (BRL)
Homemade minigel apparatus
Power packs: LKB 2103 power supply, EPS 250 power pack, GenePower
GPS 200/400 power pack.
Photographic equipment
Polaroid MP4 land camera and UV transilluminator
Photographic stills taken by the Photography Unit at CSIRO DAP
Zeiss Tessovar Macrophotography camera

Microscopy
Zeiss D-7082 Oberkochem Axiophot photomicroscope
Dissection microscope

Incubators and Ovens
A Hybird hybridisation oven was used for Southern blot incubations and
washes, 37°C incubators dry, wet and orbital shaking, 55°C dry incubator was
used for tissue digestions.

Miscellaneous Items
In situ glassware was kindly supplied by Dr N.Nishimura
DNA optical density readings were carried out on a LKB Ultraspec II
spectrophotometer.

Methods
General recombinant DNA techniques
In general, all recombinant DNA techniques were performed following published
procedures with some modification (Maniatis, 1982). Precipitation of DNA was carried
out by adding a 1/10 volume of 3M sodium acetate (pH 4.0) followed by 2 volumes of
absolute ethanol (AR grade). The precipitate was then placed at -20°C for at least 30
min or in some instances overnight. Most manipulations were done in a 1.5ml
Eppendorf tube and DNA precipitate was pelleted by centrifuging at 12000 X g (max
rpm) in a bench top microfuge for 10-15 min at room temperature. The DNA pellet was
washed briefly with 100-200µl 75% (v/v) ice cold ethanol for 2-3 min before being
removed with a drawn out pasteur pipette. The pellet was then allowed to air dry for 1-
2 hours before being resuspended in either TE or dH₂O and stored at 4°C. The
preparation of all organic solvents and their use in the various extraction steps were
carried out in a laminar flow hood.

Quantitation of nucleic acids was achieved by absorbance spectrophotometry from the
optical density at a wavelength of 260nm of 1/200 and 1/100 dilutions of stock nucleic
acid solution. The optical density of a 50µg/ml solution of DNA, a 40µg/ml solution of
RNA and a 20µg/ml solution of single strand oligonucleotide was assumed to equal 1.0
(Maniatis, 1982). Comparative intensity of ethidium bromide stained bands against a
Lambda (λ) phage concentration standard on an agarose gel following electrophoresis was also utilised for low concentration samples.

Standard electrophoresis of DNA was through 1 X TBE agarose gels (0.8% to 2.2% (w/v) depending on the size DNA fragments analysed) containing 0.5μg/ml of ethidium bromide. Samples were mixed with a 1/10 volume of neutral dye mix and pulse spun prior loading. Minigel electrophoresis was generally carried out at 100mA for 2-3 hours to resolve DNA fragments, while large gel electrophoresis for Southern blotting was carried out overnight at 30-40mA. Resolved DNA was visualised under a 260nm ultraviolet light transilluminator and a polaroid photograph was taken to record the result.

Plasmid DNA preparation
Several different size plasmid preparations were utilised depending on the expected requirement of plasmid for experimentation. Large scale maxipreps of plasmid DNA were carried out for clones required for riboprobe preparations that were required to be RNase free. The Wizard Maxiprep kit (Promega) was used following the manufacturers protocol and recovered DNA was resuspended in DEPC dH2O. Routine maxi, midi and mini plasmid preparations were carried out using modifications of the alkali lysis method (Maniatis, 1982) as described below.

Plasmid preparation
A 10ml inoculation into L-Broth at the appropriate antibiotic concentration was made with a single bacterial colony and grown overnight in a shaking incubator at 37°C. Cultures were pelleted by centrifugation at 1250 X g for 10 min in a swinging bucket rotor. Tubes were inverted to drain on tissue paper and then resuspended in 200μl of P1 by gentle agitation. 400μl of P2 was then added and the sample was transferred to a fresh 1.5 ml Eppendorf tube. 300μl of P3 was then added and the sample briefly vortexed and placed on ice. Samples were centrifuged at maximum rpm in a bench top microfuge for 10 min to pellet cell debris and the supernatant was transferred to a fresh 1.5ml Eppendorf tube. An equal volume of isopropanol was added and the tube contents were mixed by inversion and incubated at -20°C for 30 min. Precipitated DNA was pelleted by centrifugation at max rpm in a bench top microfuge for 10 min. The DNA pellet was resuspended in 50μl dH2O and 2μl of RNase A (10mg/ml) was added and incubated at 37°C for 30 min. The volume was increased to 250μl with dH2O and extracted once with an equal volume of freshly prepared phenol/chloroform (3:1) mix. Samples were briefly vortexed to mix aqueous and organic phases and then centrifuged at maximum rpm in a benchtop microcentrifuge to separate phases. The upper aqueous phase was transferred to a fresh 1.5ml Eppendorf tube and re-extracted with a chloroform/isoamyl alcohol (24:1) mix. Tubes were briefly vortexed to mix phases and re-centrifuged at maximum rpm. The upper aqueous phase was removed and plasmid DNA precipitated by the addition of a 1/10 volume of 3M Sodium
Acetate solution (pH 4.0) and 2 volumes of absolute ethanol. Samples were incubated at -20°C for 30 min and centrifuged at maximum rpm for 10 minutes to pellet the plasmid DNA precipitate. The supernatant was removed by aspiration and pellets were washed in 200μl of 70% ethanol. Ethanol was removed with a drawn-out pasteur pipette and the pellet allowed to air dry for several hours before being resuspended in 50μl dH₂O overnight at 4°C.

P1 (Resuspension solution)
50mM Tris-HCl pH 7.5
10mM EDTA pH 8.0

P2 (Lysis solution)
0.2M NaOH
1% SDS

P3 (Neutralisation solution)
2.55M potassium acetate pH 4.8. An 11.5ml aliquot of glacial acetic acid was mixed with 60ml of a 5M potassium acetate pH 4.8 stock solution.

Small scale plasmid minipreps were prepared when screening for recombinant clones following essentially the same protocol as above, except the initial starter culture was only 1ml of L Broth and the volumes of P1(100μl), P2 (200μl) and P3(150μl) were adjusted accordingly. The supernatant obtained after P3 addition and centrifugation was extracted with phenol/CHCl₃(3:1) and CHCl₃/isoamyl alcohol(24:1) then treated with 2μl RNase A (10mg/ml) for 30-45 min at 37°C. DNA was precipitated by adding 0.6 volumes of isopropanol and incubating at -20°C for 30 min followed by centrifugation for 10 min at maximum rpm. The supernatant was then removed and the pellet was washed with 200μl of 70% ethanol and allowed to air dry for several hours. The final DNA precipitate was resuspend in 20-40μl of TE or dH₂O and 10μl used for restriction endonuclease digestion.

Large scale preparations of plasmid which were not required to be RNA free were extracted from an overnight culture in 250ml L-Broth with minor variations from the above protocol. Cells were collected by centrifugation in a 250ml plastic centrifuge bottle at 15000 X g for 15 min at 4°C. The supernatant was removed and the bacterial pellet was resuspended in 10ml P1 until homogenously dispersed followed by 20ml of P2 and was swirled to mix and placed on ice. A 15ml aliquot of P3 was finally added and solution was swirled to mix before being centrifuged at 15000 X g for 30 min at 4°C. The clear supernatant was transferred to a fresh 250ml centrifuge bottle and a 0.6 volume of isopropanol was added and incubated at -20°C for 30 min. The suspension was centrifuged at 15000 X g for 20 min and supernatant was carefully removed and
the tube inverted to drain. The DNA pellet was washed with 20mls of ice cold 75% ethanol to remove salts, air dried and resuspended overnight in 3ml TE pH 8.0. Once resuspended, the DNA solution was split in 4 X 0.75ml aliquots, transferred to four fresh Eppendorf tubes for further extraction of proteins and purification of plasmid DNA via phenol/chloroform extraction and ethanol precipitation as described for the midi preparation. Samples were pooled at the end of this extraction and their concentration determined by absorbance spectrophotometry.

Preparation of Competent cells
A 0.5ml aliquot from an overnight culture of cells to be made competent (generally HB101) was used to re-inoculate a fresh 250ml of L-Broth. Cells were grown in a shaking 37°C incubator until an OD_600_ between 0.4-0.6 was reached indicative of being a mid-logarithmic phase of growth (approximately 3-4 hours of incubation). Cells were placed on ice for 30 min and then harvested by centrifugation for 5 min at 4500 x g at 4°C, resuspended in 125ml of ice cold resuspension solution (50mM CaCl_2, 10mM Tris pH 8.0) and placed on ice for another 30 min. The solution was again centrifuged at 4500 x g for 10 min at 4°C and resuspended in 30ml resuspension solution. Glycerol was added to final concentration of 25% (w/v) and 1ml aliquots were stored at -70°C.

Transformation of Competent Cells
A DNA ligation mix (approximately 15-70ng DNA in 10μl) was mixed gently with 100μl competent cells, left on ice for 30 min, heat shocked at 42°C for 2 min and then placed on ice for a further 30 min. A 1ml aliquot of L-broth was then added and incubated for 30-45 min at 37°C. After incubation tubes were pulse spun for approximately 15 sec and the volume reduced by removing approximately 500μl of L-broth. Cells were resuspended by gentle flicking of the tube before adding 20μl X-Gal (25mg/ml in dimethyl formamide) and 8 μl IPTG (0.1M) and then plated on LB agar plates containing the appropriate concentration of antibiotic. Plates were left to air dry in the laminar flow hood for 1-2 hours before covering, and inverted in a 37°C incubator for overnight incubation. The growth of transformed colonies and the development of blue/white colour selection were checked the next day with plates often left for an extra day at 4°C to allow the colour reaction to develop. White colonies were picked and streaked out on fresh LB agar plates with antibiotic selection to achieve clonal isolation.

X-Gal (25mg/ml)
Dissolved in dimethyl formamide, wrapped in aluminium foil and stored at -20°C in 1ml aliquots.

IPTG (0.1M)
Dissolved in dH_2O, filter sterilized through a 0.20μM disposable filter and stored at -20°C in 1ml aliquot's.
Subcloning of DNA Fragments

Subcloning of DNA fragments for riboprobe synthesis or as probes for Southern blot analysis was conducted as follows. The DNA fragment to be subcloned was first excised from plasmid by overnight restriction endonuclease digestion and then gel purified in order to minimise contamination by uncut DNA. Receiving vectors were linearised by overnight restriction endonuclease digestion and also gel purified. Gel purification was carried on 0.8% agarose gels in 1 X TBE, and DNA was extracted from isolated gel portions containing the fragment of interest. DNA was extracted from agarose slices by centrifugation of the diced agarose region through a Spin-X (Centricon) microcentrifuge tube according to the manufacturers protocol or as follows by using a modification of the protocol described by Wang and Rossman (1994). The isolated agarose band was diced with a sterile scalpel blade and spun through glasswool placed in the bottom of a 1.5ml Eppendorf tube with a hole pierced in its base by an 18 gauge needle. This tube was then placed inside a second 1.5ml Eppendorf tube and the agarose gel was squashed eluted through the siliconised glass wool by centrifugation at 12000 X g in a bench top microfuge for 10-15 min. The eluant was extracted once with an equal volume of phenol/CHCl₃, vortexed briefly to mix and centrifuged for 3 min to separate the phases. The aqueous layer was removed, reextracted with sevage and centrifuged to separate the phases. The aqueous layer was transferred to a fresh Eppendorf tube and DNA was precipitated and resuspended in 20µl of dH₂O.

Ligation of the linearised vector and DNA fragment to be subcloned was carried out essentially as follows. The molar ratio of DNA insert to vector was 1:1 and 3:1 in a 10µl ligation volume. Generally between 10-50ng of vector and 5-20ng of DNA insert was used depending on the yield after purification. Ligations were generally carried out overnight at 4°C or for 4 to 5 hours at room temperature in the presence of 1 U T4 DNA Ligase (NEB or Promega) and manufacturers ligation buffer. Negative controls of vector without insert and a ligation mix with no DNA were also included to determine re-ligation of linearised vector and contamination of competent cells respectively. Transformation of ligation reactions was generally carried out on the following day. For vectors which were linearised with a single restriction endonuclease and generated a high level of re-ligation, vector ends were dephosphorylated by treatment with calf intestinal alkaline phosphatase at a concentration of 2 U per 5µg of template. Reactions were incubated for 30 min at 37°C and extracted with phenol/CHCl₃, re-precipitated and resuspended in dH₂O before ligation reactions.

DNA Sequencing

Sequencing of subcloned products to determine the orientation and identity of the insert was carried out utilising an Applied Biosystems Model 373A DNA sequencer at the CSIRO DAP Sheep Gene Mapping Unit. Sequence was analysed using the ANGIS
(Australian National Genomic Information Service) database and sequence homology was determined using the BLAST or FASTA search packages.

Southern Blotting

Probe preparation
DNA probes were radiolabelled with the Gigaprin radio-labelling kit (Bresatec) following the manufacturers instructions. Briefly, 25-50ng of purified DNA fragment was denatured in a 7μl volume at 95°C for 5 min, pulse-centrifuged and placed on ice and 6μl of decanucleotide random primer, 6μl of dNTP mix and 5μl of [α-32P] dCTP (approximately 50μCi) was added. This was followed by the addition of 1μl of DNA polymerase and incubated at 37°C for 15 min. The reaction was pulse-centrifuged and placed on ice prior to a 5 min denaturation at 95°C and then added to the prehybridising nylon membrane. Periodically the incorporation of radiolabel was confirmed by running a 0.5μl aliquot of the labelling reaction both before and after the addition of enzyme on a PEI-cellulose strip in 0.75M K2HPO4, pH 3.5 followed by exposure to X-Ray film for 20-30 min at room temperature. The code of practice for persons using ionising radiations as laid out by the CSIRO DAP Health and Safety committee was followed during the handling of radioisotopes.

Gel blotting and hybridisations
Approximately 10-20μg of genomic DNA extracted from mouse tails as determined by 260/280 absorbance spectrophotometry were digested with 1 U/μg of an appropriate restriction endonuclease in a 50μl volume overnight at 37°C. Complete DNA digestion was confirmed by examining a 5μl aliquot of the digest by electrophoresis on a 0.8% 1 X TBE minigel. The observation of satellite bands was used as an indication that DNA digestion was complete and that equivalent amounts of DNA were digested. Remaining genomic DNA digests and appropriate molecular weight markers were then loaded on larger 0.8% 1 X TBE agarose gels and subjected to electrophoresis overnight at 50mA until the bromophenol blue had migrated two thirds of the way down the gel. Gels were photographed under the UV transilluminator and Southern blotted.

Digested DNA was transferred to Hybond N+ nylon membrane (Amersham) on a vacublot transfer apparatus. High molecular weight DNA was fragmented by depurination with 0.1M HCl for 20 min followed by a 120 min transfer to membrane with 0.4M NaOH. Depurination was not carried out for the transfer of DNA from gels containing PCR product, and transfer was generally only for 60 min. Once transfer was complete, the Hybond N+ membrane containing the transferred DNA was rinsed in 2 X SSPE solution and allowed to air dry prior to prehybridisation. The membrane was then rolled in nylon mesh in 2 X SSPE and placed in a hybridisation bottle. Prehybridisation was carried out for at least 2 hours at 65°C in a Hybaid rotating incubation oven prior to the addition of radiolabelled probe in 12.5mls of hybridisation solution (5 X SSPE, 5 X
Denhardt's solution (Denhardt, 1966), 0.5% SDS, 100µl denatured calf thymus or salmon sperm DNA (5mg/ml)). Denatured radiolabelled probe was added and hybridisation continued overnight at 65°C. The next day the hybridised blot was washed to a high stringency with two initial room temperature washes in 200-300mls of 2 X SSPE, 0.1% SDS followed by sequential washing at 65°C in 200-300 ml s 2 X SSPE, 0.1% SDS; 1 X SSPE, 0.1% SDS and 0.2 X SSPE, 0.1% SDS. The radioactivity of the blot was monitored with a Geiger counter between washes with a higher stringency wash of 0.1 X SSPE, 0.1% SDS used for blots of PCR product probed with homologous radiolabelled probe. Depending on the strength of signal detected by the Geiger counter, Southern blots of genomic DNA were exposed to X-Ray film with intensifying screens for periods of 5-20 days at -70°C while blots of PCR product probed with homologous probe were exposed for a period of 3-4 hours at -70°C.

Animal Experimentation

Animal experimentation was carried in accordance with Commonwealth Government AECC regulations. Experimentation described here is covered under the AECC protocol numbers 91058 and 94039.1

EGF Treatment of Merino Sheep and skin sample collection

Two sources of tissue were used in this experimental work with differing methods of treatment and fixation. In the first series of samples used to study FGF-5 expression Merino wethers were dosed with a depilatory dose of recombinant mouse EGF (100µg/kg) and skin samples from mid-flank were taken immediately prior to mEGF (1.5mg/ml in 3.5% w/v methyl cellulose) administration and at 0.5, 1, 3, 6, 12, 26, and 52 hours. Skin trephines (small, circular pieces of skin taken from the flank of the animal) were fixed in 10% phosphate buffered formalin for approximately 10 hours followed by standard dehydration and embedding in paraffin. A second series of skin samples used to study TGF-α expression was collected from two Merino wethers receiving a depilatory dose of EGF. Skin trephines for this series were taken immediately prior to EGF treatment and at 0.5, 2, 4, 6, 12, 26 and 52 hours as well as samples at 4, 8 and 14 days after EGF administration. Skin samples were fixed in Histochoice (Amresco) for approximately 10 hours followed by standard dehydration and paraffin embedding. All sheep received a local anaesthetic administered prior to the removal of trephined skin samples. Fleeces were collected from all treated animals 2 weeks after experimentation.

Mouse Crosses

Maintenance of mice

Mice were housed at 22°-24°C on a 12/12 day/flight cycle and fed ad libitum on a formulation of Gordons mouse pellets (Bargo, NSW). Pups were weaned and the sexes separated at 4 weeks postpartum, with breeding pairs established using mice of
6-8 weeks maturity. Mice were maintained by staff at the CSIRO DAP small animal colony. Euthanasia as required was by carbon dioxide asphyxiation or cervical dislocation.

Mouse strains
Mice harbouring the waved-1 (Tgfα<sup>wa-1</sup>) mutation were obtained from the Ludwig Institute, Melbourne and were generated on a C57BL/6 background (Mann et al., 1993; Dlugosz et al., 1995). Mice had been maintained as a homozygous breeding line at CSIRO DAP small animal colony for two years. Mice carrying the waved-2 (Egrf<sup>ma2</sup>) mutation were imported as heterozygous breeding pairs from Jackson Laboratory (Bar Harbor, ME; AQIS importation number 192LAB) on the genetic background (B6C3-a/A-vt/4-wa-2/4). Homozygous Egrf<sup>ma2</sup> mutants were generated and male animals were used for breeding purposes due to a lactational problem associated with Egrf<sup>ma2</sup> homozygosity in females (Fowler et al., 1995). The angora (Fgf5<sup>pp</sup>) mouse strain utilised carries the mutation initially described by Dickie (Dickie, 1983) and has been maintained as a homozygous closed line at the CSIRO DAP small animal colony for over a decade on a predominantly C57BL/6 genetic background. The molecular basis of the mutation has recently been characterised (Hébert et al., 1994). All mutant loci examined are on separate chromosomes: the Tgfα locus is on mouse chromosome 6, the Egrf locus on mouse chromosome 11 and the Fgf5 locus on mouse chromosome 5. The segregation of these unlinked loci was assumed to occur independently and therefore exhibit simple mendelian segregation. Complete expression of these recessive mutant hair phenotypes occurs when animals are homozygous for the mutation. Genotype nomenclature follows that of Sundberg and King (1998) with homozygosity for the mutation indicated by <sup>-/-</sup>, homozygosity for the wild-type allele by <sup>++/+</sup> and heterozygous animals by <sup>+-/-</sup>.

Phenotyping of mice
Mice produced during this experimental work (Chapters 3 and 4) were qualitatively assessed for changes in the epidermal phenotype. Waved and angora hair phenotypes were clearly visible at 3-4 weeks of age. The waved-hair phenotype is also discernible from vibrissae morphology in the neonate soon after birth. The F<sub>2</sub> progeny produced from the F<sub>1</sub> intercrosses were examined from birth through to two to three weeks of postpartum growth at regular intervals for comparative changes in epidermal development and growth rate. Animals were assigned a potential phenotype at birth based on the morphology of the vibrissae with a further development of the dorsal coat confirming whether the animals were expressing either wild-type, waved, angora or combined hair phenotypes. Individual animals were marked for identification by toe clipping at three to four days after birth. Pigmentation of the skin was also observed and taken as an indication of entry into anagen growth (Slominski et al.,
1994). Differences were estimated in the time taken for dorsal skin pigmentation and the eruption of dorsal guard hairs within litters by examination under an electric lamp. Animals with phenotypes that diverged from those normally seen were either culled for histology or the carcass was stored at -20°C for examination after genotyping. Tail and hair samples were taken from mice which exhibited the expected phenotypes and stored at -20°C.

Growth rate data collection
The genotyped mice were regularly weighed over the time of observation to determine if differences in the growth rate of individuals could be detected. Live body weight was recorded and growth rates (g/day) for each individual were estimated by averaging the pairwise growth rates of mice weighed every three to four days over a 21 day period to give an estimate of daily growth rate. An assumption of linear growth during this period was justified by preliminary analysis of a number of mice which had been observed over a 4-5 week period and a higher number of weighings had been recorded. It is important to note that many of the more severely affected animals were not included in this analysis due to the low number of growth rate readings and their early removal to prevent parentel cannibalism.

Tissue collection from mice
Mice exhibiting novel phenotypes were culled from litters, with a small tissue sample taken for the preparation of genomic DNA and the remaining carcass fixed in Histochoice (Amresco) overnight followed by a 70% ethanol treatment for 6-12 hours and storage in 50% ethanol as final fixative. Mld-dorsal skin samples were dissected from either fixed skin or carcasses and longitudinally mounted in paraffin blocks. Paraffin embedded samples were treated and sectioned by the Histology Unit at the CSIRO DAP.

Generation of Tgfa°sx° and Egfr°sx° double homozygotes
An intercross breeding structure was utilised to generate animals segregating for allelic combinations of both the Tgfa°sx° and Egfr°sx° mutations. A female homozygous for the Tgfa°sx° mutation and a male homozygous for the Egfr°sx° mutation were used as a founding breeding pair to generate F1 progeny heterozygous for both loci. Several breeding pairs were established from the F1 generation, and F2 progeny segregating for allelic combinations of both mutations were examined. Considering that the Tgfa and the Egfr loci are on separate chromosomes the expected frequency of genotypes in the F2 generation could therefore be determined assuming independent mendelian segregation.
Two sets of F$_2$ progeny were collected in the course of this experiment. An initial series of approximately 150 offspring was generated and observed for a period of 3 to 4 weeks to determine the range of phenotypes produced and if any effect on the length of the hair cycle occurred. Animals exhibiting unusual phenotypes were culled and tissue samples collected for histology and genotyping along with wild-type and waved littermates representative of the expected segregating phenotypes. The remaining animals in this initial series were not genotyped.

It became apparent from an observation of the initial F$_2$ series that a number of animals showed decreased viability associated with a slower growth rate. To counteract these viability effects and obtain more phenotypic data, a second series of 158 F$_2$ offspring was produced. Six F$_1$ breeding pairs were established and the F$_2$ offspring from these breeding pairs were phenotyped over a two week period before being collected. Animals which exhibited a runted phenotype or that diverged from the expected wild-type or waved coat morphology were generally taken before the end of this time period to avoid parental cannibalism. However in some cases runted animals were left with there mothers or transferred to foster mothers. All animals in this second series were genotyped.

The segregation of the *vestigial tail* (vt) mutation on the *Egf$^{ne-2}$* background was potentially problematic due to the possibility that vt may modify the phenotype of F$_2$ progeny. Although the molecular basis for *vestigial tail* is currently unknown, it has been suggested that the it may be due to a hypomorphic allele of *Wnt3a*, a candidate gene which shows very tight linkage to the vt locus (Greco et al., 1996). A decrease in *vestigial tail* frequency in *Egf$^{ne-2}$* homozygotes would be an indication that deleterious modifying effects due to this linked locus may be occurring. As a genetic distance of 26cM between the recessive mutation, *vestigial tail*(vt) and the *Egf* locus on mouse chromosome 11 has been defined (Greco et al., 1998) it is possible to determine the expected frequency of vt expressing progeny if a male of known vt genotype is used. A founding male *Egf$^{ne-2}$* mouse expressing the *vestigial tail* phenotype and therefore of a known homozygous vt genotype, was used to determine whether a significant divergence vt expression from expected frequencies was occurring in this experiment.

Generation of [Tgf$\alpha^{ae-1}$.l-; Fgf-5$^{po}$.l-] mice

The generation of mice homozygous for both the *Tgf$\alpha^{ae-1}$* and *Fgf-5$^{po}$* mutation was achieved by crossing *Tgf$\alpha^{ae-1}$* homozygous animals with *Fgf-5$^{po}$* homozygotes to generate an F$_1$ generation of double heterozygote [Fgf-5$^{po}$.l-; Tgf$\alpha^{ae-1}$.l-] offspring. Several breeding pairs were established from this generation to produce F$_2$ progeny segregating for combinations of these two mutations. Offspring were examined for expression of both the waved and angora hair phenotypes which were easily
distinguishable at four weeks postnatal development. Mice exhibiting the combined waved/angora hair phenotype indicative of the doubly homozygous [Fgf5\textsuperscript{repl.}-; Tgfa\textsuperscript{a-si.}-] genotype, were also observed (see chapter 4). Several breeding pairs of presumed double homozygote [Fgf5\textsuperscript{repl.-}; Tgfa\textsuperscript{a-si.-}] mice were paired and allowed to breed. Offspring from these animals all expressed the combined waved/angora phenotype and were maintainable as a separate breeding line. These mice were viable and produced healthy offspring which also expressed the combined waved/angora phenotype.

Generation of [Fgf5\textsuperscript{repl.-}; Egfr\textsuperscript{a-si.-}] mice

A similar breeding strategy was followed to generate mice doubly homozygous for the Fgf5\textsuperscript{repl.} and the Egfr\textsuperscript{a-si.} mutations. Initial breeding pairs were established using male Egfr\textsuperscript{a-si.} homozygotes which in this case did not express the vestigial tail phenotype, and female Fgf5\textsuperscript{repl.} homozygotes. As several intercross breedings were to be undertaken in this experiment it would not have been possible to track the vt locus through the experiment as described above. Several breeding pairs were established to generate double heterozygote [Fgf5\textsuperscript{repl.}; Egfr\textsuperscript{a-si.}] offspring. Animals were intercrossed at this generational level to produce progeny segregating for combinations of these two mutations. Progeny were examined for expression of the waved and angora hair phenotypes and viable mice expressing a waved/angora hair phenotype, and therefore a potentially double homozygous [Fgf5\textsuperscript{repl.-}; Egfr\textsuperscript{a-si.-}] genotype, were observed. However, these animals were not maintainable as a closed breeding line presumably due to the lactational abnormalities associated with the Egfr\textsuperscript{a-si.} mutation, and a heterozygous breeding strategy was used to maintain the double mutant phenotype.

Generation of [Fgf5\textsuperscript{repl.-}; Tgfa\textsuperscript{a-si.}; Egfr\textsuperscript{a-si.-}] mice

Mice expressing the combined waved/angora phenotype were presumed doubly homozygous for their respective mutations and were selected for breeding pairs to generate mice with the allelic combination [Fgf5\textsuperscript{repl.-}; Tgfa\textsuperscript{a-si.}; Egfr\textsuperscript{a-si.}]. Double homozygote [Fgf5\textsuperscript{repl.-}; Tgfa\textsuperscript{a-si.-}] females were mated with double homozygote [Fgf5\textsuperscript{repl.-}; Egfr\textsuperscript{a-si.-}] males and produced normal sized litters of healthy offspring which only expressed the angora hair phenotype. Male animals were also chosen which did not express the vestigial tail phenotype.

An initial F\textsubscript{2} series of approximately 200 F\textsubscript{2} animals was observed over a four week period to determine whether allelic combinations of these mutations resulted in a further disruption of the hair cycle. No easily observable difference was noted in progeny from this cross although a number of severely runted offspring and small animals were produced. These mice were often cannibalised at an early stage of postnatal growth.
As a consequence of the cannibalism a second F₂ series was generated and mice were observed over two week period to obtain more comprehensive phenotypic data. Eight breeding pairs were re-established to produce a second series of progeny. Offspring that exhibited a runted or less viable phenotype were often taken before the end of this period to decrease the likelihood of cannibalism. All animals from this second series were genotyped.

PCR

Oligonucleotides

Oligonucleotides were synthesised either on site using an Applied Biosystems oligonucleotide synthesiser or custom made, desalted and deprotected by GIBCO BRL, Australia. Oligonucleotides synthesised on site were deprotected and cleaved from columns by treatment with approximately 2ml of saturated NH₄OH solution. Columns were incubated at room temperature for approximately one hour with occasional agitation of the ammonium hydroxide solution through the column. The 2ml of solution containing the oligonucleotide were then placed into teflon-capped glass tubes, paraffin-sealed and incubated overnight at 55°C. The deprotected oligonucleotide solution was removed from each tube and dispensed equally into two 1.5ml Eppendorf tubes. Samples were then lyophilised on a Speedvac evaporator rotor under heat for several hours until a dried oligonucleotide pellet was visible. The lyophilised sample was resuspended in sterile dH₂O and then diluted in water to generate a working stock of 10μM which was aliquotted and stored at -20°C.

PCR Reagents

All reagents used for PCR were aliquotted into smaller volumes to avoid repeated freezing and thawing and possible cross contamination. Manufacturer’s 10 X reaction buffer and 25mM magnesium chloride solution were dispensed into 200μl aliquots. A 100mM combined nucleotide mix (25mM each dNTP)(Pharmacia or Stratagene) was prepared and diluted in water to generate 200μl aliquot’s of a 10mM combined working stock which was stored at -20°C. All dilutions and preparation of reaction mixes were carried out using sterile milliQ water (AMRESCO).

PCR procedure

All dilutions of reagents and preparations of reaction cocktail mixes were done in a separate building utilising pipettemen dedicated to PCR reagent manipulation. Aerosol-resistant pipette tips were used at all stages. Reaction cocktail mix was aliquoted into 0.2ml thin walled PCR tubes (Stratagene) and overlaid with paraffin oil (Sigma) prior to transfer to another building for the addition of template. Dilution of genomic DNA template was carried out in a laminar flow hood using a separate set of pipettemen.
Diluted template was added to the PCR reaction mix in a separate laminar flow hood with pipetmen dedicated to this procedure and tubes were recapped and placed on the ROBOCYCLER PCR machine (Stratagene). After completion of the amplification, tubes were removed and taken to another floor of the laboratory where they were opened and samples either directly analysed on gels after the addition of 10 X Loading Buffer or an aliquot taken for restriction endonuclease digestion.

Genotyping Assays

Preparation of genomic DNA

Extraction of genomic DNA from mouse tails for the preparation of PCR template was as described by Parvan et al. (1995). Approximately 1cm of mouse tail or the equivalent weight from fixed mouse tissue was removed from collected samples. Tissue was digested for 24-48 hours at 55°C in 800μl digestion buffer (10mM Tris-HCl pH 8.0, 100mM NaCl, 100mM Na2 EDTA pH 8.0, 0.5% SDS) with 20μl of Proteinase K (20mg/ml) in 1.5ml Eppendorf tubes. Digested tissue was extracted with 400μl of a 3:1 phenol/chloroform solution, vortexed briefly to mix and centrifuged at maximum rpm (12000 X g) for 5 min in a bench top centrifuge to separate the aqueous and organic phases. A 600μl aliquot of the upper aqueous phase was carefully removed without disturbing the protein rich interface and placed into a fresh Eppendorf tube containing 95% ethanol. Samples were inverted several times and centrifuged at maximum rpm for 5 min in a benchtop centrifuge to pellet the precipitate. Supernatant was aspirated with a thin glass pasteur pipette and DNA pellets were allowed to air dry for several hours before being resuspended in 100μl of water. Samples were heat treated at 65°C for 10 min, 37°C for 30 min to aid resuspension and left at 4°C overnight before a 1:100 dilution was made in water to prepare template for PCR analysis. The remaining sample was stored at -70°C. An average concentration of the 100μl resuspension of extracted gDNA from mouse tail tissue was approximately 2μg/μl as determined by absorbance spectrophotometry. A 1:100 dilution of purified genomic DNA was made in sterile water giving a solution of 20ng/μl. A 5μl aliquot (generally 100ng) of this dilution was used for PCR genotyping assays.

Genotyping of mice

Genotype analysis was carried out using appropriate-concentration agarose gels to detect the expected amplified PCR product. The Tgfa<sup>++/−</sup> PCR product was resolved on 1% agarose 1 X TBE gels containing ethidium bromide while the mobility shift utilised in genotyping of the Egfi<sup>++/−</sup> mutation after restriction endonuclease digestion was detected on 2.2% agarose 1 X TBE gels containing ethidium bromide. PCR products were generally separated by electrophoresis at 100mA for 2-3 hours prior to recording the genotypes by polaroid photography under the UV transilluminator.
Optimisation of genotyping assays

The Tgfα<sup>−−/−</sup> genotyping assay

The transgenic null allele of the Tgfα<sup>−−/−</sup> locus used in this experiment was initially generated by the targeted disruption of exon 3 by an insertion of a PGK-Neo expression cassette (Mann et al., 1993). To detect the mutant and wild-type alleles of the TGF-α locus, a genotyping assay was designed based on the published primers utilised by Mann et al. (1993). A multiplex PCR assay using all three primers P1, P2 and P3 was developed to detect both alleles in a single PCR reaction rather than the separate assays previously described by Mann et al. (1993). The P1/P2 primer combination detects the mutant transgenic allele while the P1/P3 primer combination amplifies the wild-type allele of Tgfα exon 3 (Figure 2.1.A). Optimal conditions were re-established for each of the individual genotyping assays using a simplified reaction mix and minimising the amount of thermostable DNA polymerase required. Primer and magnesium chloride concentrations as well as optimal annealing temperature were determined which amplified the wild-type product and mutant alleles in separate reaction mixes prior to developing the multiplex assay. An initial optimisation of reactions for the primer combinations P1/P2 and P1/P3 at 1μM each and Mg<sup>2+</sup> concentrations of 1.25 and 2.5mM was performed on a ROBOCYCLER PCR machine using a temperature gradient profile of 44°C-66°C. A single PCR product for each primer pair of the expected size at an annealing temperature of 66°C was produced at 2.5mM Mg<sup>2+</sup> with aberrant PCR products appearing at lower annealing temperatures. A spurious low molecular weight PCR doublet was produced with the P1/P2 primer combination in all reactions, consistent with previous observations (Mann et al., 1993). Correct amplification of the appropriate wild-type or Tgfα<sup>−−/−</sup> allele was confirmed by analysing a test panel of known wild-type homozygotes, Tgfα<sup>−−/−</sup> homozygotes and heterozygous animals. Each primer pair amplified a product of the correct size in each of the appropriate templates. The P1/P3 primer pair amplified an approximately 1kb band in animals carrying the wild-type Tgfα allele while the P1/P2 primer pair amplified an approximately 1.4kb product in animals carrying the Tgfα<sup>−−/−</sup> mutant allele.

Optimisation of the multiplex assay was determined by utilising a test panel of known homozygous and heterozygous mice. The optimal primer concentrations of P1, P2 and P3 were obtained by testing various combinations in the range of 0.5, 1.0 and 2.0μM for each primer utilising a known heterozygous animal carrying wild-type and mutant alleles for a genomic DNA PCR template. The combination of 1.0μM P1, 2.0μM P2 and 1.0μM P3 was effective in amplifying both alleles with 1.0 U of thermostable DNA polymerase. Further optimisation of these multiplex conditions was carried out to increase specificity of the PCR reaction and optimise reagents used. Decreasing the optimised primer concentration by a factor of four resulted in the amplification of the wild-type and Tgfα<sup>−−/−</sup> alleles with little or no background amplification of spurious product.
but thermostable DNA polymerase concentration could not be decreased below 1.0 U per 25 µl reaction. A test panel of several homozygous and heterozygous mice was utilised to confirm these final optimised conditions. The PCR product was resolved on a 1.0% 1X TBE agarose gel containing ethidium bromide and subjected to electrophoresis at 100mA for 2-3 hours. All samples produced the expected amplified products for their genotype (Figure 2.1.B). The final conditions for the multiplex amplification of both wild-type and mutant alleles were 0.25 µM P1, 0.5 µM P2, 0.25 µM P3, 2.5 mM Mg²⁺, 0.25 mM each dNTP, 1.0 U RED HOT thermostable DNA polymerase (Advanced Biotechnologies), 1X Reaction Buffer IV (Advanced Biotechnologies) and approximately 100 ng genomic DNA template in a 25 µl reaction volume. The PCR conditions for amplification consisted of an initial denaturation step of 3 min at 95°C, 30 sec at 64°C, 70 sec at 72°C, 30 amplification cycles of 30 sec at 95°C, 30 sec at 64°C, 70 sec at 72°C and a final extension cycle of 30 sec at 95°C, 30 sec at 64°C and 7 min at 72°C.

The \textit{Egf^{as-2}} genotyping assay

The point mutation resulting in the \textit{Egf^{as-2}} mutation introduces a restriction enzyme site for the \textit{Fok1} restriction endonuclease in exon 20 of the mouse EGFR (Luetteke \textit{et al.}, 1994). This enzyme is a remote cutting endonuclease which cleaves the DNA strand at a site 9/13 base pairs 3' from the \textit{Fok1} recognition sequence. A PCR-RFLP (Restriction fragment length polymorphism) assay was established that would allow detection of the homozygous and heterozygous genotypes segregating in the intercross breeding structure used to generate animals carrying various allelic combinations of the \textit{Egf^{as-2}} mutation (Figure 2.2). The design of primers to amplify mouse exon 20 was based upon two criteria; the length of the 5' oligonucleotide upstream of the \textit{Egf^{as-2}} point mutation which occurs 11 bp from the intron/exon boundary (Callaghan \textit{et al.}, 1993) and the amplification of a fragment that would allow a detectable mobility shift of the exon 20 PCR product when cleaved with \textit{Fok1} on a high percentage agarose gel. Three primers were designed to fulfill this criteria. Two primers were designed to bind to the exon 20 region 5' to the \textit{Egf^{as-2}} mutation. Primer WF1 is a 16mer designed to increase the length of the oligonucleotide binding region by including an extra 6 bp of putative intronic sequence of the intron/exon boundary region described in the chicken EGFR (Callaghan \textit{et al.}, 1993). Lower case letters represent putative intronic sequence incorporated in WF1. A second primer WF2, contained only the 10mer of exon 20 sequence 5' of the \textit{Egf^{as-2}} point mutation. A third primer at the 3' end of exon 20 was designed to increase the size of the amplified exon 20 product so that restriction fragments could easily be observed when the amplified product was digested with \textit{Fok1}. The expected size of amplified product from the primer pair WF1/WF1 is 180bp which when digested with \textit{Fok1} would generate a 150bp and 30bp product while the WF2/WF1 combination would amplify a 174bp product cleavable to generate a 150bp and 24bp product.
WF1
5'-ctcagGAAGCCTATG-3'

WF2
5'-GAAGCCTATG-3'

WR1
5'-CACACCAGTTGAGGAGG-3'

PCR was initially optimised using the ROBOCYCLER temperature gradient annealing profile 44°C-66°C and two magnesium concentrations, 1.25mM and 2.5mM. Reaction conditions were as described for the Tgfrα**' genotyping assay in a 25µl reaction volume with initial primer concentrations of 1µM utilised. A fragment of the correct size was amplified with the WF1/WR1 primer pair at both Mg²⁺ concentrations although background PCR products were also present at the different annealing temperatures. No band of the expected size was produced with the WF2/WR1 primer concentration. Confirmation of the amplification of the exon 20 region was determined by Southern hybridisation of these initial gels with a 690bp cDNA probe containing a region of the mouse Egfr encompassing the exon 20 region (kindly supplied by Dr M.Hibbs, Ludwig Institute of Cancer Research, Melbourne). Southern blots were washed under high stringency conditions and blots were exposed to X-ray film for 2-3 hours at -70°C. A band of the correct size which hybridised strongly with this probe was detected in the PCR reactions utilising the primer pair WF1 and WR1. No hybridisation signal was detected with the WF2 and WR1 primer combination suggesting that the amplification of exon 20 was not successfully occurring with the smaller WF2 oligonucleotide. The optimal annealing temperature and Mg²⁺ concentration for the primer pair WF1/WR1 were determined from the annealing temperature profile to be 46°C and 1.25mM Mg²⁺. Multiple background PCR product was also present under these conditions. Primer concentrations were then manipulated to decrease background amplification and increase fidelity of the exon 20 amplification by increasing or decreasing the relative primer concentrations of WF1 and WR1. Primer concentrations were varied between 1.0, 0.5 and 0.1µM in pairwise combinations and re-amplified under the reaction conditions established. This resulted in an optimal primer concentration of 1.0µM WF1 and 0.5µM WR1 which amplified a single PCR product corresponding to the correct molecular size of the expected exon 20 fragment. The amplification conditions were further manipulated to determine a threshold level of Mg²⁺ and thermostable DNA polymerase below which fidelity of amplification was compromised. This would possibly allow a more robust amplification of the exon 20 product and reduce the amount of thermostable DNA polymerase required for the genotyping assay. Optimal conditions for amplification of the exon 20 product were finalised at 1.0µM WF1, 0.5µM WR1, 1.75mM Mg²⁺, 0.25mM each dNTP, 1.0 U RED HOT thermostable DNA
polymerase (Advanced Biotechnologies), 1 X Reaction Buffer IV (Advanced Biotechnologies) and approximately 100 ng genomic DNA template in a 25 µl reaction volume. The temperature profile for amplification consisted of an initial denaturation cycle of 3 min at 95°C, 30 sec at 45°C, 30 sec at 72°C, 35 amplification cycles of 30 sec at 95°C, 30 sec at 46°C, 30 sec at 72°C and a final extension cycle of 30 sec at 85°C, 30 sec at 46°C and 6 min at 72°C.

Once optimal conditions had been established for amplification of exon 20, the FokI PCR-RFLP was optimised by determining the amount of FokI required to achieve complete digestion of the PCR product. The PCR products from a test panel of DNA templates from homozygous wild-type, homozygous Egfr<sup>−/−</sup> and heterozygous animals were digested with a range of amounts of FokI enzyme ranging from 0.5 U, 1.0 U and 2.0 U for 5 hours at 37°C. Digested PCR products were subjected to electrophoresis on 2.2% agarose 1 X TBE gels containing ethidium bromide for 2-3 hours at 100mA and examined under a UV transilluminator. A clear mobility difference and complete digestion of the PCR product was discernible in genotypes homozygous for the Egfr<sup>−/−</sup> mutation. Southern blot analysis of this optimisation assay with the Egfr cDNA probe confirmed the identity of the PCR product and its complete digestion at all enzyme levels assayed. The lower 30 bp fragment was not detectable and had likely run off the gel. A FokI concentration of 1 U per 10 µl of PCR reaction was utilised for the determination of the genotypes of animals bred during the course of this experiment. DNA from a homozygous wild-type, a heterozygote and a homozygous Egfr<sup>−/−</sup> animal were also included in each batch of FokI digested samples as positive controls (Figure 2.2A and B).

The Fgf5<sup>−</sup> genotyping assay

The Fgf5<sup>−</sup> mutation in the strain used in this experiment is due to a deletion in exon 1 of the FGF-5 gene extending at least 2kb upstream of the translation initiation site (Figure 2.3A) (Hébert et al., 1994). Animals homozygous for this deletion mutation were detected by RFLP Southern blot analysis utilising a full length mouse Fgf5 cDNA probe (kindly supplied by Dr R. Seymour, CSIRO DAP). An EcoR1 RFLP was established in which the absence of a molecular weight band of approximately 9kb was indicative of the deletion mutation and hence of homozygous Fgf5<sup>−</sup> individuals. A higher molecular weight band of approximately 19kb is apparent in all samples and contains the undeleted region of the FGF-5 gene containing exon 2 and exon 3 (Figure 2.3B).
polymerase (Advanced Biotechnologies), 1 X Reaction Buffer IV (Advanced Biotechnologies) and approximately 100ng genomic DNA template in a 25μl reaction volume. The temperature profile for amplification consisted of an initial denaturation cycle of 3 min at 95°C, 30 sec at 46°C, 30 sec at 72°C, 35 amplification cycles of 30 sec at 95°C, 30 sec at 46°C, 30 sec at 72°C and a final extension cycle of 30 sec at 95°C, 30 sec at 46°C and 6 min at 72°C.

Once optimal conditions had been established for amplification of exon 20, the Fok1 PCR-RFLP was optimised by determining the amount of Fok1 required to achieve complete digestion of the PCR product. The PCR products from a test panel of DNA templates from homozygous wild-type, homozygous Egfr<sup>−/−</sup> and heterozygous animals were digested with a range of amounts of Fok1 enzyme ranging from 0.5 U, 1.0 U and 2.0 U for 5 hours at 37°C. Digested PCR products were subjected to electrophoresis on 2.2% agarose 1 X TBE gels containing ethidium bromide for 2-3 hours at 100mA and examined under a UV transilluminator. A clear mobility difference and complete digestion of the PCR product was discernible in genotypes homozygous for the Egfr<sup>−/−</sup> mutation. Southern blot analysis of this optimisation assay with the Egfr cDNA probe confirmed the identity of the PCR product and its complete digestion at all enzyme levels assayed. The lower 30 bp fragment was not detectable and had likely run off the gel. A Fok1 concentration of 1 U per 10μl of PCR reaction was utilised for the determination of the genotypes of animals bred during the course of this experiment. DNA from a homozygous wild-type, a heterozygote and a homozygous Egfr<sup>−/−</sup> animal were also included in each batch of Fok1 digested samples as positive controls (Figure 2.2.A and B).

The Fgf5<sup>−/−</sup> genotyping assay

The Fgf5<sup>−/−</sup> mutation in the strain used in this experiment is due to a deletion in exon 1 of the FGF-5 gene extending at least 2kb upstream of the translation initiation site (Figure 2.3.A) (Hébert <i>et al.</i>, 1994). Animals homozygous for this deletion mutation were detected by RFLP Southern blot analysis utilising a full length mouse Fgf5 cDNA probe (kindly supplied by Dr R. Seymour, CSIRO DAP). An EcoR1 RFLP was established in which the absence of a molecular weight band of approximately 9kb was indicative of the deletion mutation and hence of homozygous Fgf5<sup>−/−</sup> individuals. A higher molecular weight band of approximately 19kb is apparent in all samples and contains the undeleted region of the FGF-5 gene containing exon 2 and exon 3 (Figure 2.3.B).
polymerase (Advanced Biotechnologies), 1 X Reaction Buffer IV (Advanced Biotechnologies) and approximately 100ng genomic DNA template in a 25μl reaction volume. The temperature profile for amplification consisted of an initial denaturation cycle of 3 min at 95°C, 30 sec at 46°C, 30 sec at 72°C, 35 amplification cycles of 30 sec at 95°C, 30 sec at 46°C, 30 sec at 72°C and a final extension cycle of 30 sec at 95°C, 30 sec at 46°C and 6 min at 72°C.

Once optimal conditions had been established for amplification of exon 20, the Fok1 PCR-RFLP was optimised by determining the amount of Fok1 required to achieve complete digestion of the PCR product. The PCR products from a test panel of DNA templates from homozygous wild-type, homozygous Egfr<sup>ws2</sup> and heterozygous animals were digested with a range of amounts of Fok1 enzyme ranging from 0.5 U, 1.0 U and 2.0 U for 5 hours at 37°C. Digested PCR products were subjected to electrophoresis on 2.2% agarose 1 X TBE gels containing ethidium bromide for 2-3 hours at 100mA and examined under a UV transilluminator. A clear mobility difference and complete digestion of the PCR product was discernible in genotypes homozygous for the Egfr<sup>ws2</sup> mutation. Southern blot analysis of this optimisation assay with the Egfr cDNA probe confirmed the identity of the PCR product and its complete digestion at all enzyme levels assayed. The lower 30 bp fragment was not detectable and had likely run off the gel. A Fok1 concentration of 1 U per 10μl of PCR reaction was utilised for the determination of the genotypes of animals bred during the course of this experiment. DNA from a homozygous wild-type, a heterozygote and a homozygous Egfr<sup>ws2</sup> animal were also included in each batch of Fok1 digested samples as positive controls (Figure 2.2.A and B).

The Fgt5<sup>go</sup> genotyping assay

The Fgt5<sup>go</sup> mutation in the strain used in this experiment is due to a deletion in exon I of the FGF-5 gene extending at least 2kb upstream of the translation initiation site (Figure 2.3.A) (Hébert et al., 1994). Animals homozygous for this deletion mutation were detected by RFLP Southern blot analysis utilising a full length mouse Fgf5 cDNA probe (kindly supplied by Dr R. Seymour, CSIRO DAP). An EcoR1 RFLP was established in which the absence of a molecular weight band of approximately 9kb was indicative of the deletion mutation and hence of homozygous Fgt5<sup>go</sup> individuals. A higher molecular weight band of approximately 19kb is apparent in all samples and contains the undeleted region of the FGF-5 gene containing exon 2 and exon 3 (Figure 2.3.B).
Figure 2.1 The multiplex PCR genotyping assay utilised for genotyping the Tgflα"-/-" mutation. (A) A diagrammatic representation of the neomycin (Neo) insertion into exon 3 of the mouse TGF-α locus. Primer sequence was as described (Mann et al., 1993). Amplification of the wild-type allele with primer pair P1/P3 generates a 1kb product while the mutant allele is detectable with the primer pair P1/P2 generating a 1.4kb product. (B) A 1.0% 1 X TBE agarose gel stained with ethidium bromide with a representative sample of the homozygous mutant, homozygous wild-type and heterozygous individuals. Lanes 1 and 2, homozygous Tgflα"-/-" samples expressing the waved phenotype. Lanes 3 and 4, homozygous wild-type mice. Lanes 5 and 6, heterozygous samples with both wild-type and mutant alleles. Lane 7, an Ssp1(EcoRl) size standard (Progen). PCR conditions were optimised such that both wild-type and mutant alleles are detectable in a single PCR reaction. Amplification of the primer pair P1/P3 does not occur in homozygous Tgflα"-/-" animals due to the length of the intervening neomycin expression cassette and the PCR cycling parameters used.
**Tgfαwa-1** Multiplex Genotyping Assay

A.

Exon 3

Wild-type allele (1.0 kb)

 Neo

Tgfαwa-1 allele (1.4 kb)

B.

Lane 1  Lane 2  Lane 3  Lane 4  Lane 5  Lane 6  Spp-1

1.5kb, 1.39kb
1.16kb
980bp
720bp
Figure 2.2 The PCR-RFLP used to genotype the Egrf*2 mutation. (A) A diagrammatic representation of the PCR assay developed to detect the base substitution in Egrf*2 which introduces a Fok1 restriction endonuclease cleavage site in exon 20 of the Epidermal Growth Factor Receptor. Amplification of exon 20 with the WF1/WR1 primer pair generates a 180bp product which is cleaved into a 150bp and 30bp product if the Fok1 cleavage site is present. (B) A 2.2% 1 X TBE agarose gel stained with ethidium bromide with a representative sample of homozygous wild-type, heterozygote and homozygous Egrf*2 samples. Lanes 1 and 2, homozygous wild-type samples with an intact 180bp amplified product after Fok1 digestion. Lanes 3 and 4, homozygous Egrf*2 samples after Fok1 digestion of the amplified product generating a 150bp product (the 30bp fragment is not visible). Lanes 4 and 5, heterozygous samples after Fok1 digestion. Both 180bp and 150bp amplified products are present. Lane 6, a PUC19 (HpaII) size standard (Progen).
*Egfr^wa-2* PCR-RFLP Genotyping Assay.

A. 

WF1 → Exon 20

- TGATG

WF1 → Fok-1

- GGATG

Wild type allele (180bp)

Egfr^wa^ allele (150bp + 30bp)

B.

Lane 1  Lane 2  Lane 3  Lane 4  Lane 5  Lane 6  P UC19

- 242bp
- 190bp
- 147bp
- 111bp
Figure 2.3 The Restriction Fragment Length Polymorphism (RFLP) genotyping. The Fgf5⁺⁺ mutation utilised during this experimental work is the result of a deletion mutation encompassing exon 1 of the FGF-5 gene (Hebert et al., 1994). (A). A diagrammatic representation of the deletion mutation present in the Fgf5⁺⁺ mice. The deleted region encompasses exon 1 and at least 2 kb of upstream promoter sequence (adapted from Hebert et al., 1994). (B). The autoradiograph of a Southern blot of genomic DNA isolated from Fgf5⁺⁺ (lane 1), Tgα⁺⁻⁻ (lane 2) and Egrfp⁻⁻⁻ (lane 3) homozygotes probed with a radiolabelled full length mouse FGF-5 cDNA. The Tgα⁺⁻⁻ and Egrfp⁻⁻⁻ animals were used as representatives of the wild-type Fgf5 genotype and contain the 9kb band absent in animals homozygous for the Fgf5⁺⁺ mutation (arrow head). A selection of homozygous angora animals used as breeding pairs in chapter five also exhibit the expected homozygous Fgf5⁺⁺ genotype (lanes 4, 5 and 6). Samples were digested with EcoR1 and Southern blotted as described. The deletion of the exon 1 fragment is clearly visible in lanes 1, 4, 5 and 6 by the absence of a lower molecular weight EcoR1 fragment encompassing exon 1. The wild-type Fgf5 RFLP is distinguishable in lanes 2 and 3 with two EcoR1 fragments observed.
**Fgf-5<sup>o</sup>** RFLP Genotyping Assay

A.

Wild type allele

Exon 1

Exon 2

Fgf-5<sup>o</sup> allele

Exon 1 deletion

Exon 2

B.

Lane 1  Lane 2  Lane 3  Lane 4  Lane 5  Lane 6

23.13kb

9.4kb

6.5kb
In Situ Hybridisation

Riboprobe preparation and purification

Linearisation of DNA template
A 450bp PstI/SacI fragment of mouse FGF-5 containing approximately 200bp of exon 3 (Haub et al., 1990) was directionally subcloned into pBluescript SK(+). Orientation of the subcloned fragment in the vector was confirmed by sequence analysis. Preparation of linearized template was achieved by digestion of 20-40 µg of plasmid with 10-20 U of either PstI(Promega) or SacI(Promega) restriction enzyme in the manufacturers buffer at a final volume of 400 µl. Overnight digests were further digested with 5-10 U of the appropriate enzyme and incubated at 37°C for 3-4 more hours. Digests were extracted once with a phenol/CHCl₃ (3:1) mix followed by a CHCl₃/isoamyl alcohol (24:1) extraction. Linearised template DNA was precipitated with a 1/10 volume of 3M sodium acetate (pH 7.0) and two volumes of absolute ethanol followed by an incubation at -20°C for 20-30 min. The precipitated DNA was pelleted by centrifugation for 10 min at maximum rpm in a bench top centrifuge, the supernatant was aspirated and the pellet washed briefly in 100 µl of 70% ethanol before being air dried for several hours. The DNA pellet was then resuspended in DEPC dH₂O and stored overnight at 4°C. A 1-2 µl aliquot of digested template was checked for linearisation by gel electrophoresis on a 0.8% 1 X TBE agarose gel containing ethidium bromide. The concentration of the extracted template was determined on the same gel by comparison with a λ phage DNA concentration standard.

Preparation of cRNA probe
Approximately 1 µg of linearised template was utilised for the generation of DIG-labelled riboprobes according to the manufacturers specifications (Boehringer Mannheim). In vitro transcription of cRNA was achieved utilising a final concentration of 1 µg linearised template, 1 X transcription buffer (Boehringer Mannheim or Promega), 1 X DIG RNA labelling mix (Boehringer Mannheim), 40 U of T7 or SP6 RNA polymerase (Boehringer Mannheim or Promega) and made to a final volume of 20 µl with RNase free dH₂O. The reaction mix was pulse spun and incubated at 37°C for 2 hours. Residual DNA template was removed by treatment with 10 U of RNase free DNase 1 (Promega) by incubation for 15 min at 37°C. The reaction was then stopped by the addition of 2 µl of 0.2M EDTA solution and cRNA was precipitated with 2.5 µl of 4M LiCl and 75 µl of ice cold absolute ethanol and incubated at -20°C for 2 hours. Precipitate was pelleted by centrifugation at maximum rpm in a benchtop microcentrifuge for 10 min and the supernatant removed. The pellet was washed briefly with 50 µl 70% ethanol, allowed to air dry and was resuspended in 20-25 µl of DEPC dH₂O. Concentration and integrity of the cRNA, was determined by 260/280 absorbance spectrometry and analysis of a 2 µl aliquot on a 2.0% 1 X TBE agarose gel with an appropriate size standard.
Both sense and antisense cRNA transcripts were prepared and in the case of FGF-5 cRNA the approximately 450bp product was hydrolysed under alkali conditions to generate a 200bp DIG riboprobe. The incubation time to achieve a cRNA of desired length (150-200bp) for in situ hybridisation was calculated using the following formula (Cox et al., 1984):

\[ T = \frac{(L_o - L_d)}{(0.11 \times L_o \times L_d)} \]

where:
- \( L_o \) = original length of cRNA transcript
- \( L_d \) = desired length of cRNA transcript

Hydrolysis was carried out by the addition of 100μl of hydrolysis buffer (80mM NaHCO₃, 120mM Na₂CO₃, 20mM β-mercaptoethanol, pH 10.2) to 100μl of cRNA solution and incubating at 60°C for the calculated length of time and the reaction stopped by the addition of 200μl of stop buffer (0.2M sodium acetate pH 6.0, 0.1% glacial acetic acid, 10mM DTT). Hydrolysed cRNA was precipitated by the addition of 1μl of yeast tRNA (10mg/ml) 40μl of 3M sodium acetate pH 7.0 and two volumes of absolute ethanol. Incubation was at -70°C for several hours. cRNA was pelleted by centrifugation at maximum rpm in a bench top centrifuge and the supernatant removed. The pellet was air dried briefly and resuspended in 20 μl of DEPC dH₂O. Hydrolysis of an approximate 200bp riboprobe was confirmed by running an aliquot of the reaction on a 2% 1 X TBE agarose gel under RNase free conditions with an appropriate size standard. The concentration of cRNA after in vitro transcription was determined by 260/280 absorbance spectrophotometry and appropriate dilutions were made in hybridisation solution to give a final cRNA concentration between 1-5ng/μl for in situ hybridisation.

**FGF-5 and TGF-α in situ hybridisations**

Skin samples from Merino sheep treated with a deflecceed dose of EGF were fixed for histology as described. Skin sections were analysed separately with two cRNA transcripts, one containing a portion of the mouse FGF-5 exon 3 coding sequence while the other contained a portion of the N-terminus of the sheep TGF-α cDNA encoding N-terminal amino acids of the TGF-α peptide (kindly supplied by Dr G.Cam, CSIRO DAP). Sheep TGF-α DNA template, which consisted of a 155bp fragment encompassing the 5' portion of the sheep TGF-α cDNA had been subcloned into pCRII (Supplied by Dr G.Cam, CSIRO DAP) and was linearised at the NotI and HindIII sites respectively. The orientation of the cDNA sequence had been determined previously by DNA sequencing. An initial attempt at detecting the transcript for TGF-α in the epidermis at a cRNA concentration of 1ng/μl hybridisation solution revealed low levels of TGF-α transcript that were difficult to detect with a DIG-labelled antisense
probe. However, raising the cRNA concentration to 5ng/µl in hybridisation solution in a subsequent experiment enabled TGF-α expression to be detected in EGF treated Merino epidermis. Skin sections used were from a time series encompassing a 0 min, 4 hours, 12 hours, 24 hours, 52 hours and 8 days post EGF treatment. Skin sections were fixed in Histochoice and mounted for \textit{in situ} hybridisation as described. Control sections probed with the sense strand of the sheep TGF-α probe were used to determine background due to non-specific RNA hybridisation.

The mouse FGF-5 exon 3 region clone was used to generate sense and antisense transcripts which were hydrolysed to produce fragments of approximately 200bp for use as \textit{in situ} hybridisation probes (see above). These were used at a concentration of 1 ng/µl hybridisation solution. A similar series of skin samples was used as for the detection of \textit{Tgfa} mRNA, although the time frames and method of fixation were different. As the experimental aim in this study was to determine if \textit{Fgf5} mRNA was being induced in an immediate-early response manner, the analysed time frames were limited to 0 min, 30 min, 3 hour and 6 hour periods after EGF administration. The skin sections used for this experiment were kindly supplied by Dr D.L. Adelson CSIRO DAP and had been fixed in 4% paraformaldehyde.

\textit{In situ} Hybridisations

The digoxigenin \textit{in situ} hybridisation process was undertaken over a three-day period essentially following the protocol described by Tohyama \textit{et al.} (1994). Embedded tissue was sectioned at 6µM thickness and mounted on poly-L-lysine coated slides. On the first day, paraffin was removed from embedded skin sections by washing 2 X in xylol for 10 min followed by serial rehydrations in ethanol (2 X 100%, 1 X 90% and 1 X 70%) for 3 min intervals. Slides were immersed in 0.2M HCl for 20 min at room temperature and briefly washed in 1 X PBS for 5 min. Proteinase K treatment of slides was carried out for 15 min at 37°C using 1-5µg/ml in 1 X PBS. This was followed by a 5 min wash in 1 X PBS. Slides were then immersed in 1 X PBS containing 2mg/ml glycine for two 10 min intervals followed by acetylation for 15 min at room temperature in 0.1M triethanolamine with 1.5ml of fresh acetic anhydride added per 500ml 0.1M triethanolamine. Acetylated slides were washed twice in 2 X SSC for 5 min intervals. Prehybridisation of slides was carried out using hybridisation buffer without probe. Slides were wiped to remove excess wash solution and 100-200µl of hybridisation buffer was added to cover tissue sections and placed in a humidity chamber for approximately 1-2 hours at 50°C. Prehybridisation solution was drained from slides by placing them on an angle and excess solution was removed by wiping around the slide with a lint free Kimwipe tissue. An identical hybridisation solution was then prepared containing DIG-labelled riboprobe at a final concentration of 1-5ng/µl hybridisation solution. Hybridisation solution was then applied to each slide in 150µl aliquots,
covered with paraffin strips (Nascofilm) and incubated in a humidity chamber overnight at 50°C.

On the second day hybridised slides were rinsed in 2 X SSC, 50% formamide solution to wash off paraffin strips followed by a more stringent wash in fresh solution at 50°C for 15 min. A 5 min room temperature wash in Buffer A was then followed by RNase A treatment to digest single strand RNA. A pre-warmed 50µg/ml RNase solution in Buffer A was prepared and slides were digested for 30 min at 37°C. This was followed by a brief wash in Buffer A without RNase A. Further washes were carried out in pre-warmed Solution C (1 X SSC, 50% formamide) for 10 min at 50°C followed by a 15 min wash at room temperature in fresh Solution C. A 5 min wash in Buffer B at room temperature was then followed by treatment with a blocking agent. Aliquots of a solution containing 0.5% (w/v) blocking agent were then added to each slide (approximately 200µl per slide) which were then incubated at room temperature for 30 min at room temperature in a humidity chamber. Slides were then immersed briefly in Buffer B followed by addition of 200µl of a solution containing 5% sheep serum, 1% BSA and 1X PBS. Slides were incubated in a humidity chamber for 45 min after which they were drained and wiped with a lint free tissue. Slides were then incubated with a 1/500 dilution of antibody conjugate (Boehringer Mannheim) in Buffer B with approximately 200µl aliquots added per slide for 1 hour at room temperature in a humidity chamber. Slides were then immersed twice in Buffer B for 10 min, each time followed by an equilibration in Buffer C for 5 minutes at room temperature. Development of the colour reaction was generally carried out overnight in a light-tight humidity chamber with 300µl aliquots of colour solution added per slide. The humidity chamber was equilibrated with Buffer C.

On day three, development of the colour reaction was observed and slides were removed from the humidity chamber and the colour reaction stopped by immersion of slides in Buffer D. Slides were dehydrated by sequential immersion in 1 X 70%, 1 X 90% and 2 X 100% ethanol for 3 min intervals followed by three immersions in xylene for 5 min periods. Slides were removed and the edges wiped with lint-free Kimwipe tissue before coverslipping with DPX mounting media. Coverslipped slides were left for several hours to dry before analysis and photography under the light microscope.

Solutions for In situ hybridisation:
All glassware was baked at 180°C overnight prior to use. All solutions were made with DEPC (0.1% v/v) treated dH₂O. Demineralised dH₂O received an aliquot of DEPC in the laminar fume hood and was then shaken vigorously to mix before being placed at 37°C overnight. Incubated DEPC dH₂O was then placed in a baked bottle and autoclaved before use.
0.1M Triethanolamine
500mls - 4.5g NaCl and 7.5mls Triethanolamine and DEPC dH₂O added to 500mls. Solution was filtered through 0.2μM filters and stored at room temperature. Fresh acetic anhydride was added (1.25mls) prior to use in the acetylation reaction.

Hybridisation Buffer
50% deionised formamide, 4 X SSC, 500μg/ml yeast tRNA, 1 X Denhardt’s solution and 100mg/ml Dextran sulphate, DEPC H₂O Stored at -20°C.

10 X PBS
130mM NaCl
7mM Na₂HPO₄, 12H₂O
3mM NaH₂PO₄, 2H₂O

Buffer A
10mM Tris-HCl pH 7.5
0.5M NaCl
1mM EDTA pH 8.0

Buffer B
100mM Tris-HCl pH 7.5
150mM NaCl

Buffer C
0.1M Tris-HCl pH 9.5
0.1M NaCl
50mM MgCl₂

Buffer D
0.1M Tris-HCl pH 8.0
1mM EDTA pH 8.0

Haematoxylin and Eosin staining
Mid-dorsal skin sections fixed in Histochoice and serially dehydrated were dissected from mice collected during the course of these experiments to determine epithelial morphology. Skin was longitudinally mounted in paraffin blocks and sectioned at 8μ thickness, mounted on albumin coated slides and incubated overnight at 37°C. Skin sections were deparaffinised in xylol for 2 X 5 min periods and rehydrated in a sequential series of ethanol washes. A 5 min immersion in 90% ethanol was followed by three brief immersions in 5% colloidion in equal parts absolute ethanol and ether, a 5
min immersion in 70% ethanol and a 5 min immersion 50% ethanol. Treatment of skin sections with 5% colloidion reduces section lifting and fibre loss. Slides were then rinsed in water and placed in Mayers haematoxylin for 30 min, rinsed again in water and allowed to drain briefly. This was followed by three brief immersions in 1% acid alcohol to decolourise the sections and rinsed again in running tap water. Sections were blued in alkaline solution (tap water, pH8) for 1 min and rinsed in water. This was followed by immersion in acidified eosin phloxine solution for 30 sec and a sequential dehydration by briefly dipping slides in 70%, 90%, 95% and three absolute ethanol washes. Sections were cleared in xylol for 5 min and then a glass coverslip with DPX mountant was overlaid and gentle pressure applied to remove air bubbles. Slides were then allowed to harden overnight before being photographed under a light microscope.
Chapter Three: No evidence for epistatic interaction between Tgfαwa-1 and Egfrwa-2 for viability

Introduction

The widespread expression of the EGFR and its associated ligands in a number of tissues during embryogenesis and at maturity is suggestive of a reiterated role for this RPTK subfamily during mammalian development. Sites of expression have been found at the earliest stages of embryogenesis in the unfertilised oocyte, during the peri-implantation period (Cross et al., 1994), as well as postnatally in parts of the brain, intestinal mucosa, mammary gland and the skin (Luetke et al., 1993). This diverse expression in vivo suggests that, due to the potentially pleiotropic nature of this RPTK subfamily, a range of phenotypic effects could result when normal expression patterns are genetically disrupted.

The ligands of the EGFR subfamily and the EGFR have been widely studied both in vitro and in vivo due to their potential role in the development of a transformed cellular phenotype and the subsequent progression toward cancerous growth in a variety of tissues, notably in those of epithelial origin. With the identification of both TGF-α and the EGFR as the molecules disrupted in the waved-1 and waved-2 mutations respectively, a study of the physiological effects of multiple defects in this conserved intercellular signalling pathway is possible. I have taken advantage of this information to examine a potential genetic relationship between a peptide growth factor and its receptor by generating mice with combinations of the Tgfαwa-1 and Egfrwa-2 mutations and examining their phenotypic effect on epidermal development.

The Tgfαwa-1 mutation

The original waved-1 mouse mutant, initially discovered as a spontaneous mutation (Crew, 1933), has been shown to be allelic with null mutations generated by the targeted disruption of the Tgfα locus (Luetke et al., 1993; Mann et al., 1993). The waved-1 mutation results in a viable animal although the coat morphology and vibrissae exhibit a distinctive waved appearance most easily discernible after the development of the first hair coat. Premature eyelid opening and tooth eruption as well as defects in the morphology of ear development have also been reported (Wright et al., 1996). Although the molecular basis of the original waved-1 mutation is unknown, several aberrantly sized Tgfα mRNA transcripts have been detected in vitro in keratinocytes from the original waved-1 line (Dlugosz et al., 1995). In addition to the normal 4.5 kb transcript, two other transcripts of 4.7kb and 5.2kb have been detected, suggestive of a possible impairment in the normal processing of Tgfα mRNA. Expression levels of TGF-α transcript in the original waved-1 mutation were also found to be reduced by 90% in some tissues (Luetke et al., 1993). This decrease in Tgfα
mRNA has recently been confirmed, although the mutation responsible has still not been identified (Berkowitz et al., 1996).

Mice which contain a transgenically targeted disruption of the Tgfα locus express an identical hair phenotype to that seen in the waved-1 mutant although some subtle differences, possibly dependent on the site of disruption of the wild-type gene, occur in other organs. The targeting of a neomycin expression cassette into exon 3 of the Tgfα locus, which encodes a portion of the extracellular region containing part of the conserved cysteine loop structural motif involved in receptor binding, results in transgenic mice exhibiting the waved-hair phenotype, premature eyelid opening and tooth eruption when homozygous for the null allele (Mann et al., 1993). A similar disruption, but targeting exon 4 which encodes a region containing a portion of the conserved cysteine loop motif and the transmembrane region of the precursor form, expresses a waved phenotype when homozygous but also exhibits premature eyelid opening when heterozygous for the null allele (Luetteke et al., 1993).

Whether a partially functional TGF-α protein is still produced in either strain of knock-out mouse is unclear. An aberrantly sized transcript is produced in both Tgfα<sup>−/−</sup> transgenic strains but neither seems to produce substantial amounts of translated product. Both transgenic mutations have been shown to be allelic with the original waved-1 mutation as determined by complementation analysis. The effect of these mutations on the normal cellular function of TGF-α remains unknown, although it has been suggested that the normal growth of the developing and mature hair follicle may be disrupted. This may include the orientation of follicle primordia during organogenesis and the regulation of growth in the mature follicle possibly via an interaction with the EGFR localised in the ORS (Luetteke et al., 1993).

The Egfr<sup>−/−</sup> mutation
The discovery that mutations of the TGF-α locus generate a waved-hair phenotype led to an analysis of other candidate loci for waved-hair mutations. Such an approach has revealed the molecular basis of the waved-2 mutation (Egfr<sup>−/−</sup>) whereby an analysis of the EGFR coding sequence from this mutation has shown that a point mutation in the tyrosine kinase domain of the receptor is responsible (Luetteke et al., 1994). The functional effect of the Egfr<sup>−/−</sup> mutation, which results in an amino acid substitution of a conserved valine (residue 743) for a glycine residue in subdomain III of the intracellular kinase domain (Hanks et al., 1988), is not well understood. This amino acid substitution changes a conserved hydrophobic region to a more hydrophilic state and it has been suggested that the binding and presentation of ATP to the kinase domain of the receptor may be affected, although other aspects of receptor function may also be impaired (Luetteke et al., 1994).
The mutated receptor shows a marked reduction in EGF stimulated tyrosine kinase activity in vitro when liver membrane preparations from mutant and wild-type mice are compared. A further in vitro study of transfected CHO cells, which do not produce endogenous EGFR, revealed that when the Egfr<sup>−/−</sup> single nucleotide mutation was introduced into a wild-type rat EGFR, a similar decrease in kinase activity was observed. The transfected mutant EGFR was able to be partially stimulated in a ligand dependent manner although at levels lower than that seen for the wild-type EGFR. A study of the in vivo effects of the mouse Egfr<sup>−/−</sup> mutation revealed a reduction in EGFR tyrosine phosphorylation observable after minimal EGF administration in dorsal skin samples from Egfr<sup>−/−</sup> homozygotes and heterozygous controls. This decrease in EGFR phosphorylation is not as pronounced as that noted in vitro. No impairment of transcriptional or post-translational processing was apparent as the expression levels of Egfr<sup>−/−</sup> mRNA and protein were found to be of similar levels in both mutant and control animals (Luetetteke <i>et al.</i>, 1994).

An analysis of EGF-binding in fibroblasts isolated from Egfr<sup>−/−</sup> and wild-type mice has also revealed that the Egfr<sup>−/−</sup> mutation results in the loss of high affinity binding sites for EGF when compared with wild-type fibroblast cultures which exhibit both high and low affinity EGF binding sites. Similar results were also observed in NIH 3T3 fibroblast cells lacking an endogenous EGFR and transfected with a human EGFR engineered to contain the Egfr<sup>−/−</sup> mutation (Fowler <i>et al.</i>, 1995). Transfected cells, which express large numbers of the mutated receptor, are mitogenically responsive only when treated with high levels of EGF. These cell lines also lack the high affinity EGF binding sites.

Both observations contrast with the original study done in CHO cells expressing wild-type EGFR and Egfr<sup>−/−</sup>, where no difference in EGF binding affinity was reported (Luetetteke <i>et al.</i>, 1994), suggesting that cell type differences in response to ligand stimulation of the mutated EGFR may be occurring. Fowler <i>et al.</i> (1995) have raised the possibility that other protein kinases may be involved in the modulation of EGFR activity notably, erbB2. Transphosphorylation of the EGFR by erbB2 has been demonstrated (Spivak-Kroizman <i>et al.</i>, 1992), while a kinase negative erbB2 has also been shown to exert a dominant-negative effect on EGFR signalling (Qian <i>et al.</i>, 1994). The presence of varying affinity EGF binding receptors has also been observed in vitro with cell lines expressing both EGFR and erb-B2 (Wada <i>et al.</i>, 1990). Interestingly, the erbB2 gene has been mapped to mouse chromosome 11 in the vicinity of another waved-hair mouse mutation, the semi dominant <i>Rα</i> (Rex) locus (Losi <i>et al.</i>, 1994) and may be a candidate gene for this mutation. These data raise the possibility that the normal association of erbB2 with the EGFR is altered by the Egfr<sup>−/−</sup> mutation.
Subdomain III of the tyrosine kinase catalytic domain is highly conserved between many receptor and nonreceptor tyrosine kinases including other members of the EGFR subfamily (Luetteke et al., 1994). The involvement of this tyrosine kinase domain in mediating the intracellular interactions involved in receptor homo- and heterodimerisation has been suggested with the existence of a conserved 'dimerisation motif' (Chantry, 1995). Furthermore a potential role for the kinase domain in mediating homo- and heterodimerisation of the EGFR subfamily with the possibility of tetrameric higher order agglomerations occurring has also been suggested (Murali et al., 1996).

One known aspect of signal transduction that is impaired by the Egfr<sup>neu</sup> mutation is the phosphorylation of the PKCδ isoform, a member of the Protein Kinase C family of serine/threonine kinases. In contrast to the phospholipid activation of several other PKC isoforms, PKCδ is indirectly phosphorylated when the EGFR is activated possibly via members of the src family of intracellular tyrosine kinases (Denning et al., 1996). The resulting phosphorylation of PKCδ deactivates this kinase, inhibiting the progression of keratinocytes to a terminally differentiated state. It seems likely that EGFR activation and the subsequent tyrosine phosphorylation of PKCδ from its active to its inactive state decreases the inhibitory effect on cell proliferation of active PKCδ.

An examination of the PKC signalling pathway in keratinocytes isolated from the Egfr<sup>neu</sup> mutation has revealed that the impaired EGFR is unable to phosphorylate PKCδ in response to TGF-α stimulation. This usually occurs within five minutes of EGFR activation in wild-type keratinocytes <i>in vitro</i> (Denning et al., 1996; Yuspa et al., 1998). The relationship between EGFR activation and the deactivation of PKCδ by tyrosine phosphorylation is not direct, and may involve an interaction with other kinases such as c-src and c-fyn. The resulting phosphorylation of PKCδ deactivates this kinase, thus inhibiting the progression of keratinocytes to a terminally differentiated state. PKCδ, as well as other PKC isoforms, have also been shown to exhibit a distinct distribution in the hair follicle with PKCδ present in cells of the dermal papilla and ORS keratinocytes (Hoffmann et al., 1996). It is also possible that impaired signalling via the PKC pathway may also involve erbB3, a third member of the EGFR subfamily able to heterodimerise with the activated EGFR and known to influence membrane phospholipid turnover by activation of Phosphatidylinositol-3-Kinase and the subsequent activation of PKC signalling pathways (Soltt et al., 1994).

The Egfr<sup>neu</sup> mutation, with its change of subdomain III to a more hydrophilic state, may affect the normal intracellular interactions of the EGFR with a number of other RTPK's and intracellular kinases. It is interesting to note that the Valine 743 residue which is mutated in Egfr<sup>neu</sup> is adjacent to a Tyrosine 742 residue. Although this tyrosine residue has not been reported to be autophosphorylated during EGFR activation, the mutation of an amino acid immediately adjacent to it, resulting in a change from a hydrophobic to
a hydrophilic state, may affect the specificity of binding with intracellular SH-2 domain containing proteins. A region of three amino acids located C-terminal of other known autophosphorylated tyrosine residues is important in determining this specificity of interaction (Cantley and Songyang, 1994; Marengere and Pawson, 1994). Whether Tyrosine 742 is a cryptic tyrosine autophosphorylation site with an as yet undefined role is unknown, but it must be viewed as unlikely as this tyrosine residue has not been conserved in other members of the RPTK family (Van der Geer and Hunter, 1994).

Another possible clue to the mechanism perturbed in Egfr<sup>ww2</sup> mice is the fact that the resulting amino acid substitution occurring in the Egfr<sup>ww2</sup> mutation lies three amino acid residues C-terminal of a highly conserved glutamic acid at residue 740, the first amino acid of exon 20 of the EGFR (Callaghan et al., 1993; Van der Geer and Hunter, 1994). The functional significance of this highly conserved glutamic acid residue and any possible effect that the Egfr<sup>ww2</sup> mutation may have is unknown. However the conservation of either a hydrophobic or neutral amino acid at this position is evident among other members of this RPTK subfamily implying that this domain has functional significance (Van der Geer and Hunter, 1994). Although the signalling functions disrupted by the recessive Egfr<sup>ww2</sup> mutation are poorly understood it is likely that the resulting partially functional receptor activates only a subset of the signalling molecules normally associated with the EGFR.

**Localisations of TGF-α and EGFR in the mouse epidermis**

TGF-α is expressed in a concentric layer of cells surrounding the mouse vibrissae hair shaft. A similar pattern is seen in the pelage hair follicles where TGF-α mRNA is expressed in the zone of keratinisation above the proliferative cells of the follicle bulb in the IRS and possibly in regions of the ORS (Luetteke et al., 1993). The target for TGF-α, the EGFR, is expressed in the ORS and the bulb of the anagen hair follicle (Luetteke et al., 1994). These localisations are consistent with a potential functional relationship between a ligand and its receptor in the IRS and ORS regions respectively. This potential juxtacrine relationship may be involved in the differentiation of the mature fibre as well as maintaining the proliferative activity of cells in the follicle bulb.

**A potential genetic interaction between Tgfa<sup>ww-4</sup> and Egfr<sup>ww2</sup>**

A genetic analysis of a possible interaction between several waved-hair mutations has been previously reported. This study was undertaken prior to the advent of molecular methods for defining the genotype of the mutations involved and relied instead on progeny testing to determine an individual's genetic identity (Carter, 1951). Mice with mutations in waved-1 (Tgfa<sup>ww-4</sup>), waved-2 (Egfr<sup>ww2</sup>) and the Re (Rex) loci were backcrossed to generate progeny carrying various allelic combinations of these mutations. The main aim of that study was to examine a potential genetic interaction.
between Re and either Tgfa<sup>−/−</sup> or Egfr<sup>−/−</sup> and no report of a phenotype for animals doubly homozygous for Tgfa<sup>−/−</sup> and Egfr<sup>−/−</sup> was presented. Additive gene action was noted between several of the genotypes produced-with subtle variations in the waved-hair phenotype observed.

**Aims of this Study**

To genetically investigate a functional relationship between one of the known EGFR ligands of the epidermis, TGF-α, and the EGFR. To achieve this, I have utilised the Tgfa<sup>−/−</sup> transgenic null mutation (Mann et al., 1993) and the partially functional EGFR mutation, Egfr<sup>−/−</sup> (Luetteke et al., 1994). Both mutations result in phenotypically indistinguishable waved-hair phenotypes. By generating offspring with all possible genotypic combinations of alleles at these loci and examining the epidermal phenotypes of progeny homozygous for both recessive, unlinked mutations, an understanding of their combined role in epidermal development may be possible. The in vivo result of a cumulative impairment of this conserved ligand-receptor signal transduction pathway will be examined genetically by the use of specific DNA polymorphisms associated with these mutations to track the inheritance of recessive alleles.
Results

Generation of double homozygotes

The genotype of the Tgfα locus was determined utilising a multiplex PCR assay based on the primer pairs designed by Mann et al. (1993) and optimised to detect both wild-type and Tgfα<sup>−/−</sup> alleles with a single PCR reaction (Chapter 2). Detection of the wild-type and Egfr<sup>−/−</sup> alleles required the development and optimisation of a novel PCR assay which detected the Egfr<sup>−/−</sup> point mutation by restriction endonuclease cleavage of a FokI restriction site introduced as a result of this mutation (Chapter 2). Genotyping notation used in this study is as described by Sundberg and King (1996). The wild-type allele segregating at each locus is indicated by '+' while the mutant allele at each locus (either the Tgfα<sup>−/−</sup> null allele or the partially functional Egfr<sup>−/−</sup> mutation) is indicated by '-'.

The mating structure used to generate the various genotypic combinations at these two loci is presented in Figure 3.1. It is important to note that the founding male animal expressing the vestigial tail phenotype (and therefore homozygous at the vt locus) was used. All animals used to initiate this breeding experiment expressed the appropriate waved-hair phenotype (Figure 3.2). The F₁ breeding pairs used to generate the F₂ offspring were all assayed to confirm the doubly heterozygous [Tgfα<sup>−/+</sup>; Egfr<sup>−/+</sup>] genotype (Figure 3.3). All F₁ animals carried the expected allelic combination and expressed the wild-type hair phenotype (Figure 3.2). Only offspring collected for histology (those expressing an extreme phenotype) from the initial F₂ series examined were genotyped while the second series of 158 F₂ progeny were all assayed to determine the frequency of the various genotypes segregating. The observed genotype frequencies in these 158 animals and a comparison with expected values are presented in Table 3.1. A mortality rate of 5.7% due to parental cannibalism occurred during the collection of the second F₂ series.

F₂ progeny identified as double homozygotes were repeated genotyped to confirm their identity. All of these animals reproduced the same genotype as first detected. Progeny expressing the waved-hair phenotype were all found to be homozygous for either the Tgfα<sup>−/−</sup> or Egfr<sup>−/−</sup> mutations. This internal control was a useful indication that the genotyping assays used in this study were accurate in identifying an individual's genotype. Of the second series of 158 F₂ progeny genotyped, only four double homozygote [Tgfα<sup>−/−</sup>; Egfr<sup>−/−</sup>] genotypes were uncovered (Table 3.1). Genotyping of histology samples collected from the initial F₂ series examined revealed a further two more double homozygotes, one of which expressed the vestigial tail phenotype. In total, six double homozygotes were recovered, with five expressing the vestigial tail phenotype.
Table 3.1. Observed and expected numbers of the genotypic combinations for the Tgf-α and Egfr loci assuming unlinked independent mendelian segregation. The number of animals expressing the vestigial tail phenotype in each genotype are also shown.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Number of Mice</th>
<th>Hair Phenotype</th>
<th>Expression of vestigial tail phenotype</th>
<th>Contribution to Chi-square value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tgfa&lt;sup&gt;na&lt;/sup&gt;-/+; Egfr&lt;sup&gt;ne&lt;/sup&gt;-2 +/-</td>
<td>12 (9.875)</td>
<td>wild-type</td>
<td>1</td>
<td>0.457</td>
</tr>
<tr>
<td>Tgfa&lt;sup&gt;na&lt;/sup&gt;-/+; Egfr&lt;sup&gt;ne&lt;/sup&gt;-2 +/-</td>
<td>18 (19.75)</td>
<td>wild-type</td>
<td>1</td>
<td>0.155</td>
</tr>
<tr>
<td>Tgfa&lt;sup&gt;na&lt;/sup&gt;-/-; Egfr&lt;sup&gt;ne&lt;/sup&gt;-2 +/-</td>
<td>14 (9.875)</td>
<td>waved</td>
<td>2</td>
<td>1.723</td>
</tr>
<tr>
<td>Tgfa&lt;sup&gt;na&lt;/sup&gt;-/+; Egfr&lt;sup&gt;ne&lt;/sup&gt;-2 +/-</td>
<td>18 (19.75)</td>
<td>wild-type</td>
<td>2</td>
<td>0.029</td>
</tr>
<tr>
<td>Tgfa&lt;sup&gt;na&lt;/sup&gt;-/+; Egfr&lt;sup&gt;ne&lt;/sup&gt;-2 +/-</td>
<td>46 (39.5)</td>
<td>wild-type</td>
<td>14</td>
<td>2.285</td>
</tr>
<tr>
<td>Tgfa&lt;sup&gt;na&lt;/sup&gt;-/-; Egfr&lt;sup&gt;ne&lt;/sup&gt;-2 +/-</td>
<td>19 (19.75)</td>
<td>waved</td>
<td>3</td>
<td>0.029</td>
</tr>
<tr>
<td>Tgfa&lt;sup&gt;na&lt;/sup&gt;-/+; Egfr&lt;sup&gt;ne&lt;/sup&gt;-2 -/-</td>
<td>12 (9.875)</td>
<td>waved</td>
<td>2</td>
<td>0.457</td>
</tr>
<tr>
<td>Tgfa&lt;sup&gt;na&lt;/sup&gt;-/+; Egfr&lt;sup&gt;ne&lt;/sup&gt;-2 -/-</td>
<td>11 (19.75)</td>
<td>waved</td>
<td>7</td>
<td>3.878</td>
</tr>
<tr>
<td>Tgfa&lt;sup&gt;na&lt;/sup&gt;-/-; Egfr&lt;sup&gt;ne&lt;/sup&gt;-2 -/-</td>
<td>4 (9.875)</td>
<td>waved</td>
<td>4</td>
<td>3.495</td>
</tr>
<tr>
<td>Combined χ² value</td>
<td></td>
<td></td>
<td></td>
<td>12.507</td>
</tr>
</tbody>
</table>

The linkage of the vestigial tail locus and the Egfr locus on mouse chromosome 11 would tend to generate genotypes homozygous for both Egfr<sup>ne</sup>-2 and vestigial tail. Due to the fact that a founder Egfr<sup>ne</sup>-2 male expressing the vestigial tail phenotype was used, the F<sub>1</sub> breeding pairs can be assumed to be heterozygous at both the Egfr<sup>ne</sup>-2 and vt loci. As the Egfr<sup>ne</sup>-2 and vt loci are separated by a distance of 26cM (Greco et al., 1998) the expected frequency of individuals homozygous for both Egfr<sup>ne</sup>-2 and vestigial tail can be determined and compared with the observed frequencies. The expected frequency of an F<sub>2</sub> homozygous Egfr<sup>ne</sup>-2 genotype that is also homozygous for vt is approximately 13.7% (ie the probability of an F<sub>1</sub> parental gamete that has not undergone recombination is 0.37 if a 26cM map distance is assumed between Egfr<sup>ne</sup>-2 and vt. The probability of progeny homozygous for both Egfr<sup>ne</sup>-2 and vt is therefore 0.37² or 0.1369). From Table 3.1, 27 individuals homozygous for Egfr<sup>ne</sup>-2 were uncovered in the 158 animals genotyped. Of these, 13 (46%) expressed the vt phenotype, with the remaining 14 exhibiting a wild-type tail phenotype. By examining the vestigial tail expression in individuals that are also homozygous for the Egfr<sup>ne</sup>-2 mutation it becomes apparent that the frequency of vt expression increases in two of these genotypes. The [Tgfa<sup>na</sup>-/+; Egfr<sup>ne</sup>-2 -/-] genotype has approximately 16% (2/12) expressing the vt phenotype, a value that is in agreement with the expected
occurrence of the doubly homozygous vt and Egfr<sup>ms2</sup> genotype. However, both the \([Tgfα<sup>αα<sup>-</sup></sup>^+/+; Egfr<sup>ms2-/-</sup>]\) and \([Tgfα<sup>αα<sup>-</sup></sup>-/-; Egfr<sup>ms2-/-</sup>]\) genotypes have a larger proportion of individuals expressing the vt phenotype has occurred (63% and 100% respectively).

**Statistical test of significance**

A comparison of the numbers of genotypes observed during this experiment with those expected assuming the independent mendelian segregation reveals a deficiency in the number of animals homozygous for both mutations. To determine whether this divergence is statistically significant an analysis of the genotype frequencies can be made by conducting two single locus Chi-square analysis (df=2), a contingency (homogeneity) Chi-square analysis testing the independence of genotypes at the two loci (df=4), and finally a two locus Mendelian segregation Chi-square analysis (df=8). The objective of using a variety of Chi-square analyses is to determine the individual effects of each mutation and their potential interaction with each other. All three analyses are presented in Table 3.2.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Tgf-α&lt;sup&gt;αα&lt;sup&gt;-&lt;/sup&gt;&lt;/sup&gt; +/+</th>
<th>Tgf-α&lt;sup&gt;αα&lt;sup&gt;-&lt;/sup&gt;&lt;/sup&gt; +/-</th>
<th>Tgf-α&lt;sup&gt;αα&lt;sup&gt;-&lt;/sup&gt;&lt;/sup&gt; -/-</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Egfr&lt;sup&gt;ms2&lt;/sup&gt; +/+</td>
<td>12</td>
<td>18</td>
<td>14</td>
<td>44</td>
</tr>
<tr>
<td></td>
<td>11.97</td>
<td>21.72</td>
<td>10.30</td>
<td>39.5</td>
</tr>
<tr>
<td></td>
<td>9.87</td>
<td>19.75</td>
<td>9.87</td>
<td></td>
</tr>
<tr>
<td>Egfr&lt;sup&gt;ms2&lt;/sup&gt; +/-</td>
<td>19</td>
<td>49</td>
<td>19</td>
<td>87</td>
</tr>
<tr>
<td></td>
<td>23.68</td>
<td>42.95</td>
<td>20.37</td>
<td>79</td>
</tr>
<tr>
<td></td>
<td>19.75</td>
<td>39.5</td>
<td>19.76</td>
<td></td>
</tr>
<tr>
<td>Egfr&lt;sup&gt;ms2&lt;/sup&gt; -/-</td>
<td>12</td>
<td>11</td>
<td>4</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td>7.35</td>
<td>13.33</td>
<td>6.32</td>
<td>39.5</td>
</tr>
<tr>
<td></td>
<td>9.87</td>
<td>19.75</td>
<td>9.87</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>43</td>
<td>78</td>
<td>37</td>
<td>158</td>
</tr>
<tr>
<td></td>
<td>39.5</td>
<td>79</td>
<td>39.5</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.2. Chi-square analysis for genotype frequency. Observed values (see table 3.1) are normal font in the body of the table. Single locus segregation test for 1:2:1 ratio at the \(Tgfα-α^f\) locus (expected values are in bold on the right hand side of the table; \(χ^2 =0.481, p=0.7862, df=2\)). Single locus segregation test for 1:2:1 ratio at the \(Egfrα^ms\) locus (expected values are in bold at the bottom of the table; \(χ^2 =5.278, p=0.0714, df=2\)). The homogeneity (contingency) analysis (expected values are in italics in the body of the table; \(χ^2 =8.038, p=0.0902, df=4\)). Two locus independent Mendelian segregation analysis (expected values are in bold italics in the body of the table; \(χ^2 =12.507, p=0.1299, df=8\)).

If the data for each locus are considered separately there is no statistically significant departure from the observed and expected frequencies for the \(Tgfα-α^f\) genotype.
(χ²=0.481, p=0.7862, df=2). For the Egfr<sup>as-2</sup> the χ² value, predominately due to deficiency of the recessive homozygous class approaches significant at the 5% probability level (χ²=5.278, p=0.0714, df=2). This single locus analysis suggests that homozygosity for the Egfr<sup>as-2</sup> mutation may result in a reduction of the viability of the recessive homozygotes but the effect is not statistically significant using the standard 5% significance threshold.

By analysing the multilocus genotype frequencies using either a contingency Chi-square or two locus independent Mendelian segregation Chi-square analyses, it is possible to test for interactions between the genotypes or alleles of these two loci. Both analyses revealed that there was no statistically significant departure of genotype frequencies observed from those expected (Table 3.2). Inspection of the two locus Chi-square values reveal that the deficiency of homozygous Egfr<sup>as-2</sup> genotypes was most severe in combination with the Tgfa<sup>as-1</sup> +/+ and Tgfa<sup>as-1</sup> -/+ genotypes.

**Phenotypes of Tgfa<sup>as-1</sup> and Egfr<sup>as-2</sup> double homozygotes**

Of the six double homozygous animals uncovered during the course of this experiment, four had been collected for histological analysis. Mid-dorsal skin was dissected from these mice, and from litter mates that were representative of the wild-type and waved-hair phenotypes. Dorsal skin samples were paraffin embedded, sectioned and H and E stained according to the protocol outlined in Chapter 2. Samples were examined and photographed under a Zeiss light microscope. The range of epidermal phenotypes presented by each of these double homozygotes are shown in Figures 3.4 to Figure 3.7. Epidermal samples from wild-type and waved littermates at the same stage of development are also shown.

The phenotype of the six double homozygotes uncovered ranged from severely runted to normal, healthy animals with waved coat hair. The animals that had developed normally were all the smallest in their litters and showed delayed pigmentation and development of the first hair coat when compared with their littermates, suggesting that a retardation of epithelial development had occurred in these animals. The non-significant deficiency of double homozygotes may be due to the reduced viability of Egfr<sup>as-2</sup>-/- together with a slight possible increase in inviability in combination with the Tgfa<sup>as-1</sup>-/-, and the degree of cannibalism that was occurring due to the targeting of the runt phenotype by parental animals.

Of the 158 mice collected and genotyped from the second F<sub>2</sub> series, only four double homozygotes were uncovered. Of these four, two exhibited a severely runted phenotype and were collected for histology at six and nine days postpartum growth.
The animal taken at six days postpartum exhibited a greater delay in general development compared with its littermates, weighing only 29% of the average of its littermates and it was just starting to exhibit dorsal skin pigmentation (Figure 3.4). This animal also exhibited premature eyelid opening. The animal taken at nine days postpartum growth also was 44% of the average weight of its littermates (Figure 3.5). Development of the hair coat was delayed compared to its littermates and this animal also exhibited premature eyelid opening.

The other two double homozygotes from this series were not candidates for histology. One was 72% of the average weight of its littermates and was viable when collected at 15 days postpartum growth. This animal exhibited a comparatively slower development of its first hair coat although this was not as pronounced as for the other more severely affected double homozygotes uncovered. The remaining double homozygote identified in this series was found as a half eaten four day old carcass and seemed to exhibit a retarded general development although this was difficult to discern.

The remaining two double homozygotes were from the first F₂ series where only animals exhibiting extreme phenotypes were collected. These animals were at ten and twenty days postpartum growth and both were the smallest in their litters. The ten day old animal was 42% of the average weight of its littermates and was comparatively retarded in skin pigmentation and dorsal coat development (Figure 3.6). The 20 day old double homozygote was 57% of the average weight of its littermates and was the only double homozygote that expressed a wild-type tail phenotype. A qualitative assessment of this animal also suggested that the coat morphology was unusual compared to the waved and wild-type coat phenotypes seen in its littermates and in other animals observed during the course of this experiment. Several observations of the developing coat phenotype from this individual suggested that not only did the first hair coat show delayed development but once the first hair coat had developed it appeared to be shorter, less dense and straighter than in its waved littermates. Comparative epidermal histology from this non Vf expressing double homozygote and wild-type and waved littermates revealed some unusual discrepancies in skin morphology (Figure 3.7). At twenty days postpartum growth, mice have generally passed through the first hair cycle and are in the telogen or resting phase of hair growth. Skin thickness is also at its thinnest at this stage. The dorsal skin from the double homozygote exhibited characteristics not expected for an animal in the telogen state. The characteristic thinning of the epidermis and regression of the telogen follicle toward the skin surface was not apparent in this animal. The fibre morphology and the depth of the hair follicle in the dermis suggested that this animal failed to progress into the telogen phase of the hair cycle when compared with its waved and wild-type littermates.
Figure 3.1 Diagram of intercross structure utilized to generate double homozygotes. A single founding breeding pair was utilized to generate F₁ offspring. A female Tgfa<sup>xa-4</sup> animal was mated with a male Egrf<sup>x-2</sup> animal expressing the vestigial tail phenotype. The resulting F₁ progeny were healthy and exhibited the wild-type coat phenotype and the expected doubly heterozygous genotype [Tgfa<sup>xa-4</sup> +/+; Egrf<sup>x-2</sup> +/]. Several F₁ breeding pairs were established to generate the observed F₂ progeny, including the 158 animals which were genotyped to determine the segregation frequencies of the various genotypic combinations. Presence of the mutant allele is represented by a white region on the appropriate chromosome. The Egr locus is located on mouse chromosome 11 while the Tgfa locus is located on mouse chromosome 6. Segregating genotypes are depicted in the F₂ generation with the expected hair phenotypes indicated under the appropriate genotype.
Intercross to generate $\text{Egfr}^{wa-2}$ and $\text{Tgf}\alpha^{wa-1}$ double homozygotes

$\text{Tgf}\alpha^{wa-1} \times \text{Egfr}^{wa-2 (vt/vt)}$

$\downarrow$

F1

$\downarrow$

F2

Wildtype

Waved

Chromosome 6

Chromosome 11
Figure 3.2 Photographs of the wild-type, $Tgfa^{\text{wt}}$ and $Egf^{\text{wt}}$ coat phenotypes. (A) A 4-5 week old mouse which expressed the wild-type coat phenotype. (B) A similar age mouse which expressed the $Tgfa^{\text{wt}}$ phenotype, note the undulation of the coat indicative of the adult waved phenotype. (C) A similar age mouse homozygous for the $Egf^{\text{wt}}$ mutation. The hair phenotype of this mutation was essentially identical to that seen in the $Tgfa^{\text{wt}}$ mutation and the two mutations could not be visually distinguished.
Figure 3.3 Genotypes of the six F1 breeding pairs intercrosses to generate the F2 progeny genotyped in Table 3.1. M and F indicate male and female animals respectively. All animals expressed the wild-type hair phenotype. A. Genotype at the Tgfα

locus. All breeding animals carried the expected heterozygous genotype. +/-, -/- and +/- indicate control samples from wild-type, Tgfα

homozygous mutant and heterozygous individuals. B. Genotype at the Egfr

locus. All breeding animals carry the expected heterozygous genotype. +/-, -/- and +/- indicate control samples from wild-type, Egfr homozygous mutant and heterozygous individuals.
Figure 3.4 H and E stained longitudinal dorsal skin sections of a wild-type, waved and doubly homozygous mouse at six days postpartum development. All animals are from the same litter (A) An example of a wild-type skin morphology, note the straight fibres in an anagen growth state. Genotype [Tgfa<sup>±1</sup> +/+; Egfr<sup>±2</sup> +/−]. (B) A waved epidermal phenotype. Genotype [Tgfa<sup>±1</sup> +/+; Egfr<sup>±2</sup> −/−]. (C) An example of a doubly homozygous [Tgfa<sup>−1</sup> −/−; Egfr<sup>−2</sup> −/−] animal. This animal was the most severely affected double homozygote uncovered and expressed a runted phenotype. Note the immature epidermis when compared with wild-type and waved littermates in A and B. The developing follicles are jumbled and disorganised. Follicle development was markedly retarded with poor development of the hair canal and penetration of the developing fibre through the epidermis. A thick cornified layer of epidermis was also present, this could be embryonic periderm which is usually absent by this stage of development. The dermal layer of the skin was also much thinner than usual when compared with littermates. Scale bar = 25μM.
Figure 3.5 H and E stained longitudinal dorsal skin sections of a wild-type, waved and doubly homozygous mouse taken at nine days postpartum development. All animals were from the same litter. (A) An example of a wild-type skin phenotype at this stage of development. Genotype \[ Tgf\alpha^{ab2-1} +/++; Egrf^{ab2-2} +/- \]. (B) A waved-hair phenotype, note the irregularity of follicle development is not as pronounced in this animal compared to other waved coat animals. Genotype \[ Tgf\alpha^{ab2-1} +/++; Egrf^{ab2-2} -/- \]. (C) A doubly homozygous mutant with the disorganised follicle development and the waved-hair phenotype. This animal was the next most severely affected animal and although not runted it was the smallest in its litter. Follicle development was delayed in this animal when compared with its littermates although fibres had penetrated the skin surface. An increase in follicle density may also have occurred along with a noticeably thinner dermal layer than that seen in its littermates in A and B. Scale Bar = 25µM.
Figure 3.6 H and E stained longitudinal dorsal skin sections of a wild-type, waved and doubly homozygous mouse taken at ten days postpartum development. All animals were from the same litter. (A) An example of wild type skin at this stage of development. Genotype [tgfa<sup>wt<sup>+/−</sub>; egfr<sup>wt<sup>+/−</sub>]. (B) An example of the waved-hair phenotype. Genotype [tgfa<sup>wt<sup>+/−</sub>; egfr<sup>wt<sup>−/−</sub>]. (C) A doubly homozygous animal with disorganised follicle development. This animal was developing a waved dorsal coat and was the smallest in its litter. The thinning of the dermis was not as pronounced in this animal, and a possible increase in follicle density may have occurred along with a denser looking dermis.
Figure 3.7 H and E stained longitudinal dorsal skin sections of a wild-type, waved and doubly homozygous mouse taken at twenty days postpartum development. All animals were from the same litter. (A) An example of wild-type skin at this stage of development. Note that the hair follicles had progressed to the telogen stage of the hair cycle with the concomitant regression of the follicle and thinning of the dermis. Genotype [Tgfα1+/+; Egfrα2+/−]. (B) The waved phenotype at this stage of development is also exhibited the morphological signs of the telogen stage of the hair cycle. Genotype [Tgfα1−/−; Egfrα2−/−]. (C) A doubly homozygous mouse at twenty days postpartum development. This was the oldest double homozygote uncovered during the course of this experimental work and was the only animal which did not express the vestigial tail phenotype. The morphology of the dorsal skin from this animal was noticeably thicker than its wild-type and waved littermates and did not exhibit the same regression of hair follicles associated with the telogen stage of the hair cycle. The hair follicles were also present in the lower regions of the dermis, suggesting that this animal may have been delayed in its entry into telogen. This animal was also phenotypically distinct from its wild-type and waved litter mates with an intermediate degree of coat waviness. Scale bar = 25μM.
Discussion

A study of interaction between mutant loci should ideally be carried out using congenic mouse strains (Sundberg and King, 1996). Congenic strains have an identical genetic background differing only at the mutant loci to be examined. Therefore the variation in phenotype is constrained due to the elimination of unwanted effects and interactions with the genetic background. As the mutations used in this study were not introgressed onto a congenic background, the effects of background loci and other epigenetic factors that may have affected the variation in phenotype need to be considered. The existence of genetic modifiers of the EGFR signalling pathway has recently been suggested from the expression of an EGFR null mutation which varies depending on the mouse strain harbouring the null mutation (Miettinen et al., 1995; Sabilla et al., 1995; Threadgill et al., 1995). The phenotypic variation observed in the compound homozygotes generated in this study, although not exhibiting the same severity as the EGFR null mutant, is consistent with the assumption that genetic modifiers are likely to be segregating on the genetic backgrounds utilised to generate these individuals.

The linkage of the vestigial tail locus on mouse chromosome 11 is an example of one known background gene that may affect the expression of the compound mutant phenotype. Of the six double homozygotes uncovered during this experimental work, five expressed the vestigial tail phenotype. The expected frequency of the vestigial tail genotypes can be calculated since a founder Egrf$^{a2}$ male, expressing the vestigial tail phenotype, was used to generate the F1 breeding pairs and the map distance between vestigial tail and Egrf$^{a2}$ loci is known (Greco et al., 1996). Thus the influence of this locus on viability can be determined by calculating the expected frequency of individuals homozygous for both vt and Egrf$^{a2}$ (see results). Although the frequency of recombinants between these two loci is low it is not a statistically significant departure from expected ($\chi^2 = 1.9774, p = 0.1597, df = 1$). The neutrality of vt is also supported by the observation that many double homozygotes expressed vestigial tail and it is therefore unlikely to be having a deleterious modifying effect on viability.

The variation in epidermal and viability phenotypes observed may also be the result of an increased sensitivity of the compound mutant to environmental stressors. Certain strains of inbred mice have been shown to be very sensitive to environmental effects which result in considerable within-strain phenotypic variability (Falconer and Mackay, 1996). Although the strains used in this study were not inbred and the progeny examined were the second generation of a cross between two closed breeding strains (and as such would be expected to exhibit heterosis), the compound mutants could have been more sensitive to environmental factors. As a result, individuals of the same mutant genotype would be expected to express a wider range of phenotypes.
Considering these caveats regarding background modifier loci and epigenetic effects, the reduced frequency of certain genotypes observed in this study have shown that there is a mild viability effect associated with homozygosity at $Egrf^{na}$-2. A deficiency of $Egrf^{na}$-2/- genotypes that approached statistical significance was supported by a single locus Chi-square analysis. A similar analyses of the $Tgfz^{na}$-1 genotype frequency revealed no significant deficiency of mutant homozygotes. Both the contingency Chi-square and two-locus Chi-square analyses failed to detect any significant deviations of genotype frequencies that may be indicative of an interaction effect between these two loci. However, a closer examination of the contingency and two-locus Chi-square analyses reveals that the deficiency of $Egrf^{na}$-2 homozygotes was most severe when combined with either the heterozygous or homozygous $Tgfz^{na}$-1 mutation.

The peri-natal viability effect of $Egrf^{na}$-2 homozygosity in this study contradicts several reports in the literature which have examined the phenotypic effects of this locus either in isolation or in combination with other loci that may also be involved in the EGFR signalling cascade. A study on the viability effects associated with $Egrf^{na}$-2 homozygosity by Fowler et al. (1995) lead to the conclusion that the viability effects were associated with the maternal genotype and impaired lactation rather than the genotype of the progeny. Of more interest is a recent study by Wang et al. (1997) who have reported a similar attempt to genetically disrupt the mammalian EGFR signalling cascade in the mouse. By using the partially functional $Egrf^{na}$-2 mutation in combination with a heterozygous null allele of the $Sos1$ gene, an intracellular component of the EGFR signalling cascade (See Figure 1.3), a subtle enhancement of the sporadic $Egrf^{na}$-2 "eyes open at birth" phenotype was observed. A deficiency of $Egrf^{na}$-2/$Sos1$ genotypes was noted that was similar to that observed in the study presented in this chapter and which the authors interpreted as evidence for a "strong genetic interaction" between $Egrf^{na}$ and $Sos1$. However Chi-Square analysis of the genotype frequency data presented by Wang et al. provides no statistical support for such an interaction. The deficiency in $Egrf^{na}$-2 mutant homozygotes found by Wang et al. can be solely attributed to a viability effect at this locus alone. Therefore, considering the results obtained in the study by Wang et al. (1997) together with the data presented in this chapter, any attempt to genetically dissect the mammalian EGFR signalling cascade using the partially functional $Egrf^{na}$-2 mutation must take into account this mild viability effect before postulating evidence for any genetic interaction.

The epidermal phenotypes observed in double homozygotes have also indicated that the combined effect of these mutations on epidermal development was variable. Although slower epidermal development was noted in several double homozygotes, a retardation of general development and growth rate was also observed (see Chapter 5) suggesting that the combined effects were pleiotropic and not restricted to epidermal development. The oldest double homozygote examined, which at 20 days was
developing normally when collected for histology, was phenotypically distinct from its littermates at this stage and the hair follicles were qualitatively less waved and had not entered telogen (see Figure 3.7). This animal also exhibited an retarded early coat development compared with its littermates and the epidermal phenotype observed at 20 days may indicate an initially slower entry into anagen growth and a subsequently delayed passage into the catagen and telogen stages of the normal hair cycle. However, considering the waved and wild-type phenotypes normally observed, the atypical phenotype of this 20 day old double homozygotes could be an indication of an overlapping functionality between members of EGFR subfamily. Other EGFR ligands such as betacellulin, amphiregulin and HB-EGF are all produced in the epidermal keratinocytes and may be able to compensate for the loss of TGF-α and EGFR activity. Other members, such as erbB2, could also be involved. The complex associations known to occur between these ligands and receptors suggest that a buffering capacity may be inherent in this group of growth factor molecules (Chapter 1). A similar mechanism has been suggested to occur within the myogenic family of transcription factors. A transgenic null mutation in the MyoD gene results in a normal healthy mouse indicative of functional redundancy in this gene family. However, this normality was actually due to the fact that, Myf-5, another myogenic transcription factor was upregulated in the MyoD mutant (Rudnicki et al., 1992). The correlated response of members of the EGFR subfamily to mutational disruption of other members may also be an avenue of experimentation that is worthwhile pursuing.

Many studies of a potential interaction between mutations affecting development have relied on an inferred functional relationship based on the similarity of mutant phenotypes or the co-localisation of gene products during development (Helwig et al., 1995; Estibeiro et al., 1993). By attempting to gain an understanding of the likely phenotypic consequences of combining two functionally related mutations (a ligand and its receptor), an effect on viability has been demonstrated. While there appears to be no evidence supporting an interaction between these two loci, there is a mild viability effect related to Egfr<sup>ax<sup>2</sup></sup> homozygosity that approaches statistical significance. Therefore, it appears that the effect on viability of combining these two mutations is essentially additive. This interpretation of lack of interaction is supported by an analyses of another trait, growth rate (see Chapter 5). Additive gene effects between the Tgfα<sup>ax<sup>1</sup></sup> and Egfr<sup>ax<sup>2</sup></sup> loci agree with a previous report examining the phenotypic effects of combining several other waved-hair mutations, where additive gene effects were also concluded (Carter, 1951). In this study where it was possible to utilise well defined genotyping assays for each of these phenotypically indistinguishable mutations, a more accurate assessment of the potential interaction between the Tgfα<sup>ax<sup>1</sup></sup> and Egfr<sup>ax<sup>2</sup></sup> mutations has been possible.

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(Pennycuik and Raphael, 1984). Although no other hair follicle abnormalities have been observed in the angora mutation an increase in DETC (Dendritic epidermal T-Cells) has been reported in both the angora and hairy ears mutations (Tigelaar and Lewis, 1995). Unpublished data suggests that the angora mutation may result in structural defects of the hair fibre and defects of the immunoregulation of hair follicle growth (Sundberg, Homepage).

The Fgf5<sup>op</sup> mutation
The genetic basis of the angora mutation was uncovered by the mutational analysis of a member of the FGF growth factor family, FGF-5 (Hébert et al., 1994). The phenotype expressed by mice homozygous for a targeted disruption of exon 1 of Fgf5 is identical to that described for the angora mutation. Complementation analysis has revealed that the defective Fgf5 locus was an allele of the angora mutation and that the Fgf5 locus in the original angora mutation was the result of a deletion of the promoter sequence and a portion of exon 1 (Hébert et al., 1994). The angora mutation has since been renamed Fgf5<sup>op</sup> (Sundberg and King, 1995). An analysis of Fgf5 mRNA from the original Fgf5<sup>op</sup> mutant has revealed that the deletion mutation results in a null allele of the gene with no transcript being produced (Hébert et al., 1994).

FGF-5
The FGF family of peptide growth factors share a 30-70% amino acid homology and currently consists of ten members which have been implicated in the development and growth of a wide range of tissues. FGF-5 was initially discovered as a transforming oncogene isolated from a human bladder carcinoma cell line (Zhan et al., 1987) but, as with other FGF family members, a wide range of functions have been described. The mature FGF-5 polypeptide is 97% conserved at the amino acid levels between human and mouse and 30-50% conserved within a core 120 amino acid region between all FGF family members (Goldfarb et al., 1991). The presence of two open reading frames, one upstream from that coding for the mature peptide, may also indicate a mechanism of translational control. A hydrophobic N-terminus also suggests that the mature peptide is secreted. The FGF-5 gene is coded for by 3 exons in the mouse with a splice variant lacking exon 2 having been reported in the rat although its functional significance is currently unknown (Hattori et al., 1996).

Spatially restricted Fgf5 mRNA expression occurs in a wide range of tissues during development (Haub and Goldfarb, 1991; Hébert et al., 1991) and has been utilised as a marker to monitor early embryonic stem cell differentiation (Conover et al., 1993). Post-implantation development and differentiation are also marked by a rapid increase in epiblastic Fgf5 transcription (Hébert et al., 1991) suggestive of a role for this growth factor in the gastrulating stages of embryogenesis. Fgf5 mRNA is also expressed in somatic lateral mesoderm coincident with myotome formation and may play a role in myogenesis (Cossu et al., 1996) as well as being a potential angiogenic and
neurotrophic factor (Giordano et al., 1996, Lindholm et al., 1994). Low levels of transcript for FGF-5 are found in the adult brain with sites of expression observed in the hippocampus, cerebral cortex and thalamus (Goldfarb et al., 1991).

A coordinated regulation of Fgf5 expression may also be occurring in several tissues. Kainic acid induction of epileptiform activity results in a transient increase in hippocampal Fgf5 mRNA after 3 hours, reaching a peak by 12 hours and returning to normal levels by 24 hours, an observation consistent with a primary transcriptional response for this gene. An increase in hippocampal expression levels of Fgf2 and Fgfr1 mRNA after kainic acid treatment was also noted (Gómez-Pinilla et al., 1995). A coordinated expression of Fgf-5 and other members of the FGF and FGFR family is also apparent from the study of prostate cancer progression (Yan et al., 1993). The binding affinity of FGF-5 protein with members of the FGFR subfamily has been determined. High affinity binding sites to both FGFR1 and FGFR2 have been reported in vitro (Ornitz and Leder, 1992; Clements et al., 1993).

Considering the expression pattern of Fgf5 from the earliest stages of embryogenesis and throughout development, it is somewhat surprising that the only currently known phenotypic effect of an FGF-5 null mutation is an aberrant regulation of the hair cycle. Localisation of Fgf5 transcript in the mouse epidermis during the various stages of the anagen to catagen transition of the hair cycle has revealed that transcript is expressed only in the lower third of the ORS during late anagen VI, prior to catagen transition (Hébert et al., 1994). It should be noted that mouse dorsal skin was analysed at 6 days, 12 days and 18 days of the hair cycle. The expression of Fgf5 mRNA in the lower third of the ORS is also distinct from the expression of FGFR1 in the dermal papilla, a known high affinity FGF-5 binding site (Rosenquist and Martin, 1996).

An functional relationship between Tgfαwe-1, Egrfrwe-2 and Fgf5we
The mitogenic stimulation of fibroblast cultures in vitro by members of the EGF ligand family, EGF and TGF-α, results in an immediate-early transcriptional response of FGF-5 (Weber et al., 1991a and references therein). This observation is suggestive of a relatively direct transcriptional induction of this FGFR ligand when the EGFR is activated. Activation of this transcriptional response reaches its transcriptional peak 3 hours after the addition of EGF and TGF-α. This transcriptional activation was not affected by the addition of the protein synthesis inhibitor cycloheximide adding further supporting evidence that the induction of Fgf5 expression is as an immediate-early response to EGFR activation.

The involvement of the EGFR in the growth regulation of the hair follicle has been determined by the use of transgenically engineered EGFR mutations which express only in the epidermis. Dominant-negative EGFR mutants, which lack the cytoplasmic domain of the EGFR and express in epidermal regions similar to that seen for normal
EGFR expression, have implicated the EGFR in the regulation of the anagen to catagen transition of the mouse hair cycle (Murillas et al., 1995). These mice express a waved-hair phenotype consistent with the waved-hair mutations of Tgfa<sup>−/−</sup> and Egrf<sup>−/−</sup> mice. Other epidermal phenotypes were also expressed, including a disruption of hair growth control.

Animals hemizygous for the dominant-negative mutation also expressed a waved-hair phenotype. This semi-dominant mode of expression may be similar to that noted for several Rex alleles, another semidominant waved-hair mutation. Homozygotes also expressed the waved-hair phenotype but also failed to enter the catagen stage of the hair cycle. A continuation of anagen growth occurred during the first hair cycle resulting in a phenotype of long waved hairs. Follicles eventually underwent necrotic cell death (Murillas et al., 1995). This result suggests that a functional cytoplasmic domain of the EGFR is required for the transition of the actively growing hair follicle from the anagen to catagen states during the normal hair cycle in mice. Collectively this data is strongly suggestive of a functional interrelationship between epidermally expressed ligands for the EGFR such as TGF-α and EGF, EGFR activation and the subsequent induction of immediate-early response genes such as FGF-5 during the regulation of the anagen to catagen hair growth transition.

In contrast, when a general null mutation of the EGFR was generated the mutant phenotype exhibits a strain dependent expressivity varying from embryonic lethality to a postnatal loss of fitness. This variation in phenotype has been suggested to involve modifying alleles present on the different genetic backgrounds utilised which determine the expressivity of the null mutation (Miettinen et al., 1995; Threadgill et al., 1995; Sibilia and Wagner, 1995). Epidermal development in EGFR null mutant animals which survive postnatally is delayed. These animals exhibit a general epidermal hypoplasia and a retardation of hair follicle development. The epithelial development of many organs is affected in the EGFR null mutants while the epidermally expressed dominant-negative mutants specifically affected hair follicle development and control of the hair cycle. This phenotypic difference between a general EGFR null mutation and an epidermally targeted disruption has revealed that the EGFR functions in many contexts during epithelial development in mammals.

**Aims of this study**

Considering the experimental result indicating that Fgf5 mRNA may be inducible as an immediate-early response transcript in vitro (Werner et al., 1991a), a potential functional relationship between three mutations affecting hair growth control was investigated. The analysis of this potential functional relationship between an extracellular ligand, its receptor and an immediate-early response gene was undertaken by utilising the mouse mutations Tgfa<sup>−/−</sup>, Egrf<sup>−/−</sup> and Fgf5<sup>−/−</sup>. Mice homozygous for all three mutations were
generated by the consecutive intercrossing of the laboratory strains carrying each of these recessive, unlinked mutations. A phenotypic and genotypic characterisation of these animals with respect to epidermal development and hair follicle growth was undertaken to determine if allelic combinations of these three mutations exhibit a synergistic effect, that is there combined phenotypic effect is greater than that expected from the summation of their individual effect. Considering the known biochemical interaction between the gene products of the Tgfa<sup>+/−</sup> and Egf<sup>+/−</sup>, a further disruption of epidermal development might be expected to occur in animals homozygous for all three recessive mutations.
Results

Generation of triple homozygotes

The intercross structure used to generate the triple homozygotes is outlined in Figure 4.1. As the results from Chapter 3 have indicated, it seems likely that the effects of vt segregation are neutral and in this experiment no attempt was made to keep track of the vestigial tail locus due to the complexity of the intercross structure. Founder Egfr<sup>wa-2</sup> males were chosen which did not express the vt phenotype and were therefore of a heterozygous or wild-type vt genotype. Mice doubly homozygous for either the [Fgf5<sup>SPA</sup>+/<sup>-</sup>; Tgfa<sup>wa-1</sup>+/<sup>-</sup>] or [Fgf5<sup>SPB</sup>+/<sup>-</sup>; Egfr<sup>wa-2</sup>+/<sup>-</sup>] genotypes were produced as described (see Chapter 2) and expressed both the combined waved and angora hair phenotypes. These animals were easily discernible from their waved or angora littermates at four weeks of age due to the long waved guard hairs and underfur (Figure 4.2). The phenotype of either double homozygote genotype was essentially identical and could not be distinguished. The subsequent crossing of these presumed double homozygotes produced F<sub>1</sub> offspring which expressed the angora phenotype and developed normally. The eight breeding pairs used to generate the second F<sub>2</sub> series extensively examined in this chapter were genotyped to confirm homozygosity of the Fgf5 loci and the heterozygosity of both the Tgfa<sup>wa-1</sup> and Egfr<sup>wa-2</sup> loci (see Chapter 2). All F<sub>1</sub> animals utilised as breeding pairs carried the expected genotype [Fgf5<sup>SP</sup>+/<sup>-</sup>; Tgfa<sup>wa-1</sup>+/<sup>-</sup>; Egfr<sup>wa-2</sup>+/<sup>-</sup>] (Figure 4.3). Intercrossing of these animals therefore generated progeny segregating for allelic combinations of Tgfa<sup>wa-1</sup> and Egfr<sup>wa-2</sup> on a homozygous Fgf5<sup>SP</sup>+/<sup>-</sup> background.

The initial experimental aim in constructing these crosses was to determine whether combinations of mutant alleles known to affect hair growth regulation and the hair cycle would result in a further disruption of the normal growth and cycling of the hair follicle. An initial F<sub>2</sub> series was generated and observed over a period of 4 weeks postnatal growth to determine if any further extension of the hair cycle was occurring. These mice did not reveal any obvious effect on hair growth control beyond that seen with the angora mutation. Animals which survived to the end of this 4 week period expressed either the expected angora or combined waved/angora hair phenotypes. A number of animals also exhibited a delayed epidermal and general development with several severely runted animals observed during the early postnatal period. Often these animals were cannibalised by their parents soon after birth. An increased severity of runting relative to that seen in progeny segregating for the experiment described in Chapter 3 also appeared to be occurring.

During the course of this study, experimental data were simultaneously published by several laboratories which suggested that the targeted disruption of the Egfr locus
resulted in mutant animals exhibiting a similar severely runted phenotype with variable penetrance depending on the genetic background of the donor transgenic strain used (Miettinen et al., 1995; Sibilia and Wagner, 1995; Threadgill et al., 1995). As a result of these reports the experimental design was altered to determine if the progeny exhibiting the runted phenotype were due to the segregation of genotypic combinations of the Tgfα<sup>−/−</sup>, Egf<sup>−/−</sup> and Fgf5<sup>0/0</sup> mutations. As a large number of runted offspring had been cannibalised in the first F<sub>2</sub> series examined, a second F<sub>2</sub> series was generated with close attention being paid to animals exhibiting delayed epidermal development and a severely runted phenotype. Animals expressing this phenotype were easily discernible from their healthy littermates at two or three days postnatal development due to slower skin pigmentation, absence of milk in their stomachs and were often severely dehydrated. Unfortunately these animals were often cannibalised before they could be collected.

Table 4.1 Observed and expected frequencies of the genotypic combinations of Tgfα and Egf loci assuming unlinked independent mendelian segregation. All genotypes are also homozygous for the Fgf5<sup>0/0</sup> mutation.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Number of Mice</th>
<th>Hair Phenotype</th>
<th>Contribution to Chi-square value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Observed (Expected)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tgfα&lt;sup&gt;−/−&lt;/sup&gt;; Egf&lt;sup&gt;−/−&lt;/sup&gt; /+</td>
<td>22 (16.75)</td>
<td>angora</td>
<td>1.645</td>
</tr>
<tr>
<td>Tgfα&lt;sup&gt;−/−&lt;/sup&gt;; Egf&lt;sup&gt;−/−&lt;/sup&gt; /+</td>
<td>46 (33.5)</td>
<td>angora</td>
<td>4.664</td>
</tr>
<tr>
<td>Tgfα&lt;sup&gt;−/−&lt;/sup&gt;; Egf&lt;sup&gt;−/−&lt;/sup&gt; /+</td>
<td>21 (18.75)</td>
<td>waved /angora</td>
<td>1.078</td>
</tr>
<tr>
<td>Tgfα&lt;sup&gt;−/−&lt;/sup&gt;; Egf&lt;sup&gt;−/−&lt;/sup&gt; /+</td>
<td>35 (33.5)</td>
<td>angora</td>
<td>0.067</td>
</tr>
<tr>
<td>Tgfα&lt;sup&gt;−/−&lt;/sup&gt;; Egf&lt;sup&gt;−/−&lt;/sup&gt; /+</td>
<td>71 (67)</td>
<td>angora</td>
<td>0.239</td>
</tr>
<tr>
<td>Tgfα&lt;sup&gt;−/−&lt;/sup&gt;; Egf&lt;sup&gt;−/−&lt;/sup&gt; /+</td>
<td>40 (33.5)</td>
<td>waved /angora</td>
<td>1.157</td>
</tr>
<tr>
<td>Tgfα&lt;sup&gt;−/−&lt;/sup&gt;; Egf&lt;sup&gt;−/−&lt;/sup&gt; /+</td>
<td>11 (16.75)</td>
<td>waved /angora</td>
<td>1.974</td>
</tr>
<tr>
<td>Tgfα&lt;sup&gt;−/−&lt;/sup&gt;; Egf&lt;sup&gt;−/−&lt;/sup&gt; /+</td>
<td>19 (33.75)</td>
<td>waved /angora</td>
<td>8.446</td>
</tr>
<tr>
<td>Tgfα&lt;sup&gt;−/−&lt;/sup&gt;; Egf&lt;sup&gt;−/−&lt;/sup&gt; /+</td>
<td>3 (16.75)</td>
<td>waved /angora</td>
<td>11.267</td>
</tr>
</tbody>
</table>

Progeny were therefore observed over a shorter 2 week period in the second series with animals exhibiting severe runting culled and fixed for histology. Smaller animals which were less severely affected and still feeding were left either with their mothers or placed with foster mothers (either Quackenbush or an F1 hybrid line females) and allowed to develop for several days before collection for histology and genotyping. Littermates of animals exhibiting the runted phenotype were also taken at the same stage of development to allow a comparison with the expected angora and
waved/angora epidermal phenotypes. A record of the number of offspring cannibalised was noted and revealed that approximately 10% of the animals born were cannibalised soon after birth. A series of 286 F\textsubscript{2} offspring was collected and genotyped to determine the frequency of the segregating genotypes and the potential phenotypic effects of these genotypes (Table 4.1).

Statistical tests of significance

Analyses of the observed genotype frequencies revealed highly statistically significant differences from expected for the segregating Tgfα\textsuperscript{−/−} and Egfr\textsuperscript{−/−} genotype combinations. A similar set of Chi-square analyses was conducted as for Chapter 3. The results of all three analyses are presented in Table 4.2. If the genotype frequency data are considered separately for either the Tgfα\textsuperscript{−/−} and Egfr\textsuperscript{−/−} loci, there appears to be no significant departure from the expected 1:2:1 genotype frequencies for the Tgfα\textsuperscript{−/−} genotype ($\chi^2=0.179$, p=0.9144, df=2). However, for the Egfr\textsuperscript{−/−} locus there is a highly statistically significant departure from expected values ($\chi^2=25.552$, p<10\textsuperscript{-4}, df=2) primarily due to a deficiency of mutant homozygotes. This single locus analysis suggests that homozygosity for the the Egfr\textsuperscript{−/−} mutation, on a homozygous Fgf5\textsuperscript{−/−} background, causes a highly statistically significant depression of viability.

Analysing the multi-locus genotype frequencies using either the contingency or two locus independent Mendelian segregation Chi-square analyses, permits a test for the interaction between the Tgfα\textsuperscript{−/−} and Egfr\textsuperscript{−/−} loci. The contingency Chi-square analyses reveals that that there is no significant departure from expected values ($\chi^2=5.181$, p=0.2692, df=4). The result of this analyses therefore suggests that there is no evidence for an interaction (i.e. non-independence) between the Tgfα\textsuperscript{−/−} and Egfr\textsuperscript{−/−} loci. Inspection of the two locus test for independent Mendelian segregation reveals a highly statistically significant departure from expected values ($\chi^2=28.557$, p=0.0004, df=8). However it is obvious that this departure from expectation is entirely due to single locus viability effects caused by homozygosity for Egfr\textsuperscript{−/−}, which are enhanced or amplified on the homozygous Fgf5\textsuperscript{−/−} background. The results provide no statistical support for interaction between the loci. Nevertheless it is interesting to note that although the two locus Chi-square analysis reveals a deficiency of Egfr\textsuperscript{−/−} -/- genotypes for all Tgfα\textsuperscript{−/−} genotypes it is most extreme in the Tgfα\textsuperscript{−/−} +/- and -/- genotypes, a situation similar to that described in Chapter 3.

Clearly the reduced viability of the Egfr\textsuperscript{−/−} mutant homozygotes is still the major contributing factor to the departure from expectation. Consistent with this, the frequency of parental cannibalism increased in this cross to approximately 10% relative to the figure of 5% reported in Chapter 3 were the reduction in viability of the Egfr\textsuperscript{−/−} homozygotes was less severe. Clearly the runts represented predominantly Egfr\textsuperscript{−/−}
mutant homozygotes. Prenatal mortality may also be responsible for some of the deficiency of this mutant class. Growth rate data from all these genotypes are presented in Chapter 5.

Table 4.2. Chi-square analyses of single and two locus genotype frequencies. Observed values (see table 4.1) are normal font in the body of the table. Single locus segregation test for 1:2:1 ratio at the Tgfa<sup>wa</sup>- locus (expected values are in bold on the right hand side of the table; $\chi^2 = 0.179$, p=0.9144, df=2). Single locus segregation test for 1:2:1 ratio at the Egfr<sup>wa</sup>- locus (expected values are in bold at the bottom of the table; $\chi^2 = 25.552$, p<10<sup>-4</sup>, df=2). The contingency analysis (expected values are in italics in the body of the table; $\chi^2 = 5.181$, p=0.2692, df=4). Two locus independent Mendelian segregation analysis (expected values are in bold italics in the body of the table; $\chi^2 = 28.557$, p=0.0004, df=8).

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Tgfa&lt;sup&gt;wa&lt;/sup&gt;-/+</th>
<th>Tgfa&lt;sup&gt;wa&lt;/sup&gt;-+/ -</th>
<th>Tgfa&lt;sup&gt;wa&lt;/sup&gt;-/-</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Egfr&lt;sup&gt;wa&lt;/sup&gt;-/+</td>
<td>22 (22.58, 16.75)</td>
<td>46 (45.16, 33.5)</td>
<td>21 (21.25, 16.75)</td>
<td>89 (87)</td>
</tr>
<tr>
<td>Egfr&lt;sup&gt;wa&lt;/sup&gt;-/+</td>
<td>35 (37.04, 33.5)</td>
<td>71 (74.09, 67.0)</td>
<td>40 (34.87, 33.5)</td>
<td>146 (134)</td>
</tr>
<tr>
<td>Egfr&lt;sup&gt;wa&lt;/sup&gt;-/-</td>
<td>11 (8.37, 16.75)</td>
<td>19 (16.75, 33.5)</td>
<td>3 (7.88, 16.75)</td>
<td>33 (67)</td>
</tr>
<tr>
<td>Total</td>
<td>68 (67)</td>
<td>136 (134)</td>
<td>64 (67)</td>
<td>268</td>
</tr>
</tbody>
</table>

Phenotype of [Fgf5<sup>oo</sup>-/-; Tgfa<sup>wa</sup>-/-] and [Fgf5<sup>oo</sup>-/-; Egfr<sup>wa</sup>-/-] double homozygotes

The hair phenotype of both the [Fgf5<sup>oo</sup>-/-; Tgfa<sup>wa</sup>-/-] and the [Fgf5<sup>oo</sup>-/-; Egfr<sup>wa</sup>-/-] genotypes was essentially a combined effect of the two mutations (Figure 4.2). This additive phenotype was most easily visible by three to four weeks of postnatal growth when the Fgf5<sup>oo</sup> phenotype is fully expressed after completion of the extended first hair cycle. Both double homozygous phenotypes were essentially identical although a thorough examination of the duration of the hair cycle was not undertaken and subtle differences in the duration of anagen may be occurring between the two genotypes. A number of [Fgf5<sup>oo</sup>-/-; Egfr<sup>wa</sup>-/-] double homozygotes did express a wavy/diagonora phenotype that seemed to be qualitatively longer although this was not quantitated. A more severe eye phenotype was also noted sporadically in several of these animals that was distinct from the "open eye phenotype" and cataract
development seen in the \textit{Egf}^{ma-2} homozygotes. These animals failed to open their eyes and were blind.

Phenotype of \{\textit{Fgf5}^{wa-1}; \textit{Tgf}^{wa-1}; \textit{Egf}^{wa-2}\} triple homozygotes

Of the 268 animals collected as part of the genotyped series, only three animals were homozygous for all three mutations. The collection of extreme animals for histology from the first \(F_2\) series revealed the presence of one other triple homozygote. In total, four triple homozygous animals were uncovered. The oldest triple homozygote exhibiting a severely runted phenotype was collected at 7 days from the first series of animals and expressed an open eye phenotype at birth with no dorsal hair growth apparent although the skin was pigmented. This animal exhibited a normal tail phenotype and was 40\% of the average weight of its littermates when collected for histology. Histological examination of the dorsal skin from this animal revealed that the hair follicles were generally at the stage 7 or stage 8 growth phase (Hardy, 1949) with the hair fibres still in the hair canal (Figure 4.4). Dorsal skin from \textit{angora} and \textit{waved/angora} littermates exhibited a more advanced epidermal development with hair follicles having emerged from the hair canal and penetrated the skin surface equivalent to a stage 10 growth phase. The epidermis also exhibited a marked contouring with a range of relative dermal-epidermal thickness observed. The fat layer of the dermis was considerably less developed than in the \textit{angora} and \textit{waved/angora} littermates at the same stage of development. Due to the dramatic reduction in thickness of the skin, it was difficult to discern whether follicle size and density were affected.

The three remaining triple homozygotes were all collected from the second series of mice examined. Two of these triple homozygotes, collected at two days postpartum development, were from the same litter and exhibited extreme runting, premature eyelid opening, a normal tail phenotype and \textit{waved} vibrissae. No signs of dorsal skin pigmentation were observed and these animals were 50\% and 62\% of the average weight of their littermates. A histological analysis of dorsal skin revealed a similar, although less pronounced, immature skin phenotype (Figure 4.5). The characteristic contouring of the epidermis with a varying dermal thickness is evident although a retardation of hair follicle development is less pronounced than in the seven day old triple homozygote.

Suppression of the triple homozygote phenotype

The final triple homozygote collected was also part of the second \(F_2\) series. This animal was the smallest in its litter and displayed slower hair coat development. However, it did not exhibit the severity of runting noted in the other triple homozygotes and was healthy when collected at 18 days of postnatal development. Once this animal had
been repeat genotyped (including an analysis of the Fgf5po RFLP) and reconfirmed as a triple homozygote, a closer inspection of the hair coat revealed that the expression of the angora phenotype was largely suppressed. This animal was phenotypically distinct from the usual angora and waved/angora phenotypes and was initially thought to be the result of a spontaneous mutation affecting hair development, as the atypical hair phenotype was not observed in over 500 mice examined in the course of this experimental work. Dorsal guard hairs appeared straighter than those seen in the waved/angora littermates, although expression of the waved hair phenotype was observed from the animals vibrissae. A comparison of the hair coat phenotype of this triple homozygote with doubly homozygous [Fgf5po-/-; Tgfαax-1/-] and [Fgf5po-/-; Egfrax2-/-] animals at the same age revealed that the waved/angora phenotype of double homozygotes was markedly different from the hair phenotype expressed by this triple homozygote (Figure 4.6 and 4.7). For the purposes of discussion, I will refer to this phenotype as a "suppression" of the angora phenotype, although it should be noted that angora expression may only be delayed due to the age of the animal when harvested. A comparison with the phenotypes of the other triple homozygotes examined suggests that the occurrence of this phenotype was in every 4 triple homozygous animals.

Other Phenotypes

A number of runted animals were observed during the collection of this F2 series which were assigned to various genotypic categories. Not all runts were confined to the triple homozygote genotype. The genotypic category [Fgf5po-/-; Tgfαax+/-; Egfrax2-/-] also had approximately 20% of animals expressing a runted phenotype. The [Fgf5po-/-; Tgfαax+; Egfrax2-/-] genotype had two of the eleven animals expressing a runted phenotype. It is interesting to note that all runts were homozygous for the Fgf5po and Egfrax2 mutations. The hair phenotypes of these three mutations were essentially additive with complete expression of the waved/angora hair phenotype occurring when animals where homozygous at either the Tgfαax or the Egfrax2 loci. Premature eyelid opening was also frequently noted in these animals. No heterozygous effect on hair fibre morphology was evident in the [Fgf5po-/-; Tgfαax-1+/-; Egfrax2-2+/-] category which expressed the expected angora phenotype. However, a small proportion of these animals did exhibit premature eyelid opening (approximately 8%). Considering the degree of intercrossing and that founder Egfrax2 males were used which did not express vestigial tail it was unlikely that a large number of vestigial tail expressing animals would be observed. No vestigial tail expressing offspring occurred in the genotyped F2 series, suggesting that only the heterozygous or homozygous wild-type vestigial tail genotype was present in the founder Egfrax2 male animals.
Figure 4.1 Diagram representing the combined intercross structure utilised to generate the triple homozygotes. Initially $Fgf5^{oo}$ homozygous animals were crossed to either $Tgfau^{oo+1}$ or $Egf^{no+2}$ homozygous animals respectively. F$_1$ offspring from either cross were then intercrossed to generate the respective doubly homozygous genotypes. Only the doubly homozygous genotypes are depicted for diagrammatic clarity. An example of the waved/angora hair phenotypes of these individuals is presented in Figure 4.2. Mice expressing the waved/angora phenotype from each cross were mated to produce an F$_1$ generation. Progeny from this generation were used as breeding pairs to generate F$_2$ animals segregating for the triply homozygous genotype. All F$_1$ breeding pairs used to establish this F$_2$ generation were of the expected $[Fgf5^{oo+1}; Tgfau^{no+1+4}; Egf^{no+2+3}]$ genotype. A representative sample of genotypes is shown for the F$_2$ offspring together with their expected hair phenotype. The mutant locus is indicated on each respective chromosome by white hatching. The $Fgf5^{oo}$ locus is located on mouse chromosome 5 (yellow), the $Tgfau^{oo+1}$ locus on chromosome 6 (red) and the $Egf^{no+2}$ on chromosome 11 (green).
Intercross to generate $Egfr^{wa-2}$, $Tgf\alpha^{wa-1}$, and $Fgf-5^{oo}$ triple homozygotes.

$Fgf-5^{oo}$ $\times$ $Tgf\alpha^{wa-1}$

$F_1$ $\rightarrow$ Double Homozygotes

$F_1$ $\rightarrow$ $F_2$

$Waved/Angora$ $\quad$ $?$$\quad$ $Angora$
Figure 4.2 An example of the hair phenotype of animals segregating in this cross. A. An Fgf5<sup>o+</sup> mouse expressing the angora phenotype. Note the elongation of the coat hairs as a result of the delayed entry into the catagen stage of the hair cycle. B. An example of a doubly homozygous [Fgf5<sup>o+</sup>; Tgif<sup>tm</sup>-/-] animal. The waved/angora phenotype is clearly discernible. C. An example of a doubly homozygous [Fgf5<sup>o+</sup>; Egfl<sup>tm</sup>-/-] animal. Expression of the waved/angora phenotype is very clearly discernible.
Figure 4.3 Genotypes of the eight breeding pairs intercrossed to generate the progeny genotyped in Table 4.1. M and F indicates male and female animals respectively. A. Genotype at the Fgf5 locus. All breeding animals were homozygous for the deletion mutation in Fgf5 and expressed the angora phenotype. +/-, +/- are control samples from angora and wild-type individuals. B. Genotype at the Tgfa^wa-1 locus. All breeding animals carry the expected heterozygous genotype. +/-, +/- and +/- are control samples from wild-type, Tgfa^wa-1 homozygous mutant and heterozygous individuals. C. Genotype at the Egf^m2 locus. All breeding animals carry the expected heterozygous genotype. +/-, +/- and +/- are control samples from heterozygous, wild-type, Egf^m2 homozygous mutant individuals.
Figure 4.4 The oldest triple homozygote uncovered which exhibited the severely runted phenotype. Mice were harvested at 7 days post partum. A. Gross phenotype of a triple homozygote (top), waved/angora (middle) and angora (bottom) littersmates. Note the severely runted phenotype of the triple homozygote. B. Dorsal longitudinal skin sections from the angora littermate. Fibres are regularly arranged and in mid-anagen growth. Genotype [Fgf15<sup>+/--</sup>; Tgfa<sup>+/--</sup>; Egfr<sup>+/--</sup>]. C. Skin sections from the waved/angora littermate also in mid-anagen and showing some irregularity in arrangement typical of the waved phenotype. Genotype [Fgf15<sup>+/--</sup>; Tgfa<sup>+/--</sup>; Egfr<sup>+/--</sup>]. D. Dorsal skin sections from the triply homozygous runt animal. Note the marked decrease in skin thickness especially in the dermis. The epidermis is also more contoured. Follicle development is retarded with most fibres yet to emerge from the hair canal through the skin surface. Follicles also exhibit the irregular arrangement indicative of the waved phenotype. Scale bar = 25µM.
Figure 4.5 H and E stained longitudinal dorsal skin sections from two day old animals at higher magnification (20 X). Two triple homozygotes were uncovered in a single litter at two days post-partum development. A. An angora littermate exhibiting early follicle development. Genotype [Fgf5"-/-; Tgfα"-/-; Egrf"-/-]. B. A waved/angora littermate also exhibiting early follicle development. Genotype [Fgf5"-/-; Tgfα"-/-; Egrf"-/-]. C and D. Two triple homozygotes which were both in the same litter. Note the thinner dermis and contoured epidermis compared with A and B. The fibrous epidermal layers in D are likely to be embryonic periderm which has usually sloughed at this stage. The immature skin phenotype of these triple homozygotes is not as pronounced as in the seven day old animal shown in Figure 4.4. Scale bar = 50μM.
Figure 4.6 Suppression of the angora phenotype A. The 18 day old suppressed angora triple homozygote uncovered during the course of this study (middle) compared with a doubly homozygous [Fgf5ln; Tgfα+1/--; Egf+1/+] animal (left) and a doubly homozygous [Fgf5ln; Tgfα+1/++; Egf+1/] animal both at 18 days post partum development. B and C. A comparison of the extremes of phenotype presented in the triple homozygote. The severely runted phenotype generally seen in most triple homozygotes and the viable suppressed angora phenotype.
Figure 4.7 Macrophotography of the dorsal hair phenotype of animals presented in Figure 4.6 emphasising the suppression of the angora phenotype A and B. 18 day old double homozygotes for Fgf10<sup>–/–</sup> and Tgfα<sup>−/−</sup> or Fgf10<sup>−/−</sup> and Egfr<sup>−/−</sup> respectively. Thewaved/angora hair phenotype is clearly observable. C. Suppression of angora expression in the 18 day old triple homozygote. Note the much shorter dorsal hairs and the absence of the undulation in the dorsal coat indicating that thewaved phenotype is also partially suppressed.
Discussion

Pairwise allelic combinations of the three mutant loci Tgfox<sup>wa<1></sup>, Egfr<sup>wa<2></sup> and Fgf5<sup>po</sup> generally resulted in the expected combined phenotypes for development of the hair follicle. This simple combination was evident in the doubly homozygous [Fgf5<sup>po</sup>-/-; Tgfox<sup>wa<1></sup>/-] and [Fgf5<sup>po</sup>-/-; Egfr<sup>wa</sup><sup>2</sup>-/-] genotypes initially generated in the construction of this combined intercross. These observations are consistent with the combination of phenotypes reported for doubly homozygous [Fgf7-/-; Tgfox<sup>wa<1></sup>/-] mice (Guo et al., 1998). Whether more subtle effects on the hair cycle and hair fibre morphology are occurring in these double homozygote genotypes is uncertain and was not investigated in this study. Interbreeding of these double homozygotes produced [Fgf5<sup>po</sup>-/-; Tgfox<sup>wa<1></sup>/-; Egfr<sup>wa</sup><sup>2</sup>/+] F<sub>1</sub> offspring which were healthy, developed normally and expressed the angora phenotype. These F<sub>1</sub> animals were used for the production of two series of F<sub>2</sub> progeny segregating for the potential triple homozygote genotype. One of these series was genotyped.

Qualitative and quantitative differences in epidermal development, viability and growth rate phenotypes (see Chapter 5) were observed with a larger number of consistently runted offspring occurring than seen in the progeny segregating for the Tgfox<sup>wa</sup><sup>1</sup> and Egfr<sup>wa</sup><sup>2</sup> mutations described in Chapter 3. Therefore it appears that the viability effect observed in chapter 3 has been amplified (or enhanced) in this study when the phenotypic effects of allelic combinations of Tgfox<sup>wa</sup><sup>1</sup> and Egfr<sup>wa</sup><sup>2</sup> are examined on a homozygous Fgf5<sup>po</sup>-/- background. The Chi-Square analyses have revealed a highly statistically significant deficiency of Egfr<sup>wa</sup><sup>2</sup> mutant homozygotes indicating that the mild viability effect previously observed (Chapter 3) has been significantly enhanced when combined with Fgf5<sup>po</sup> homozygosity. The contingency Chi-square analyses has not found evidence for any significant interaction between Tgfox<sup>wa</sup><sup>1</sup> and Egfr<sup>wa</sup><sup>2</sup>. The highly significant two-locus Chi-square value is entirely attributable to the single locus viability effect at the Egfr<sup>wa</sup><sup>2</sup> locus and provides no evidence for interaction with Tgfox<sup>wa</sup><sup>1</sup>. Homozygosity for Fgf5<sup>po</sup>-/- has also resulted in a significant effect on the growth rate of the progeny genotyped in this study (see chapter 5).

This viability effect is surprising considering that viable [Fgf5<sup>po</sup>-/-; Egfr<sup>wa</sup><sup>2</sup>-/-] double homozygotes were initially generated for use in this breeding experiment and an initial inspection of the phenotype of this gene combination suggested an additive gene effect (see figure 4.6). As a combined (additive) hair phenotype was observed when generating this genotype, a thorough investigation of relative viability was not undertaken. Considering the data obtained in this study, it appears that further investigation of the viability effect of the Egfr<sup>wa</sup><sup>2</sup> and Fgf5<sup>po</sup> mutations is definitely warranted. This would require the molecular characterisation of the deletion mutation at the Fgf5<sup>po</sup> locus allowing the development of a novel genotyping assay to detect
heterozygosity and examining the potential interaction between Fgf5<sup>P</sup> and Egfr<sup>rs-2</sup> more thoroughly. The viability effect effect associated with Egfr<sup>rs-2</sup> homozygosity, which contradicts published data suggesting that the maternal genotype is responsible for the viability effects of this mutation (Fowler et al., 1995), also needs to be revisited.

Of considerable interest is that the most severe decrease in Egfr<sup>rs-2</sup> genotypes has again occurred when in combination with either the Tgfa<sup>rs-1</sup> +/- or -/- genotypes, a situation similar to that observed in chapter 3. Although the analysis did not reveal any statistical support for an interaction between these two loci, it is noteworthy that there was a consistent deficiency of double homozygous mutants in two independent breeding trials. Therefore, it may be that the Tgfa<sup>rs-1</sup> mutation is not neutral and is in fact mildly enhancing the viability effect of Egfr<sup>rs-2</sup> either singly or more dramatically, when combined with Fgf5<sup>P</sup> homozygosity. These effects on viability and growth were observed in animals of the genotype [Fgf5<sup>P</sup>-/-; Tgfa<sup>rs-1</sup> +/-; Egfr<sup>rs-2</sup>-/-] with a greater degree of runting (~20%) evident. A more extreme viability effect was observed in the triple homozygotes which also affected epidermal development. Homozygosity for each of the three mutations generally resulted in a greater severity of runting compared to the double homozygote [Tgfa<sup>rs-1</sup>-/-; Egfr<sup>rs-2</sup>-/-] genotype described in Chapter 3, with three of the four animals expressing a severely runted phenotype.

The epithelial immaturity generally evident in these triple homozygotes is similar to that described for several of the transgenic strains which carry null alleles of the Egfr locus (Miettinen et al., 1995; Sibilia and Wagner, 1995; Threadgill et al., 1995). As can be seen from mid-dorsal skin sections, the epidermis of these animals is thinner and less well developed than their waved/angora and angora littermates (Figure 4.4 and 4.5). A comparison of follicle growth between the angora, waved/angora and the 7 day old triple homozygote reveals that epidermal development is significantly delayed in the triple homozygote. This skin phenotype is very similar to that reported for a 6 day old EGFR null mutant (Miettinen et al., 1995) with a similar hairless flaky dorsal skin and an almost identical histological epidermal phenotype.

It has been suggested by Miettinen et al. (1995) that a common denominator of the EGFR null mutation phenotype is a defective or delayed epithelial development of several tissues, a phenotype that is consistent with observations of a retarded epidermal development in these triply homozygous mice. It is also important to note that even within strains, the expression of the EGFR null phenotype varied suggesting that considerable genetic variation was still present on the strain background. This phenotypic variation ranged from animals that died or were cannibals early after birth to those which survived for longer periods and exhibited a delayed epidermal development and a failure to thrive and was suggested to be the result of segregating
modifier loci affecting the activity of the impaired EGFR pathway (Miettinen et al., 1995; Sibilia and Wagner, 1995; Threadgill et al., 1995).

The importance of modifier genes, genes with small effect that may interact with other loci by either enhancing or suppressing a particular phenotype, is becoming evident from the study of traits which do not exhibit simple mendelian inheritance (Frankel, 1995). The focus of mammalian genetics has often centred on single gene mutations although it has been recognised that even simple single gene mutations can be affected by the presence of modifying loci, so that a number of loci may be involved in determining the expression of even a simple mendelian phenotype. I have approached the issue of potential genetic modifiers by postulating the existence of a functional relationship between Tgfa<sup>−/+</sup>, Egrf<sup>−/−</sup> and Fgf5<sup>−/−</sup>. This hypothesis was based on experimental data suggesting that these three molecules may interact in vitro and from their effects on the regulation of mouse hair growth. Moreover, as the normal products of these loci interact biochemically and functionally, it is expected that mutations in these loci may interact genetically.

The enhanced severity of growth and viability effects noted in animals carrying all three mutations suggest that they may be potential modifiers of each other. The Egrf<sup>−/−</sup> and Fgf5<sup>−/−</sup> mutations clearly interact, with this study providing strong statistical support. However the spectrum of epidermal and viability phenotypes observed suggests that there may also be other modifiers segregating. The apparently increased severity of the Egrf<sup>−/−</sup> viability effect observed in the triple homozygote genotype suggests that the combination of these three mutations has had a greater effect on viability than the effects of Egrf<sup>−/−</sup> and Fgf5<sup>−/−</sup> alone. The repeatable disruption in Egrf<sup>−/−</sup> genotype frequencies in combination with Tgfa<sup>−/1</sup>, +/− and −/− genotypes is evidence for this. Therefore, the effects of these three mutations in combination appears to be consistent with Fgf5<sup>−/−</sup> acting as a strong modifier to enhance the Egrf<sup>−/−</sup> viability effect whereas the enhancing effects of Tgfa<sup>−/1</sup> are much milder.

The oldest triple homozygote recovered revealed a remarkably different phenotype to the other severely runted triple homozygotes. This individual had largely suppressed the angora phenotype and developed a dorsal coat which was shorter and less waved than expected for an animal of this genotype. The suppression of the angora phenotype and the apparent viability of this animal emphasises the relevance of background modifier loci on the expression of these mutations. The viability effects that were observed in the other triple homozygotes seem to have been largely suppressed so that an animal with the same recessive mutations has developed more normally. Interestingly, a milder example of suppression is possibly evident in one of the double homozygotes described Chapter 3. The longest surviving double homozygotes (see Figure 3.7) was also phenotypically distinct from its waved and wild-type littermates.
and showed a partial suppression of the waved-hair phenotype and a delayed hair cycle.

In considering a possible molecular basis for this suppression, it is important to note that, although angora mice have an extendedagen phase, they do eventually undergo catagen. This suggests that another FGF-5 independent pathway is involved in the control of hair follicle growth and may have been upregulated in this suppressed animal. Other components of these conserved RPTK signalling cascades may be potential candidates. For instance, a gain of function mutation in MAPK can bypass multiple mutated RPTK pathways (Brunner et al., 1994) while a suppression of the vulvaless mutations in the \textit{lin3} and \textit{let 23} mutations (the \textit{C. elegans} homologues of TGF-\textit{u} and EGFR) can occur if a constitutively activated form of MAPK Kinase, a downstream component of the MAPK phosphorylation cascade, is expressed (Koga and Oshima, 1995).

The suppression of mutant phenotypes is being studied intensely in a number of genetically well defined organisms. This has been the basis for the genetic dissection of developmental pathways affecting well defined developmental systems such as the \textit{C. elegans} vulva and the \textit{Drosophila} eye. Often the genetic screening of a large number of mutant offspring has been undertaken to isolate individuals with either a suppression or enhancement of a mutant phenotype. This has resulted in the identification of gene products involved in the regulatory pathway mutated. The genetic dissection of the EGFR signalling cascade in \textit{C. elegans}, \textit{Drosophila} and recently in the mouse (see Chapter 1) has identified both suppressors and enhancers which interact epistatically with mutations in the EGFR pathway (Sundberg, 1994; Simon et al., 1991; Rogge et al., 1991; Wang et al., 1997). The genetic identification of these interacting components is, when combined with molecular biology, revealing a highly conserved signalling paradigm.

I am interested in developing a similar understanding of the EGFR signalling pathway in the mouse. I have uncovered preliminary evidence for the existence of two modifiers of the epidermal and viability phenotypes of the \textit{Egf}\textsuperscript{max} mutation. Clearly the\textit{Fgf}\textsuperscript{max} mutation strongly enhances the \textit{Egf}\textsuperscript{max} mutation, to influence viability. A much milder enhancement by \textit{Tgf}\textsuperscript{max} is suggested from the disruption in \textit{Egf}\textsuperscript{max} frequencies. Other uncharacterised modifier loci (or possibly environmental factors) have suppressed expression of the epidermal and viability phenotypes in one compound homozygote. The results generated in this study have genetically confirmed the identity of two other molecular components of the mammalian EGFR signalling pathway. Therefore the use of an inferred biochemical relationship between three loci has been a successful approach toward genetically dissecting the EGFR signalling pathway in the mouse.
Chapter Five: A statistical analysis of the effects of mutations at the Tgfα and Egfr loci on growth rate

Introduction

The rationale for establishing an in vivo analysis of the combined phenotypic effects of the three mutations; Tgfα<sup>−/−</sup>, Egfr<sup>−/−</sup> and Fgfs<sup>−/−</sup>, was based upon published data suggestive of a signal transduction pathway between an extracellular ligand, its receptor and a downstream primary response gene. The breeding of mice with various combinations of these three mutations has revealed evidence for an effect on epidermal development and viability. The deficiency of multiple homozygous genotypes from mendelian expectation provide evidence for this (Figure 5.1A). The viability effects of these mutations is evident in a number of genotypes although it is most apparent in the decreased number and severe runting of the triple homozygote (Figure 5.2B). An analyses of the growth rate data collected from these same progeny has also revealed that these mutations have an effect on growth rate. Unfortunately the growth rate data are somewhat compromised since the most severely stunted animals (multiple mutant homozygotes) tended to be cannibalised and thus are not included in this analysis of growth.

Results

An analysis of growth rate data with respect to genotype was undertaken, although the small sample sizes of several genotypes, notably those with the greatest number of mutations, means that only tentative conclusions can be drawn. Mean growth rates are presented in Tables 5.1 and 5.2 for animals discussed in Chapters 3 and 4 respectively with respect to epidermal and viability phenotypes. Analyses of Variance (ANOVA) under assumptions of a General Linear Model (GLM) were undertaken on these data (see acknowledgments) to determine if significant genotypic effects at the Tgfα and Egfr loci were present and if any significant interactions were occurring between these loci on either a wild-type Fgfs<sup>+/+</sup> or Fgfs<sup>−/−</sup> genetic background. Growth rate means were assessed for both main effects and interactions with regard to the Tgfα<sup>−/−</sup> and Egfr<sup>−/−</sup> genotypes on both genetic backgrounds. The four models used to analyse the data are presented in Tables 5.3-5.7.

As the standard deviations of each genotype category appeared fairly constant an ANOVA was undertaken. Since the experimental design was unbalanced terms may be removed sequentially, as would be done in a multiple regression analysis. Interactions were tested before main effects. In Model 1 (Table 5.3) effects of genotypes at the two loci nested within genetic background were examined for an interaction effect between the Tgfα<sup>−/−</sup> and Egfr<sup>−/−</sup> loci. No significant interaction effect
was seen between these loci on the Fgf5\(^\circ\) +/+ or Fgf5\(^\circ\) -/- genetic backgrounds (p=0.214). In Model 2 (Table 5.4) the interaction effect has been removed as it is not significant and the main effect of genotype at each locus analysed. Both genotypic main effects are significant (p<0.0005). Model 3 (Table 5.5) was used to determine if genotypic main effects varied with genetic background. This revealed that the interaction effects of each of these loci with the genetic background (Fgf5\(^\circ\) +/+ or Fgf5\(^\circ\) -/-) were not significant (p=0.501 and 0.617), but as expected from the model 2 analysis, significant main effects of each locus are still evident. As the results were the same as for Model 2 and no significant interaction effect was seen with genetic background, the genotype by genetic background effect can be removed. Model 4 (Table 5.6) examines the main effects of genotype with no distinction between the genetic background of each cross. This reveals significant main effects of the Tgfa\(^{ws-1}\) and Egfr\(^{ws-2}\) genotypes and for the Fgf5\(^\circ\) genetic background (p<0.0005).

A slightly different picture emerges if growth rate data for each experiment are analysed separately without regard to the genetic background (Table 5.7 and 5.8). Here a significant interaction effect is seen between the Tgfa\(^{ws-1}\) and Egfr\(^{ws-2}\) loci in the progeny genotyped from Chapter 3 (p=0.031) but not for those genotyped in Chapter 4 (p=0.949). The interaction effect observed is for an inflated mean for the [Tgfa\(^{ws-1}\) +/+; Egfr\(^{ws-2}\) -/-] genotype and does not seem to be experimentally relevant. As the three way model (Model 1) did not reveal a significant interaction effect between Tgfa\(^{ws-1}\) and Egfr\(^{ws-2}\), a conclusion that can be drawn from these analyses is that it is only the main effects of each genotype which are evident and there is no significant interaction effect between Tgfa\(^{ws-1}\), Egfr\(^{ws-2}\) or Fgf5\(^\circ\) genotypes in either experiment. Therefore, although model 3 has shown that there appears to be no significant interaction effect with Fgf5\(^\circ\), model 4 has shown that there is a significant main effect of this locus with animals homozygous for Fgf5\(^\circ\) -/- consistently having a lower mean growth rate (compare 5.2 A with 5.2 B). An overall conclusion from this quantitative study may be that mean growth rate varies significantly across the three Tgfa\(^{ws-1}\) genotypes (+/+; +/--; -/-) and across the three Egfr\(^{ws-2}\) genotypes (+/+; +/--; -/-). The genetic background (either Fgf5\(^\circ\) +/+ or Fgf5\(^\circ\) -/-) also has a significant main effect suggesting that each locus contributes in an additive manner to a depression of mean growth.
Figure 5.1 Diagrammatic representations of the deficiencies of homozygous mutant classes at the Tgα<sup>ms-1</sup> and Egfr<sup>ms-2</sup> loci when the background is either homozygous wild-type or homozygous for Fgf5<sup>pro</sup>. A. A histogram showing the decrease in frequency of multiply homozygous genotypes. Observed frequencies for each cross has been extracted from Table 3.1 and Table 4.1. Expected frequencies have been calculated from mendelian expectations for two unlinked loci. B. A viability plot from the same data. Viability values for each genotype were calculated by dividing the observed frequency by the expected value. A viability score of 1 indicates that there is no change from the expected frequency. Viability scores greater than 1 indicate that these genotypes were more prevalent than expected while values less than 1 suggest that the viability is affected. Note the decrease in viability once homozygosity for Egfr<sup>ms-2</sup> has been established.
A. Expected vs Observed Genotypes

- Expected Frequency

B. Viability vs Genotype

- Viability
### Table 5.1 Growth rates by genotype. An analysis of growth rates based on individual genotype for the progeny examined in Chapter 3. Segregation of the Egfr<sup>ws2</sup> and Tgf-α<sup>ws1</sup> mutations are on a wild-type background. Mean growth rates (gram/day), minimum (Min) and maximum (Max) values, variance (Var) and standard error of the mean (SEM) for progeny genotyped from this study. N is sample size while N/A are the number of individuals not included in this analysis due to an insufficient number of weighings.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Min</th>
<th>Mean</th>
<th>Max</th>
<th>Var</th>
<th>SEM</th>
<th>N</th>
<th>N/A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tgfα&lt;sup&gt;ws1&lt;/sup&gt;+/+; Egfr&lt;sup&gt;ws2&lt;/sup&gt;+/+</td>
<td>.4336</td>
<td>.5582</td>
<td>.6622</td>
<td>.0079</td>
<td>.0256</td>
<td>12</td>
<td>0</td>
</tr>
<tr>
<td>Tgfα&lt;sup&gt;ws1&lt;/sup&gt;+/+; Egfr&lt;sup&gt;ws2&lt;/sup&gt;+/+</td>
<td>.4258</td>
<td>.5726</td>
<td>.7025</td>
<td>.0071</td>
<td>.0199</td>
<td>18</td>
<td>0</td>
</tr>
<tr>
<td>Tgfα&lt;sup&gt;ws1&lt;/sup&gt;-/-; Egfr&lt;sup&gt;ws2&lt;/sup&gt;+/+</td>
<td>.3414</td>
<td>.4786</td>
<td>.7003</td>
<td>.0107</td>
<td>.0298</td>
<td>12</td>
<td>2</td>
</tr>
<tr>
<td>Tgfα&lt;sup&gt;ws1&lt;/sup&gt;+/+; Egfr&lt;sup&gt;ws2&lt;/sup&gt;-/-</td>
<td>.3683</td>
<td>.5402</td>
<td>.6972</td>
<td>.0062</td>
<td>.0185</td>
<td>16</td>
<td>1</td>
</tr>
<tr>
<td>Tgfα&lt;sup&gt;ws1&lt;/sup&gt;+/+; Egfr&lt;sup&gt;ws2&lt;/sup&gt;-/-</td>
<td>.2157</td>
<td>.5260</td>
<td>.8400</td>
<td>.0151</td>
<td>.0178</td>
<td>48</td>
<td>1</td>
</tr>
<tr>
<td>Tgfα&lt;sup&gt;ws1&lt;/sup&gt;-/-; Egfr&lt;sup&gt;ws2&lt;/sup&gt;+/+</td>
<td>.1186</td>
<td>.4656</td>
<td>.8260</td>
<td>.0227</td>
<td>.0345</td>
<td>19</td>
<td>0</td>
</tr>
<tr>
<td>Tgfα&lt;sup&gt;ws1&lt;/sup&gt;+/+; Egfr&lt;sup&gt;ws2&lt;/sup&gt;-/-</td>
<td>.4499</td>
<td>.5342</td>
<td>.6290</td>
<td>.0045</td>
<td>.0224</td>
<td>9</td>
<td>3</td>
</tr>
<tr>
<td>Tgfα&lt;sup&gt;ws1&lt;/sup&gt;+/+; Egfr&lt;sup&gt;ws2&lt;/sup&gt;-/-</td>
<td>.2251</td>
<td>.3704</td>
<td>.5973</td>
<td>.0177</td>
<td>.0421</td>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td>Tgfα&lt;sup&gt;ws1&lt;/sup&gt;-/-; Egfr&lt;sup&gt;ws2&lt;/sup&gt;-/-</td>
<td>.1740</td>
<td>.1740</td>
<td>.1740</td>
<td>N/A</td>
<td>N/A</td>
<td>1</td>
<td>3</td>
</tr>
</tbody>
</table>

### Table 5.2 Growth rates by genotype. An analysis of growth rate based on genotype for progeny examined in chapter 4. Segregation of the Egfr<sup>ws2</sup> and Tgf-α<sup>ws1</sup> mutations are on a homozygous Fgf-5<sup>−/−</sup> background. Statistical nomenclature as for Table 5.1.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Min</th>
<th>Mean</th>
<th>Max</th>
<th>Var</th>
<th>SEM</th>
<th>N</th>
<th>N/A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tgfα&lt;sup&gt;ws1&lt;/sup&gt;+/+; Egfr&lt;sup&gt;ws2&lt;/sup&gt;+/+</td>
<td>.2083</td>
<td>.4357</td>
<td>.6650</td>
<td>.0155</td>
<td>.0278</td>
<td>20</td>
<td>2</td>
</tr>
<tr>
<td>Tgfα&lt;sup&gt;ws1&lt;/sup&gt;+/+; Egfr&lt;sup&gt;ws2&lt;/sup&gt;+/+</td>
<td>.2067</td>
<td>.4225</td>
<td>.6704</td>
<td>.0146</td>
<td>.0199</td>
<td>37</td>
<td>9</td>
</tr>
<tr>
<td>Tgfα&lt;sup&gt;ws1&lt;/sup&gt;-/-; Egfr&lt;sup&gt;ws2&lt;/sup&gt;+/+</td>
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<td>.3319</td>
<td>.5942</td>
<td>.0276</td>
<td>.0402</td>
<td>17</td>
<td>4</td>
</tr>
<tr>
<td>Tgfα&lt;sup&gt;ws1&lt;/sup&gt;-/-; Egfr&lt;sup&gt;ws2&lt;/sup&gt;+/+</td>
<td>.1673</td>
<td>.4224</td>
<td>.6142</td>
<td>.0123</td>
<td>.0206</td>
<td>29</td>
<td>6</td>
</tr>
<tr>
<td>Tgfα&lt;sup&gt;ws1&lt;/sup&gt;-/-; Egfr&lt;sup&gt;ws2&lt;/sup&gt;-/-</td>
<td>.1370</td>
<td>.4251</td>
<td>.6537</td>
<td>.0121</td>
<td>.0144</td>
<td>58</td>
<td>13</td>
</tr>
<tr>
<td>Tgfα&lt;sup&gt;ws1&lt;/sup&gt;-/-; Egfr&lt;sup&gt;ws2&lt;/sup&gt;-/-</td>
<td>.0475</td>
<td>.3296</td>
<td>.5279</td>
<td>.0154</td>
<td>.0239</td>
<td>27</td>
<td>13</td>
</tr>
<tr>
<td>Tgfα&lt;sup&gt;ws1&lt;/sup&gt;-/-; Egfr&lt;sup&gt;ws2&lt;/sup&gt;-/-</td>
<td>.1300</td>
<td>.2828</td>
<td>.3900</td>
<td>.0089</td>
<td>.0334</td>
<td>8</td>
<td>3</td>
</tr>
<tr>
<td>Tgfα&lt;sup&gt;ws1&lt;/sup&gt;-/-; Egfr&lt;sup&gt;ws2&lt;/sup&gt;-/-</td>
<td>.1483</td>
<td>.3007</td>
<td>.5443</td>
<td>.0131</td>
<td>.0361</td>
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<td>9</td>
</tr>
<tr>
<td>Tgfα&lt;sup&gt;ws1&lt;/sup&gt;-/-; Egfr&lt;sup&gt;ws2&lt;/sup&gt;-/-</td>
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<td>.2842</td>
<td>.2842</td>
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<td>N/A</td>
<td>1</td>
<td>2</td>
</tr>
</tbody>
</table>
Table 5.3. Model 1: Genetic background, two genotypes nested within genetic background, + interaction. DF degrees of freedom, Seq SS (sequential sum of squares), Adj SS (adjusted sum of squares), Adj MS (adjusted mean squares), F (F-statistic), P (probability value).

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Seq SS</th>
<th>Adj SS</th>
<th>Adj MS</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genetic Background(GB)</td>
<td>1</td>
<td>1.20992</td>
<td>0.32080</td>
<td>0.32080</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tgα−/− (GB)</td>
<td>4</td>
<td>0.35905</td>
<td>0.23818</td>
<td>0.05954</td>
<td></td>
<td>4.33</td>
</tr>
<tr>
<td>Egfr+/− (GB)</td>
<td>4</td>
<td>0.50311</td>
<td>0.27490</td>
<td>0.06873</td>
<td></td>
<td>5.00</td>
</tr>
<tr>
<td>Tgα−/−Egfr+/− (GB)</td>
<td>8</td>
<td>0.14937</td>
<td>0.14937</td>
<td>0.01887</td>
<td>1.38</td>
<td>0.214</td>
</tr>
<tr>
<td>Error</td>
<td>338</td>
<td>4.62248</td>
<td>0.01376</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>353</td>
<td>6.84392</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 5.4. Model 2: Genetic background, two genotypes nested within genetic background

<table>
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<tr>
<th>Source</th>
<th>DF</th>
<th>Seq SS</th>
<th>Adj SS</th>
<th>Adj MS</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genetic Background(GB)</td>
<td>1</td>
<td>1.20991</td>
<td>0.93146</td>
<td>0.93146</td>
<td>67.15</td>
<td>0.000</td>
</tr>
<tr>
<td>Tgα−/− (GB)</td>
<td>4</td>
<td>0.35905</td>
<td>0.49035</td>
<td>0.12259</td>
<td></td>
<td>8.84</td>
</tr>
<tr>
<td>Egfr+/− (GB)</td>
<td>4</td>
<td>0.50311</td>
<td>0.50311</td>
<td>0.12578</td>
<td></td>
<td>9.07</td>
</tr>
<tr>
<td>Error</td>
<td>344</td>
<td>4.77184</td>
<td>4.77184</td>
<td>0.01387</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>353</td>
<td>6.84392</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 5.5. Model 3: Genetic Background, two genotypes and their interactions with the genetic background.

<table>
<thead>
<tr>
<th>Source</th>
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<th>Seq SS</th>
<th>Adj SS</th>
<th>Adj MS</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genetic Background (GB)</td>
<td>1</td>
<td>1.20991</td>
<td>0.93148</td>
<td>0.93146</td>
<td>67.15</td>
<td>0.000</td>
</tr>
<tr>
<td>(Tgf\alpha^{+/+})</td>
<td>2</td>
<td>0.33976</td>
<td>0.45363</td>
<td>0.22881</td>
<td>16.35</td>
<td>0.000</td>
</tr>
<tr>
<td>GB * (Tgf\alpha^{+/+})</td>
<td>2</td>
<td>0.01929</td>
<td>0.01919</td>
<td>0.00960</td>
<td>0.59</td>
<td>0.501</td>
</tr>
<tr>
<td>(Egr^{+/+})</td>
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<td>0.45966</td>
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<td>0.24793</td>
<td>17.87</td>
<td>0.000</td>
</tr>
<tr>
<td>GB * (Egr^{+/+})</td>
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<td>0.01343</td>
<td>0.00672</td>
<td>0.48</td>
<td>0.617</td>
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<tr>
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<td>0.01987</td>
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<td></td>
</tr>
<tr>
<td>Total</td>
<td>353</td>
<td>6.84392</td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

Table 5.6. Model 4: Genetic background, two genotypes.

<table>
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<tr>
<th>Source</th>
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<th>Seq SS</th>
<th>Adj SS</th>
<th>Adj MS</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genetic Background (GB)</td>
<td>1</td>
<td>1.20991</td>
<td>1.32731</td>
<td>1.32731</td>
<td>96.08</td>
<td>0.000</td>
</tr>
<tr>
<td>(Tgf\alpha^{+/+})</td>
<td>2</td>
<td>0.33976</td>
<td>0.46762</td>
<td>0.23381</td>
<td>16.92</td>
<td>0.000</td>
</tr>
<tr>
<td>(Egr^{+/+})</td>
<td>2</td>
<td>0.48870</td>
<td>0.48870</td>
<td>0.24335</td>
<td>17.62</td>
<td>0.000</td>
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<td>Error</td>
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<td>4.80755</td>
<td>0.01381</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>353</td>
<td>6.84392</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 5.7 Model 5: Two genotypes + interaction (Chapter 3 progeny)

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Seq SS</th>
<th>Adj SS</th>
<th>Adj MS</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tgfa\textsuperscript{wt}</td>
<td>2</td>
<td>0.12441</td>
<td>0.20404</td>
<td>0.10202</td>
<td>8.07</td>
<td>0.000</td>
</tr>
<tr>
<td>Egr\textsuperscript{wt}</td>
<td>2</td>
<td>0.22510</td>
<td>0.19633</td>
<td>0.09817</td>
<td>7.76</td>
<td>0.001</td>
</tr>
<tr>
<td>Tgfa\textsuperscript{wt} Egr\textsuperscript{wt}</td>
<td>4</td>
<td>0.13895</td>
<td>0.13895</td>
<td>0.03474</td>
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<td>0.031</td>
</tr>
<tr>
<td>Error</td>
<td>138</td>
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<td>1.74496</td>
<td>0.01284</td>
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<td></td>
</tr>
<tr>
<td>Total</td>
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<td>2.23342</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 5.8 Model 6 Two genotypes + interaction (Chapter 4 progeny)

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Seq SS</th>
<th>Adj SS</th>
<th>Adj MS</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tgfa\textsuperscript{wt}</td>
<td>2</td>
<td>0.23464</td>
<td>0.03414</td>
<td>0.01707</td>
<td>1.17</td>
<td>0.311</td>
</tr>
<tr>
<td>Egr\textsuperscript{wt}</td>
<td>2</td>
<td>0.27801</td>
<td>0.07857</td>
<td>0.03928</td>
<td>2.7</td>
<td>0.069</td>
</tr>
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<td>Tgfa\textsuperscript{wt} Egr\textsuperscript{wt}</td>
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<td>0.01042</td>
<td>0.00250</td>
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<tr>
<td>Egr\textsuperscript{wt}</td>
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<td>2.87752</td>
<td>0.01453</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tgfa\textsuperscript{wt} Egr\textsuperscript{wt}</td>
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<td>3.40059</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 5.2 A and B A three dimensional surface plot of mean growth rates based on genotype. Mean growth rates for each genotype (Table 5.1 and Table 5.2) were graphed with regard to the genotype at the Tgfa** and Egfr** loci for each of the three allelic combinations (+/+; wild-type; +/- heterozygote; -/- mutant homozygote). Growth rate (g/day) is on the z-axis with Egfr and Tgfa genotypes on the x and y axis respectively. A clear decrease in growth rates is seen in multiple homozygous genotypes. A difference in the overall mean growth rates of these genotypes on either A, the wild-type (Fgf5** +/+ ) background or B, the angora (Fgf5** -/- ) background is also evident. It is important to note that in both graphs only one doubly homozygous individual is included in this analysis and in the case of the angora background this individual showed a significant suppression of the compound mutant phenotype.
Discussion

The statistical detection and interpretation of genetic interaction has been recognised as a difficult issue due to problems associated with sample size and the confounding effects of genetic background (Frankel and Schork, 1996). These problems are accentuated when the viability of individuals is compromised within certain mutant combinations. In genotyping two series of mice segregating for the Tgfa<sup>−/−</sup> and Egfr<sup>−/−</sup> loci on either a wild-type Fgf5<sup>−/−</sup> +/+ or homozygous Fgf5<sup>−/−</sup> -/- genetic background deficiencies and excesses of certain genotypes have been observed (Figure 5.1A and B). In general, there is an increasing tendency for deficiency of homozygous mutant classes with each additional mutation which is added. This decrease in viability is especially evident for Egfr<sup>−/−</sup> -/- homozygotes on the angora background. It is also important to note that certain genotypes were selectively affected by parental cannibalism. Offspring exhibiting a runted phenotype were often eaten soon after birth, making the collection of growth data impossible. This means that for some genotypic categories, particularly the double homozygotes, growth rate is overestimated.

Although there appears to be little or no evidence for interaction effects between the loci, both the Tgfa<sup>−/−</sup> and Egfr<sup>−/−</sup> loci have a consistent, cumulative, and highly statistically significant effect on growth. Indeed mutation at the Fgf5<sup>−/−</sup> locus also appears to significantly depress growth rate. This difference however needs to be viewed with caution and may be due to other loci segregating on the angora genetic background. However, considering the phenotypic effects of the triple homozygote and their general decrease in viability, it is likely that differences in mean growth rate between these F<sub>2</sub> series are the result of Fgf5<sup>−/−</sup> homozygosity.

Growth rate decreases as the number of mutant alleles at both the Tgfa<sup>−/−</sup> and Egfr<sup>−/−</sup> loci increases, and is clearly seen in Figure 5.2A when compared to Figure 5.2B. It is also important to note that in both the Fgf5<sup>−/−</sup> +/+ and Fgf5<sup>−/−</sup> -/- backgrounds only one animal homozygous at both the Tgfa<sup>−/−</sup> and Egfr<sup>−/−</sup> loci has been included in this analysis of growth rate. This was because only animals with two or more weighings could be included for the derivation of individual daily growth rates. In one case the doubly homozygous individual exhibited signs of suppressing the mutant phenotype (the triple homozygote uncovered in Chapter 4). As growth rate data was only examined for animals which were viable this means that the depressive effects of these mutations on growth rate is likely to be underestimated in the least viable categories. For a number of animals, especially those Tgfa<sup>−/−</sup>-/- and Egfr<sup>−/−</sup>-/- homozygotes segregating on the homozygous Fgf5<sup>−/−</sup> -/- background, an analysis of growth rate was not possible due to their runted phenotype which necessitated their early collection to prevent parental cannibalism. Taking this into consideration, it is likely that the combined depressive effect on growth rate is much larger.
A clearly negative slope of the surface plot is evident in both Figure 5.2A and 5.2B. The differences in mean growth rate between the genotyped progeny of chapter 3 and those analysed in chapter 4 is also obvious. However, the effects of angora can only be inferred from model 3 and 4 where the main effects of genetic background are highly significant. This is apparent from the greater mean growth rate for most genotypes on the Fgf50 +/+ background when compared to those on the Fgf50 -/- background. Considering the effects on viability of increasing homozygosity at these mutant loci it is possible that a threshold level of activity through the signal transduction pathway is occurring in these multiple homozygous individuals. This decrease in activity below a certain threshold level may result in the novel phenotype expressed by the triple homozygotes which precludes an analysis of growth rate for these genotypes.
Chapter Six: Temporally specific induction of Fgf5 and Tgfα in the epidermis of EGF treated Merino sheep

Introduction

The epidermis of Merino sheep produces many long thin wool fibres, the result of many generations of selective improvement which is likely to involve numerous genetic factors. Wool fibre growth in the Merino sheep is a largely continuous process without regular progression through the hair cycle although the inhibition of wool growth can be induced under certain circumstances. These include periods of environmental, physiological and nutritional stress which can change wool follicle diameter and affect the tensile strength of the fibre. The resulting phenomena, described as ‘tender’ wool (Lang, 1945), has important implications for the wool industry with the reduction in tensile strength associated with tender fibres decreasing its utility in the manufacturing process. Other qualities of the fibre such as the degree of crimping or ‘waviness’ may also be a consideration. An understanding of the changes in gene activity associated with wool fibre growth and its morphological characteristics in the Merino may contribute to the identification of animals less susceptible to undesirable traits and exhibiting qualities likely to add value for the producer.

The treatment of the Merino sheep with purified mouse EGF results in a temporary synchronised inhibition of wool follicle growth (Moore et al., 1985). This inhibition of cellular growth in the anagen wool fibre is considered to be similar to the growth inhibitory state entered into by animals producing ‘tender’ wool. The decrease in fibre diameter induced by EGF allows a break to develop in the fibre allowing the wool fibre to be removed. The morphological changes associated with this growth inhibition are also similar to the initial stages of the anagen to catagen transition seen in the hair cycle of mice although a complete passage through all catagen phases to telogen does not occur (Hollis et al., 1983).

By utilising this synchronous inhibition of wool follicle growth induced by EGF and its resemblance to the early stages of catagen, an investigation of peptide growth factors associated with epidermal growth regulation is possible. The catagen-like morphological changes observed in EGF treated sheep may involve peptide growth factors implicated in mouse epidermal growth if their function has been conserved. A study of these molecules could allow a spatial and temporal understanding of the molecular biology of mammalian epidermal growth control.
EGF and the inhibition of wool follicle growth

The purification of EGF from the sub-maxillary glands of male mice and its subsequent administration to newborn mice was the first documented evidence that a peptide growth factor could influence the development of the hair follicle (Levi-Montalcini and Cohen, 1960; Cohen 1962). A more detailed study revealed a decrease in the growth rate of the hair fibre as well as a reduction in fibre diameter and length of the proliferative hair bulb (Moore et al., 1981). This reduction in bulb length was associated with a decrease in the number of proliferative cells in the bulb region (or hair matrix) of the anagen follicle. The number or size of cells in the dermal papilla showed no significant change in number following EGF treatment (Moore et al., 1983). Similar effects on the first hair coat in new born mice injected with purified TGF-α have also been reported (Tam, 1985).

The absence of EGF during development has also been associated with changes in normal epidermal growth. Anti-EGF sera administered to newborn mice resulted in a reversal of the phenotype associated with EGF treatment (Zschiesche et al., 1988). Mice exhibited delayed eyelid opening and tooth eruption and an acceleration of hair growth. This has also been examined in utero using female rats which produce EGF auto-antibodies. Progeny were produced which exhibited a thinner, dry and wrinkled epidermis and immature hair follicle development (Raaberg et al., 1995).

In contrast to the mouse, where the inhibitory effects of EGF on hair growth are seen only in the first hair coat of newborns, EGF treatment in the adult Merino sheep has a major effect. A significant change in the proliferative state of the bulb cells occurs 24 hours after EGF infusion with the lowest mitotic indices observed after 48 hours. The proliferative rate of the bulb cells returns to normal approximately 8 days after EGF infusion (Moore et al., 1985). Interestingly, the effect of EGF on the basal epidermal cells of the interfollicular regions and the cells of the sebaceous gland was proliferative rather than inhibitory with a resulting thickening of the interfollicular epidermis. This paradoxical effect of EGF on the sheep epidermis is unresolved.

EGF treated wool follicles exhibit characteristics similar to that described during the anagen to catagen transition of the mouse hair cycle (Hollis et al., 1983, Hollis and Chapman, 1989). Morphological changes comparable to those seen in the catagen stages II-VI of the mouse hair cycle (Straille, 1961) have been observed in the regressing wool follicle (Figure 6.1). However the disruption of the fibre and IRS (a follicle lesion that occurs in some fibres) and the sebaceous gland hyperplasia following EGF infusion have not been reported in the mouse and are considered to be a pathological response to EGF treatment in the sheep (Hollis et al., 1987).
Apoptotic cell death is also evident in the differentiating cells of the proximal region of the follicle bulb, an observation consistent with the induction of a catagen-like state while sebaceous gland hyperplasia also eventually returns to normal by an apoptotic mechanism (Hollis et al., 1987). Retraction of the dermal papilla to a position beneath the fibre also does not occur as is usual in the normal telogen hair follicle, but remains separated from the fibre by a column of ORS cells. The dermal papilla exhibits an initial elongation and narrowing with the ORS increasing in thickness around the regressing follicle bulb in contrast with the shortened ball shaped dermal papilla normally seen in the hair cycle (Straile, 1981). The usual 'brush end' of a typical telogen follicle is also absent in regressed wool follicles which develop a more tapered end enclosed by a distorted IRS. Although a complete transition of the wool follicle through all stages of catagen does not occur, the inhibitory effects of EGF may share similarities with the initial catagen stages of the mouse hair cycle.

Figure 6.1 A diagrammatic representation of the catagen-inducing effects of EGF treatment in Merino wool follicles (reprinted with permission from Hollis and Chapman, 1989). The stages shown represent the morphological changes induced by EGF administration after 1-12 hours through to 1, 2, 3 and 4 days respectively. Initial changes in fibre morphology were similar in all follicles until 2 days when fibres either formed fibre ends or a thinned fibre. A- E represent the bulb, lower, mid and upper keratogenous zone (KZ) respectively. The zone of hardening is represented by E. The shaded region marks the dysjunction between the fibre and IRS observed in some regressing wool follicles (B, bulb; D, Dermal papilla; E, epidermis; F, fibre; IRS, inner root sheath; ORS, outer root sheath; SG, sebaceous gland).
EGFR, EGF and TGF-α localization in the wool follicle

The detection of an EGF-like molecule in the periderm of embryonic skin, the ORS of wool follicles and the sebaceous glands of Merino epidermis may be indicative of a role for this growth factor both during development and in the adult. EGF immunoreactive material is detected in regions of the epidermis associated with cellular proliferation. This was predominantly in the keratogenous zone of the ORS and also in the sebaceous glands where it may be produced and secreted (du Cros et al., 1992). It is interesting to note that in vitro both human and mouse keratinocytes do not produce detectable EGF transcript when stimulated with other EGFR ligands although other EGFR ligands are upregulated (Barnard et al., 1994; Dlugosz et al., 1995).

TGF-α immunoreactivity has been reported in the ORS and cortical cells of the keratogenous zone of anagen wool follicles while no immunoreactive signal is detectable in the IRS, follicle bulb and dermal papilla regions (Nixon et al., 1996). During a photoperiod induced catagen and telogen stage, immunostaining decreased and was not re-established in the ORS of the wool follicle until late anagen. TGF-α was also absent from the cortical cells of wool follicles which had resumed early anagen growth. Epidermal expression of TGF-α remained intense in the epidermis and the sebaceous glands during all stages of this photoperiod manipulation of wool follicle growth. The authors have suggested this may indicate an autocrine or juxtacrine mode of action for this growth factor which may be predominantly membrane bound.

The EGFR has been localised in both the embryonic and adult Merino epidermis by radio-labelled EGF detection of putative EGFR sites (Wynn et al., 1989; Wynn et al., 1995). During embryogenesis, EGFR binding sites were detected throughout the epidermis including the epidermal cell layer juxtaposed to the dermis and in the developing epithelial aggregations of wool follicle primordia. A general decrease in binding affinity was noted in the epidermis around the time of follicle initiation, although a specific decrease in areas overlying the dermis of the developing follicle was not observed. This contrasts with a localised decrease of EGFR overlying the presumptive mesenchymal condensate observed in the rat epidermis around the time of follicle initiation (Green and Couchman, 1984). In the mature wool follicle, EGF binding sites are observed throughout the basal keratinocyte layer of the epidermis with particular aggregations noted in the bulb matrix, ORS, sebaceous and sweat glands. Binding sites may also be present in the IRS around the zone of keratinisation in the IRS/ORS juncture, an area which can be disrupted after EGF treatment.
FGF localisation in the wool follicle

Compared to the mouse hair follicle, relatively few members of the FGF family of peptide growth factors have been analysed in the wool follicle. Only FGF-1 (acidic FGF) and FGF-2 (basic FGF) have been detected by immunohistochemistry in the embryonic and mature wool follicle (du Cros et al., 1993) with the location of receptors and other ligands of this growth factor family unknown. During embryogenesis, only FGF-2 was observed in the periderm and intermediate layers of the epidermis as well as in the dermal-epidermal junction. Developing wool follicle primordia express both FGF-1 and FGF-2 with a discrete localisation of FGF-1 to the epidermal placode and the basal keratinocyte layer. Epidermal expression in the germinal epithelial layers is also evident for both growth factors during follicle formation.

In the mature follicle, a more restricted pattern of expression is observed. FGF-2 is mainly present in the suprabasal layers of the epidermis, the ORS and the basement membrane zone surrounding the upper bulb matrix. FGF-1 was apparent in the epidermis, pilary canal and the cells of the upper bulb matrix in the zone of keratinisation. A potential regulatory role for FGF-2 has been suggested based on its juxtaposition to proliferating cells of the hair matrix, while FGF-1 may be involved in the differentiation of the elongating wool fibre.

Aims of this study

The synchronous induction of a catagen-like state in the EGF treated Merino epidermis may provide an opportunity to examine the role of epidermally expressed peptide growth factors in another mammal. This may indicate a conservation in epidermal function between species. An in situ transcriptional analysis of two epidermally expressed genes, Fgf5 and Tgfo, aims to determine whether any change in the transcriptional regulation of these peptide growth factors occurs after EGF administration. The assumption that these genes may be involved in an EGF-induced catagen-like state is based on a potential inter-relationship between these two molecules in vitro and their inhibitory effect on murine hair growth in vivo (Chapter 1). The phenotypic enhancement observed in the compound mutants uncovered has also suggested that these molecules may be a components of a hair growth regulatory pathway.
Results

TGF-α Induction

Basal levels of Tgfα mRNA were evident in the proliferating follicle bulb with a more intense signal observable in the hair matrix of the bulb overlying the dermal papilla in some follicles while in others the signal was detected throughout the follicle bulb (Figure 6.2 A and Figure 6.3 A and C). This signal also progressed into the differentiating cortical cells of the keratogenous zone of the wool follicle and is possibly also present in the lower regions of the IRS. A faint, more diffuse signal is also present in the lower ORS region. No expression of a putative Tgfα mRNA is observable at this stage in the basal keratinocyte layer and the sebaceous glands of the epidermis. Expression levels remained unchanged at the 4 hour time period examined after EGF administration (see Table 6.1). A marked increase in epidermal expression was apparent 12 hours after EGF treatment in a number of epidermally derived tissues (Figure 6.2 B). This was observed in the differentiating cells of the keratogenous zone and IRS region of the wool follicle. An increase in Tgfα mRNA expression is observed in the entire ORS, the basal keratinocyte layer and the sebaceous glands (Figure 6.3 D). The elevated expression Tgfα mRNA in epithelial tissue was apparent after the 12 hour, 26 hour, 52 hour and the final time period examined at 8 days post EGF treatment (see table 6.1). No detectable background signal was apparent either prior to the elevation of Tgfα mRNA expression levels or after its increase in expression levels (Figure 6.2 C). From the results of this in situ analysis, it can be inferred that an increase in Tgfα mRNA expression levels has occurred between 4 and 12 hours after EGF infusion, a time frame consistent with a delayed-early transcriptional response. This elevation in epidermal transcriptional activity also appears to last for at least 1 week after EGF treatment.

FGF-5 Induction

Skin sections at time zero revealed a low level of detectable DIG-labelled antisense signal in the base of the follicle bulbs which may be represent basal levels of Fgf5 mRNA in the anagen wool follicle (Figure 6.4 A and Figure 6.5 A). The brown reaction product is likely due to the paraformaldehyde fixation of skin sections used in this time course study. No signal was detectable in any other epidermal tissue at this time frame suggesting that Fgf5 mRNA is not normally present. By 30 min post-EGF treatment, a detectable antisense signal is observable in the hair cone region of the bulb matrix overlying the dermal papilla. Signal is possibly present in the nuclei of cells in the lower region of the ORS (Figure 6.5 B). An increase in Fgf5 mRNA is clearly detectable 3 hours after EGF treatment in the proliferating cells of the follicle bulb. Cells in this region exhibit an intense signal in the hair cone region overlying the dermal papilla (Figure 6.4 B and Figure 6.5 C). This is clearly seen to encompass the dermal papilla at the 3 hour
time frame with the dermal papilla faintly visible (Figure 6.5 E). Antisense signal also extends into the lower regions of the ORS at this time period although DIG labelling is not as intense as that seen in the follicle bulb. A significant decrease in signal is apparent in the 6 hour time frame with a faint signal detectable generally in the hair cone region and at the base of the bulb (Figure 6.5 D). Sense strand cRNA of the subcloned mouse FGF-5 fragment did not detect non-specific RNA hybridisation at any of the time frames examined suggesting that the DIG-labelled signal detected is specific to the antisense mouse FGF-5 exon 3 fragment used (Figure 6.4 C). The elevation in DIG labelled signal is discernible in the cells of the proliferating hair matrix surrounding the dermal papilla and the lower region of the ORS of the wool follicle 3 hours after EGF treatment (Data are summarised in table 6.1). This time frame of transcriptional induction is consistent with Fgf5 mRNA acting as an immediate-early response gene following EGF treatment in the Merino epidermis however an analysis of further time frames was not undertaken in this study and its response at periods later than 6 hours is not known.

Table 6.1. Spatial and temporal expression of either TGFα or FGF-5 transcript in the EGF-treated Merino. Structures where expression was detected are in the left hand column. The following symbols imply: ‘-‘ no expression, ‘+‘ basal levels of expression, ‘++‘ elevated expression, ‘+++‘ highest levels of expression, N/A sample at this time point was not analysed.
Figure 6.2 Induction of Tgfα mRNA in the epidermis of the Merino after EGF administration (10 X magnification, scale bar = 25μM). (A) Time zero Merino epidermis taken coincident with the administration of a defleecing dose of EGF and hybridised with the antisense cRNA probe for sheep Tgfα mRNA. Basal levels of Tgfα mRNA are detectable in the bulb of the wool follicle as a blue alkaline phosphatase reaction product. Intensity of signal is more restricted to the upper region of the wool follicle bulb. (B) An elevation of Tgfα mRNA expression 12 hours after EGF treatment. A marked increase in the signal intensity is observable in all epidermally derived tissues with a more intense signal present in the IRS/ORS, sebaceous glands and basal keratinocyte layer. (C) Sense strand controls at time zero indicating that the specificity of DIG reaction product is due to the binding of the antisense TGF-α cRNA riboprobe. A negative sense strand signal was also observed at 12 hours post EGF treatment.

B= bulb, F= fibre.
Fig 6.3 Induction of Tgfa mRNA in the epidermis of the Merino after EGF administration (20 X magnification, scale bar = 50μM). A closer examination of the elevation of Tgfa mRNA. (A) Basal levels of Tgfa mRNA in the wool follicle bulb coincident with the administration of a defleecing dose of EGF. The localisation of Tgfa mRNA to the upper region of the wool follicle bulb is clearly visible. A much lower signal intensity may also be visible in the outer root sheath. (B) Elevation of Tgfa mRNA 12 hours after EGF administration in the wool follicle bulb. Signal intensity has increased and is now clearly detectable in the ORS and follicle bulb. (C) A higher magnification of the epidermal-dermal junction of Merino skin at time zero. No DIG reaction product is visible at this stage in any epidermally derived tissue. (D) After 12 hours a significant increase in signal intensity is clearly visible in the ORS, sebaceous gland and the basal keratinocyte layer of the interfollicular epidermis. DP= demal papilla, ORS= outer root sheath, SG= sebaceous gland, E= interfollicular epidermis.
Fig 6.4 Induction of Fgf5 mRNA in the epidermis of the Merino after EGF administration (10 X magnification, scale bar = 25µM). An elevation of Fgf5 mRNA is apparent 3 hours after EGF administration. The brown alkaline phosphatase reaction product indicates antisense riboprobe binding. The brown reaction product is likely due to the paraformaldehyde fixation of skin sections utilized for this in situ series (A) Skin sections from a Merino sheep taken coincident with EGF administration. A low level basal signal may be present at the base of the bulb. (B) An increase in signal intensity is apparent in the follicle bulb 3 hours after EGF administration. An elevated expression of Fgf5 mRNA is restricted to the follicle bulb with no staining evident in the other components of the epidermal or dermal layers. (C) Sense strand control hybridisation at 0 min indicating that non-specific background hybridisation is not occurring. A similar negative sense strand signal was observed at all stages of EGF treatment. B= bulb, F= fibre
Fig 6.5 Induction of Fgf5 mRNA in the epidermis of the Merino after EGF administration (20 X magnification, scale bar = 50μM). (A) A higher magnification of epidermis taken from EGF treated Merino sheep coincident with EGF administration. A low level of antisense hybridisation is detectable in the base of the wool follicle bulb. (B) 30 min after EGF administration an increase in alkaline phosphatase signal is apparent in the hair cone of the follicle bulb (arrowhead). A possible increase in staining is also apparent in the nuclei of cells of the ORS although levels are low. (C) Increased expression of Fgf5 mRNA 3 hours after EGF administration. Antisense signal is apparent in the follicle bulb of the wool follicle, intensely staining the hair cone and the lower regions surrounding the dermal papilla. Low levels of antisense signal are also apparent in the lower region of the ORS. (D) A rapid decrease in signal intensity has occurred 6 hours after EGF treatment to basal levels. (E) A 40 X magnification of the 3 hour induction of Fgf5 mRNA using Normarski optics. Scale bar = 100μM. The expression of antisense signal in the follicle bulb and in the lower regions of the ORS can be clearly seen. The dermal papilla is faintly visible surrounded by the intensely staining epithelial cells of the follicle bulb. B= bulb, F= fibre, ORS= outer root sheath.
Discussion.

The *in situ* expression results obtained in this study have shown that a basal level of Tgfα mRNA is expressed in the hair matrix of the follicle bulb. This signal also extends into the keratogenous zone of the wool follicle. A diffuse signal is also detectable in the ORS of the wool follicle prior to EGF treatment. No detectable signal is observed in the epidermis or the skin glands at this stage. Increased expression of Tgfα mRNA occurred between 4 and 12 hours in all epidermally derived tissue after EGF treatment. This is evident in the epidermis, ORS and the skin glands when compared to basal expression levels (Figure 6.3A and C). The elevation of Tgfα expression was also detectable one week after EGF administration suggesting that epidermal growth regulation is disrupted for a considerable period (data not shown). Observations of hyperplasia in the epidermis and sebocytes indicate that increased proliferation of these epidermal populations occurs after EGF administration (Moore et al., 1985).

Increased expression of TGF-α has also been noted in several hyperproliferative skin disorders such as psoriasis (Elder et al., 1989) and Bullous congenital ichthyosiform erythroderma (Finzi et al., 1991). It seems likely that the induction of TGF-α mRNA in the epidermis of EGF treated Merino epidermis is involved with this increase in epidermal mitotic activity.

The cross-induction of Tgfα mRNA after EGF treatment is also consistent with the autocrine stimulation of Tgfα transcription when keratinocytes are treated with EGF or TGF-α *in vitro* (Coffey et al., 1987). Is a similar autocrine stimulation occurring in the epidermis of Merino sheep after EGF treatment? Transcriptional cross-regulation of EGFR ligands when the EGFR is activated has been observed *in vitro* and may be a mechanism by which EGF treatment can result in the upregulation of Tgfα mRNA *in vivo* (Barnard et al., 1994; Diugosz et al., 1995). An autocrine cleavage of membrane bound TGF-α when stimulated with diffusible EGF or TGF-α is another potential mechanism that may result in an autocrine response and increased Tgfα mRNA expression (Baselga et al., 1996).

The basal and elevated expression patterns of Tgfα mRNA agree with previous localisations of EGFR populations in the Merino epidermis. Expression of Tgfα mRNA in the hair matrix region of the anagen wool follicle coincides with EGF-binding sites in the follicle bulb (Wynn et al., 1995; Green and Couchman, 1984). EGF binding sites are also present in the interfollicular epidermis, ORS and skin glands of adult sheep skin. The increased expression of Tgfα mRNA in these tissues after EGF treatment suggests that diffusible EGF may act by binding to, and activating EGFR containing cells. It has previously been suggested that infused EGF may exert its effects by interacting directly with the EGFR population of the follicle bulb although the activation
of epidermal EGFR after EGF infusion requires experimental confirmation (Moore et al., 1985).

A broader range of interactions between members of the EGFR subfamily and their associated ligands may also be disrupted by the presence of diffusible EGF. The presence of EGF binding sites with varying affinities in cell lines containing both EGFR and erb-B2 has been shown, in vitro, to result in a cellular response that can be either proliferative or apoptotic when stimulated with EGF at various concentrations (Wada et al., 1990). The EGFR subfamily member, erb-B2, has been localised in the rat epidermis and the ORS of the human hair follicle (Table 1.1). A differential cellular response, which may be mediated by the formation of aberrant EGFR heteromers in response to diffusible EGF, could explain the paradoxical effects of EGF treatment on the Merino epidermis. Different epithelial cell populations may vary in the concentration and combination of EGFR subfamily members present which, when activated by exogenous EGF, may activate intracellular signaling pathways with opposing effects on cellular growth (Spivak-Kroizman et al., 1992).

Unlike the photoperiod-induced catagen state and the associated down regulation of immunoreactive TGF-α, a decrease in Tgfα mRNA was not seen in the EGF-treated sheep. This suggests that the resulting inhibition of follicle growth may occur via differing mechanisms. In the sheep, TGF-α protein has been detected in the differentiating epidermal cell layers but not in proliferative regions of the basal keratinocyte layer, the follicle bulb and ORS (Nixon et al., 1996). However, TGF-α protein is present in the inner region of the ORS, skin glands and the general epidermis. In the anagen wool follicle, TGF-α protein is detected in the ORS in the region in closest contact with the IRS. Protein is also detected in the follicle bulb and the cortical cells of the keragenous zone which ceases once these cells become fully differentiated (Nixon et al., 1996). Immunoreactive TGF-α has been detected in the ORS of the human hair follicle, the epidermis and sweat glands (Finzi et al., 1991) with some studies also reporting immunoreactive material in the follicle bulb (Akiyama et al., 1996).

The in situ results obtained in this study reveal detectable basal levels of Tgfα mRNA in the hair matrix and the differentiating cells of the keragenous zone. Considering these locations of TGF-α protein and mRNA the following question can be raised: Is expression of Tgfα mRNA generally initiated in the follicle bulb and the TGF-α protein presented on the cell surface as these cells migrate and assume their differentiated cell fates? A juxtacrine/paracrine relationship between membrane-bound TGF-α in the IRS and the EGFR containing ORS has been proposed in the mouse hair follicle with a disruption of this interaction being suggested as a cause of the mutant waved hair phenotypes (Luetteke et al., 1993). It is interesting to note that in some wool fibres
treated with EGF a separation of the fibre and IRS occurs in this region (Hollis and Chapman, 1989, see Figure 6.1).

Immediate-early response genes (also referred to as primary response genes) are characterised by a transient increase in expression levels which are inducible by a wide range of factors including peptide growth factors, hormones, ionophores and environmental treatments (Sassone-Corsi, 1994). This inducible transcriptional response is insensitive to the presence of an inhibitor of protein translation, cycloheximide, which also acts to increase the half life of these quickly degraded transcripts. This insensitivity to cycloheximide distinguishes the immediate-early response genes from the delayed-early response genes which are induced within a few hours of stimulation but are sensitive to the effects of protein synthesis inhibitors (Sassone-Corsi, 1994). The induction of an immediate-early response gene can occur within minutes after stimulation in vitro with longer periods of induction associated with the progression toward a transformed cellular state. The protein products from immediate-early genes may be the initiators of a cascade of downstream events that result in changes in cell growth and differentiation.

An immediate-early response reported for Fgf5 mRNA expression in fibroblast cultures was associated with the mitogenic stimulation of cell growth by the serum growth factors EGF, TGF-α and PDGF (Werner et al., 1991a). After the stimulation of quiescent fibroblast cultures, Fgf5 mRNA increased eightfold within 2.5 hours and 12 fold after 5 hours with levels remaining elevated at 24 hours. Other members of the FGF family were also examined and did not exhibit a similar response. This suggests that the immediate-early response may be unique to this FGF family member. The in situ hybridisation result observed in the EGF treated Merino epidemis is in agreement with an immediate-early response of Fgf5 expression in vivo. A peak signal was detectable after 3 hours in the proliferative cells of the follicle bulb which decreased six hours after EGF treatment. This induction of Fgf5 mRNA reveals a potentially novel role for FGF-5 in the wool follicle that is consistent with its in vitro transcriptional regulation and inhibitory effects on hair growth in the mouse.

Whether an immediate-early response is the normal mode of action of Fgf5 in vivo is a point worth considering and may have implications for the role of FGF-5 in the mouse hair cycle. Fgf5 mRNA in the mouse hair follicle has been detected in the lower third of the ORS during late anagen VI (Hébert et al., 1994) a position that is removed from a known high affinity receptor for FGF-5, FGFR-1 in the dermal papilla (Rosenquist and Martin, 1996). An EGF induced expression of Fgf5 mRNA in the hair matrix surrounding the dermal papilla of the wool follicle is consistent with dermally expressed FGFR1 being a potential target for epidermally expressed FGF-5. Recombinant FGF-5 protein
also has an effect on dermal papilla cells when cultured in vitro supporting the role of the dermal papilla as a target tissue for FGF-5 in vivo (Rosenquist and Martin, 1996).

Is the immediate-early response of Fgf5 mRNA observed in the EGF treated sheep related to the partial catagen state induced? As the generally continuous anagen wool growth in the Merino sheep is the result of many years of selective breeding, it is likely that several aspects of the anagen to catagen transition mechanism may be modified relative to unselected species like the mouse. These may include Fgf5 itself which may not produce a functional protein or other upstream and downstream events regulating Fgf5 transcription.

It is important to note that mice homozygous for the Fgf5<sup>es</sup> mutation still eventually undergo catagen, an indication that other independent mechanisms are involved in regulating the anagen to catagen transition of the murine hair cycle. The localisation of Fgf5 mRNA in the lower third of the ORS in the mouse hair follicle around the time of catagen transition contrasts with the wool follicle bulb expression noted in this study. The time frames utilised by Hébert to study Fgf5 mRNA expression during the mouse hair cycle were several days apart (Hébert et al, 1994) and it is possible that an immediate-early response of Fgf5 mRNA in the hair follicle bulb has been overlooked. A more comprehensive analysis of the expression pattern of Fgf5 mRNA during the murine hair cycle may be useful in determining whether a similar primary inductive response is also occurring in the mouse.

The induction of the catagen-like state in the Merino wool follicle requires an EGF infusion over a time period longer than that necessary to induce an Fgf5 immediate-early response, suggesting that other EGF-induced events are likely to be occurring. Considering the complex signaling network regulated by the EGFR subfamily, a variety of downstream events may be induced by the activation of parallel signaling cascades. The role of FGF-5 is also unlikely to be simple with a splice variant of FGF-5 existing which may vary in function possibly between active and inactive states (Hattori et al., 1996). One member of the FGF family, FGF-2, exhibits an antisense regulation of protein translation in vivo, a regulatory mechanism that may be applicable to other members of the FGF ligand family (Savage and Fallon, 1995).

Reported changes in epidermal mitotic activity after EGF treatment are in agreement with the temporal induction of Fgf5 mRNA and Tgfα mRNA observed in this study (Moore et al., 1985). A decrease in the mitotic indices (MI) of the follicle bulb population has been observed 24 hours after EGF treatment while an increase in MI occurs in the interfollicular basal keratinocyte layer and sebocytes 48-72 hours after EGF administration. The temporal distinction observed between Fgf5 mRNA induction at 3 hours, which may contribute to the inhibition of follicle bulb proliferation, and the...
induction of Tgfrα mRNA 12 hours after EGF infusion, which may be involved in the
increased proliferation of epidermis, is consistent with the change in MI of these
epidermal regions.

The DNA sequence for exon 1 of the Merino FGF-5 gene has been obtained and
confirmation of the EGF induced primary response has been achieved by RT-PCR
using sheep specific primers (K. Ward pers.comm.). Other epidermally expressed
members of the EGF ligand family such as amphiregulin, betacellulin, HB-EGF and
receptors from the EGFR subfamily may also be similarly coordinated in the regulation
of their transcriptional response to EGF treatment. These molecules may be useful
candidates for further study.

Although the effects of EGF on epidermal cellular proliferation in the Merino sheep vary
from those observed in the normal murine hair cycle, a number of similarities may exist
in the fundamental regulation of cellular growth control. This study has investigated a
potential interrelationship between two peptide growth factor molecules involved in
murine epidermal growth regulation. A spatial and temporal induction of mRNA
expression has been established that is consistent with other reported observations
regarding the localisation and potential function of these growth factor molecules in
regulating hair growth. This may indicate that the epidermal function of these growth
factors has been conserved in mammals. A more detailed analysis of the spatial and
temporal regulation of epidermal growth control mechanisms may be possible in the
EGF treated Merino sheep with the utility of this model system resting on the
synchronised changes induced in the epidermis. The dissection of these events may
help to reveal the in vivo morphological changes initiated by growth factors.
Chapter Seven: General Discussion

Summary
The initial aim of this study was to determine whether three mutations affecting the development and growth of the mouse hair follicle, when combined, resulted in a deficiency of certain genotypes and the generation of novel phenotypes that would be considered as evidence for interaction. As stated in the null hypothesis presented in Chapter 1, evidence for no interaction between these loci would be the expression of a combined or additive compound mutant phenotype with no statistically significant deficiency in genotype frequencies. It is useful at this point to briefly summarise the evidence for either accepting or rejecting the null hypothesis.

The characterisation of the phenotypes; epidermal development, growth and viability and a Chi-square analyses of genotype frequencies conducted in Chapter 3, 4 and 5 have revealed that the effects of combining a mutation in a ligand (TGF-α) and its receptor (EGFR) are additive. A mild viability effect due to homozygosity for the Egfr<sup>ma2</sup> mutation was uncovered which accounted for the divergence of genotype frequencies. Further evidence supporting this additivity was the significant main effects that each locus contributed to a depression in growth rate. The epidermal phenotypes of the double homozygotes also appeared to be a simple combination of mutant epidermal phenotypes. In contrast, the study in Chapter 4 revealed a strong enhancement of the Egfr<sup>ma2</sup> viability phenotype when combined with the homozygous Fgfs<sup>o</sup> genotype. However, an analyses of growth rate suggested that only the main effects of these two loci act to depress growth rate with no evidence found for an interaction. This suggests that although their was a dramatic enhancement of the viability phenotype the growth rate data support an additive interpretation. Interestingly, a repeatable disruption in the frequency of Egfr<sup>ma2</sup> genotypes was also observed in two independent breeding experiments (Chapter 3 and 4) that was co-related with the genotype of the Tgfa<sup>la1</sup> locus. In both experiments a deficiency in Egfr<sup>ma2</sup> homozygous genotypes was more pronounced (although not statistically significant) when Egfr<sup>ma2</sup> homozygosity was combined with either the Tgfa<sup>la1</sup> +/− or −/− genotypes.

The divergence of genotype frequencies uncovered in this study indicate that Fgfs<sup>o</sup> strongly enhances the viability phenotype of the Egfr<sup>ma2</sup> mutation. The repeatable disruption of the homozygous Egfr<sup>ma2</sup> genotype when in combination with either Tgfa<sup>la1</sup> +/− or −/− genotypes also suggests that this locus mildly enhances the viability effect of Egfr<sup>ma2</sup>. The more severe retardation in epidermal development and viability phenotypes observed in the triple homozygotes could also be considered as evidence for this combined enhancement. Clearly, there has been the generation of novel phenotypes although this may not necessarily be an indication of interaction. The
dramatic effect on viability observed in the compound mutant could be attributable solely to the additive effects of each mutation. It is possible that both the Tgfa<sup>ns-1</sup> and Fgf5<sup>ns</sup> mutations have a very mild viability phenotype which is statistically undetectable in an experiment of this size. When these mutations are combined with the more easily detectable Egfr<sup>ns-2</sup> viability effect an enhancement occurs that is the result of the additive effects of each mutation on viability.

An additive interpretation of this data would suggest that homozygosity for the Egfr<sup>ns-2</sup> mutation is the major contributor to the viability phenotype with significant effects from Fgf5<sup>ns</sup> while Tgfa<sup>ns-1</sup> appears to contribute the least. However, before accepting this interpretation it is important to consider several points. The single-locus Chi-square analysis presented in Chapter 3 revealed no significant change in the frequency of Tgf<sup>ns-1</sup> genotypes suggesting that viability effects attributable to this locus were not detected in this experiment. Of more interest is that both the Tgfa<sup>ns-1</sup> and Fgf5<sup>ns</sup> mutations are maintainable as homozygous closed breeding lines as is the double homozygous [Tgfa<sup>ns-1</sup>-/-, Fgf5<sup>ns</sup>-/-] genotype. This would suggest that even the combined effects of these two mutations on viability are negligible. It is also important to note that non-viable animals were excluded from the analyses of the growth rate data. Considering these caveats it seems less likely that the dramatic effects on viability observed in the compound mutant can be easily explained by the simple additivity of each mutation. As both the Fgf5<sup>ns</sup> and Tgfa<sup>ns-1</sup> mutations appear to have a negligible effect on viability either singly or in combination the viability phenotype of the compound mutants should be closer to that observed in the Egfr<sup>ns-2</sup> homozygote, the major contributor to the additive equation. Overall it appears that the data generated in this study has not provided sufficient evidence to conclusively reject the null hypothesis in favor of alternative hypothesis however there does appear to be tentative evidence suggesting that the combined effects of these three mutations is not strictly additive.

**Synthetic Enhancement and Genetic Modifiers**

The phenotype of a partially functional EGFR mutation, Egfr<sup>ns-2</sup>, when combined with two other potentially interacting mutations was modified to produce a phenotype essentially equivalent to that observed in the EGFR null mutation. This is consistent with a genetic effect which has been termed synthetic enhancement (Guarente, 1993). Synthetic enhancement, where one mutation increases the severity of another, may indicate whether mutations encode interacting gene products. The overall phenotypic similarity of the triple homozygotes generated in this study with that of the EGFR null mutation suggest that Tgfa<sup>ns-1</sup>, Egfr<sup>ns-2</sup> and Fgf5<sup>ns</sup> act in a common pathway, an assumption that was made from experimental data prior to establishing an in vivo genetic analyses. The synthetic enhancement of a partially functional mutant
phenotype to that of the null mutant may be due to the reduced metabolic flow through a common or parallel pathway. The effects on viability indicate that a change in pathway activity has resulted when these mutations have been combined. Considering the complex nature of the potential signal transduction cascade that may have been disrupted, a simple linear relationship between these mutations is unlikely. This potential interaction may be better understood as a genetic or developmental network, an assumption that is based on the known multiple ligands for the EGFR, the complex signalling cascade that can be initiated when the receptor is activated, and the multiple targets of this activation which include immediate-early response genes. Disrupting several nodes of this network may have decreased the activity of this signalling cascade to the same extent as an EGFR null mutation.

Modifier effects are expected to be a natural consequence of an interconnected network of enzymatic activities and as such, modifiers are all other "enzymes" which are in the developmental pathway. Modifier mutations of RPTK signalling pathways have been found in *Drosophila* and *C. elegans*, an example being a dominant gain-of-function mutation in the *Drosophila* MAP kinase sevenmaker, an allele of the loss of function rolled mutation (Brunner *et al.*, 1994). Similar modifiers have also been found in the mouse such as the *Mom1* dominant modifier which suppresses the multiple intestinal neoplasia phenotype of the *Apc* mutation (Dietrich *et al.*, 1993; MacPhee *et al.*, 1995). These modifiers may be neutral or even deleterious on the wild-type background but gain functional significance depending on other mutated loci present. The flexibility of a network of genetic interactions and the realisation that modifier loci may be other components of a common biochemical pathway allows a useful conceptual framework to be established in understanding how functionally related loci could be effective in modifying the expression of a mutant phenotype.

**Functionally Related Loci and Developmental Sets**

How might mutations of small phenotypic effect, when combined with mutations at other functionally related loci, result in a much larger effect on phenotypic variation than expected from the individual effects of each mutation? This phenotypic enhancement has been attributed to cryptic gene effects whereby the potential effect of some loci may remain hidden until they are combined with functionally related loci of the same developmental set (Moreno, 1994). A developmental set may be a group of functionally interacting loci that contribute to a common developmental process such as bristle formation in *Drosophila*, or skin pigmentation in mice. The *Tgfα*+, *Egf*++ and *Fgf5* loci may constitute part of a developmental set that controls epithelial development and growth of the hair follicle. When functionally related loci of a developmental set are present in certain allelic combinations a greater capacity for phenotypic variation may occur.
Figure 7.1: A diagrammatic representation of the increasing phenotypic effect as a result of changes in the activity of two functionally related loci, α and β (Reprinted with permission from Annual Review of Ecology and Systematics, Vol 25, copyright 1994, by Annual Review Inc). Due to the non-linear relationship between gene activity and its phenotypic effect a change in the activity or dosage of locus α only results in a small effect on phenotype, P. If this genetic effect is combined with a change in activity of another functionally related locus β in the same developmental set a greater effect on phenotype, P', occurs.

Such phenotypic enhancement has been suggested to be the result of a non-linear relationship between gene activity and phenotype (Figure 7.1) (Moreno, 1994). Changes in gene activity at one locus α, are buffered as a result of this nonlinearity allowing a phenotype to be maintained even though a change in an underlying molecular component of that phenotype has occurred. By combining two functionally related loci, α and β, a greater phenotypic change can be effected than by changes in the activity of α or β alone. This may change a common molecular activity resulting in a greater effect on phenotypic variation. The molecular activity of a particular developmental set is unable to be maintained within its wild-type plateau and a threshold level gene activity may be crossed resulting in a dramatic effect on phenotypic variation.

It is worthwhile to think about the three loci used in this study which can reasonably be considered to constitute part of an epithelial development set in the context of Moreno’s (1994) model. An initial analysis of double homozygotes carrying the functionally
related (ligand and receptor) Tgfr\^++ and Egfr\^+/- mutations revealed an additive effect with homozygosity for the Egfr\^+/- mutation affecting viability. The absence of a significant interaction between a mutation in a ligand and its receptor is surprising. However considering the existence of multiple ligands for the EGFR, the absence of one member may be efficiently compensated for by others. The enhanced deficiency of homozygous Egfr\^++ genotypes when combined with Tgfr\^+/- or Tgfr\^/- genotypes also indicates that the Tgfr\^+/- mutation is not entirely neutral. The severity of the viability effect of the Egfr\^+/- mutation increases substantially when segregating on the Fgf-5\^o-/- genetic background.

When all three mutations of this epithelial developmental set are present a novel phenotype is generated which is not expected from a combination of individual mutant phenotypes. This is revealed by a greater reduction in viability, observed as a statistically significant departure from expectation of genotype frequencies. By combining all three mutations, the expression of mutations in a ligand and its receptor is exacerbated (or enhanced) by the introduction of a null allele in a downstream immediate-early response gene. This viability effect was most severe in the triple homozygotes providing further support for the assumption that these three loci may constitute a functionally related developmental set. Therefore the phenotypic potential revealed by combinations of these functionally related loci has effectively identified other loci constituting the postulated developmental set.

A functional relationship between two of these loci was also observed in the epidemics of an EGF treated Merino (Chapter 6). An in situ analysis of transcription has supported the in vitro observation of an immediate-early response for FGF-5 and the involvement of an autocrine response for TGF-\(\alpha\) expression, possibly via activation of the EGFR (see Figure 7.2). This cross-induction of another member of the EGF ligand family is also in agreement with in vitro observations suggesting that a complex regulatory network may exist within this ligand family (Barnard et al., 1994; Dlugosz et al., 1995). A functional role for the EGFR in regulating the anagen to telogen transition of the hair cycle in mice has also been demonstrated (Murillas et al., 1995) while a down-regulation of the EGFR has been observed during the rat hair cycle with cells of the follicle bulb exhibiting a decrease in \([^{32}\text{P}]\) EGF binding sites in the hair matrix region surrounding the upper portion of the dermal papilla from mid-anagen to early catagen (Green and Couchman, 1984). The EGFR is also the only member of the EGFR subfamily which can undergo ligand induced internalisation (Baulida et al, 1996) and this may be a potential control point for a ligand-receptor interaction.
Figure 7.2. A representation of the potential functional relationship between EGF, Tgfα, the EGFR and Fgf5 in the EGF treated Merino epidermis.

Genetic Networks and Epigenetic Stability

Genetic networks of interacting gene products are becoming evident from the study of a wide variety of developmentally significant molecules (Hunter, 1997). The complex interactions which can occur between members of the EGFR subfamily and their associated ligands in vitro may be a reflection on the range of interactions that are also occurring in vivo. Some interesting biochemical relationships have been found from studies of modifier loci in the mouse which affect skin pigmentation. Modifiers of the white spotting phenotype produced by the piebald mutation have been shown to localise to chromosomal regions which have been implicated in other white spotting phenotypes including Kit+, Mgf1 and possibly other loci involved in skin pigmentation (Parvan et al., 1995). The modification of the piebald phenotype by other functionally related loci may indicate that a developmental network exists between these gene products (Barsh, 1996). The Tgfα*, Egr* and Fgf10* mutations also suggest that modifiers of a particular phenotype may be biochemically (and therefore functionally) related since combined disruptions of these loci result in novel phenotypes.

The regulation of homeotic genes and their evolutionary role in the formation of the metazoan body plan may also be the result of interactions between networked members (Carroll, 1995). This potential for hierarchical networked genetic relationships which can be recombined in alternate configurations may be an important evolutionary mechanism and could be related to the larger size of developmental gene families observed in more complex metazoans (Carroll, 1995). A theoretical study of genetic networks and their role in generating epigenetic stability has suggested that a larger number of interacting gene products may be more effective in buffering mutational perturbation (Wagner, 1996). A complex network with a larger number of regulatory
interactions tends to result in a greater degree of stability than one with fewer connections. Whether these theoretical assumptions regarding genetic networks and epigenetic stability are valid requires an empirical experimental evaluation of the effects of disturbing such regulatory networks.

By disrupting three functionally related components of a potential network of interacting gene products in the mouse a form of epigenetic stability may have been demonstrated. The loss of viability associated with the compound homozygotes suggests that a wild-type genetic network configuration has been disrupted. The observation of a triple homozygote which had managed to compensate for this disruption may be an example of the epigenetic stability of the mammalian genome. The known interactions and cross-regulation which can occur between the EGFR subfamily members and their ligands such are consistent with the possibility of epigenetic stability. Whether similar interactions are also occurring in the FGFR subfamily and associated ligands is currently unknown although a potentially coordinated expression of FGF receptors and ligands has been noted in the hippocampus, prostate and the hair follicle (Gómez-Pinilla et al., 1995; Yan et al., 1993; Rosenquist and Martin, 1996). Functional redundancy within the FGFR subfamily may also occur due to the broad binding affinities of various FGF’s to a number of FGF receptors (Givol and Yayon, 1992).

An Evolutionary Mechanism?

Genetic Canalisation of Developmental Pathways

The canalisation of developmental pathways is a term which describes the resistance of the wild-type phenotype to change caused by genetic or environmental factors. Initially proposed by Waddington to account for the genetic assimilation of environmental effects in perturbing wing vein formation, further study of this phenomenon has focused on the development of bristle number in Drosophila and vibrissae formation in the mouse (Scharloo, 1991 for review). Genetic canalisation, the buffering or self-stabilising capacity of the genome to genetic disruption is however poorly understood from a molecular viewpoint. A molecular mechanism for genetic canalisation involving co-expressed paralogous genes which may fulfil functionally redundant roles and act to stabilise developmental pathways has recently been postulated (Wilkins, 1997).

This potential molecular mechanism for genetic canalisation could be useful in explaining the phenotypes of the triple homozygotes observed in this study. The severely decreased viability of the triple homozygote could be the result of disrupting a genetically canalised state. These multiply homozygous animals may have a more severe effect on the canalised pathway resulting in a greater disruption of the canalised
state. The suppressed triple homozygote with more normal growth and hair development could be an example of the self-stabilising capacity of a genetically canalised developmental system which has managed to buffer this genetic disruption and retain viability.

A molecular mechanism which may account for this ability to compensate or buffer genetic disruption could involve the functional redundancy existing within the EGFR and FGFR subfamilies. Members of these subfamilies could be the co-expressed paralogous genes (potentially functionally related loci) proposed by Wilkins as a molecular mechanism for genetic canalisation. It has been suggested that functional redundancy could increase the fidelity of a developmental process allowing its completion to occur even when major elements are defective (Thomas, 1993). Functional redundancy could therefore be the result of a molecular mechanism which has evolved to maintain a genetically canalised state. By increasing the functional redundancy of the developmental process a buffering of genetic disruption may be possible by providing alternate pathways for achieving the same end point, a viable organism.

The Shifting Balance Process

Genetic interaction and its effects on phenotypic variation remain a controversial point in genetics (Barton and Turelli, 1989). The combined approaches of molecular biology and genetics are starting to reveal that interrelationships between loci may play a much greater role in determining phenotypic variability than has been previously realised (Frankel, 1995, MacKay, 1995). Genetic interactions are also the basis of the Shifting Balance Process (SBP) formulated by Sewall Wright (Wright, 1982, Wright, 1980). Phase zero, a genetic reconfiguration required to generate novel phenotypes with a potential selective advantage, has been identified as a primary requirement for the SBP (Philips, 1996).

The main difference between the concept of genetic canalisation and the genetic reconfiguration required by the SBP is whether multiple fitness peaks exist. The theory of genetic canalisation is based on the Fisherian assumption of an additive polygenic system under stabilising selection and a single fitness peak. Any selection pressure away from the genetic equilibrium is increasingly difficult to achieve, this is the canalised state. The SBP assumes that multiple fitness peaks can exist, widespread non-additive effects, and the heterogeneous genetic nature of the population. This would theoretically allow many stable equilibrium states to evolve with a differing selective advantage depending on the genetic configuration fixed in a particular subpopulation. However, genetic canalisation and the initial phase zero of the SBP may be hypotheses that are similar at a mechanistic level.
Could the suppressed triple homozygote be an example of phase zero of the SBP? If the potential identity of a "suppressor of angora" locus is considered, some obvious candidates may be other paralogous members of the EGF or FGF ligand families. Several of the EGF and FGF growth factor ligand family members are tightly linked on two mouse chromosome's and syntenically conserved in other species. The FGF-5 gene is within 3cM of betacellulin and amphiregulin on mouse chromosome 5 while HB-EGF is linked to FGF-1 on mouse chromosome 18 (Pathak et al., 1995). This close linkage may be of functional significance and it is interesting to note that under mathematical models of the SBP in diploid organisms tight linkage of epistatic loci greatly decreases the time required for a fitness peak shift (Phillips, 1996).

Conclusion

By utilising a mixed genetic background to analyse a potential genetic interrelationship between the Tgfα*−, Egfr*− and Fgf-5* mutations, effects on viability have been observed. This study has also revealed the potential functional significance of the genetic background in compensating for mutational disruption. Although the use of congenic strains to determine the phenotypic effect of a mutant locus may generate a more consistent phenotype, this approach does have limitations. The congenic background is randomly fixed for a subset of potential modifier loci and as a result, the variation in phenotype would be constrained. The introgression of a mutation onto a congenic background may also inadvertently result in the fixation of modifier loci which may significantly affect the expression of the mutant phenotype. By utilising the approach presented here a range of phenotypes have been observed which became more severe as the combined mutant genotype was imposed. An animal which had modified the generally deleterious phenotype of the triple homozygote was also observed. The use of a mixed genetic background has therefore facilitated the identification of a variety of phenotypic effects that can occur when potentially interacting gene products are genetically disrupted.

The existence of genetic modifiers has been demonstrated in the study of relatively simple eukaryotes such as C. elegans and Drosophila. These genetic screens have been useful in identifying interacting components of genetic pathways by their ability to either enhance or suppress a mutant phenotype produced by a partially functional EGFR mutation (Brenner et al., 1991; Sterneberg et al., 1995; Simon et al., 1991; Rogge et al., 1991; Doyle and Bishop, 1993; Wang et al., 1997). The suppression of the triple homozygote hair and viability phenotype may be due to another interacting component of the mouse EGFR signalling pathway. The ability to genetically identify interacting components of the EGFR signalling pathway in the mouse is still in its infancy. The widespread use of such genetic screens for suppressor and enhancer mutations in other genetically well studied eukaryotes has been very successful suggesting that a
similar approach in the mouse may be fruitful. The genetic dissection of the EGFR signalling pathway in other organisms, notably *C. elegans*, may give an indication as to the types of molecules that could be involved in mediating this suppression.

An identification of suppressors of a partially functional EGFR mutation in *C. elegans* has revealed that several belong to the tumor suppressor genetic category (Stenberg *et al.*, 1994; Stemberg *et al.*, 1995). These include the negative regulators *lin-15A*, *lin-15B*, *unc101*, *sli-1* and *rok-1*, several of which have mammalian homologues with a known role in regulating EGFR signaling. The *sli-1* gene codes for a protein product with homology to the human *cbl* proto-oncogene which interacts directly with the EGFR and is phosphorylated when the EGFR is activated (Bowtell and Langdon, 1995). The *unc101* mutation encodes an adaptin type molecule which may mediate internalisation of the EGFR, while *rok-1*, *lin-15A* and *lin-15b* encode functionally uncharacterised proteins. In *Drosophila*, the genetic dissection of the EGFR pathway is not as advanced although a negative regulator, *argos*, has been uncovered by a similar genetic screen for mutations which suppress a partially functional EGFR (Schweitzer *et al.*, 1995).

Considering the results generated in other organisms and the likelihood that these sorts of modifiers may be tumor suppressors, the locus(loci) responsible for the suppressed triple homozygote may be relevant to an understanding of the negative regulatory elements of the mammalian EGFR pathway. An upregulation in expression of EGFR subfamily members and their ligands has been observed during the progression to a cancerous state in a wide variety of tissues including; skin (Greenlaugh *et al.*, 1996), breast (Chryogeles *et al.*, 1994) and pancreas (Korc *et al.*, 1992). A better understanding of the EGFR signalling cascade in mammals and the identification of potential negative regulatory controls could have medical relevance. A combinatorial approach using several mutations with small individual effect, which interact when combined, could be a novel approach to genetically dissect signalling pathways in the mouse. This potentially increased sensitivity to the effects of modifier loci may be more efficient than a genetic screen for suppressor activity using a single major mutation of large effect which may be more resistant to modification.

The genetic screens carried out in *Drosophila* and *C. elegans* often involve the screening of thousands, if not tens of thousands of individuals in the search for suppressor or enhancer loci, a strategy that is not feasible in the mouse. An effective way of reducing the number of mice that need to be screened to look for similar modifiers may allow a similar genetic strategy to be undertaken. Genetic screens also often use an easily observed phenotypic change. This is one major advantage of using mouse hair mutations as an indicator of the underlying genetic variation, as the modified phenotype is easily distinguishable. The molecular identification of the modifier
locus(loi) responsible is still a daunting task although the increasing ease of carrying out genome wide scans combined with new technology for genetically isolating such loci could make this an achievable proposition (Lisitsyn et al., 1994). An analysis of potential candidate genes for transcriptional upregulation in the suppressed or enhanced individual could also be informative by an analysis of labelled cDNA from suppressed or enhanced individuals to filter arrays of candidate genes. In the case of the suppressed triple homozygote other members of the EGFR and FGFR subfamilies and their ligands may be involved, especially paralogues co-expressed in the hair follicle such as FGF-2. These may be further members of the postulated epithelial developmental set.

The experimental data presented here has demonstrated the feasibility of utilising the mouse as a mammalian model system for the genetic dissection of the EGFR signaling pathway in a manner similar to that described in C. elegans and Drosophila. As the genetic basis for further mouse hair mutations will likely be revealed by the targeted disruption of other growth factor genes, utilising easily distinguishable hair phenotypes to identify interacting gene products may be possible. The study of mouse hair mutations is rapidly becoming a significant contributor toward our understanding of the complexity of multicellular organisms. The reiteration of conserved developmental mechanisms are becoming apparent with the realisation that the fundamental processes underlying organogenesis of other multicellular structures are also occurring in the hair follicle. The mouse hair follicle, and the many catalogued mendelian mutations affecting hair growth may therefore provide a useful developmental model for the genetic dissection of mammalian growth factor signalling pathways.
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Compound Mutations in the Mammalian EGFR Signalling Pathway Affect Epidermal Development, Growth and Viability

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Thesis submitted for the degree of Doctor of Philosophy
October, 1997
PLEASE NOTE

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This document constitutes a thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy. I declare that this work has not been submitted for a higher degree in any other university or institution and that the thesis contains the results of my own work, except where otherwise indicated.

Bruce Paul Davidson
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Abstracts

Complex genetic interaction between $Tgf\alpha^{rs1}$, $Egrl^{rs2}$ and $Fgf5^{po}$ affecting epidermal development. B.P. Davidson, D. Adelson, K.A. Ward. Presented at the 15th Annual Meeting of the Australian and New Zealand Society for Cell Biology, Sept 30-Oct 2, 1996, Brisbane.

Epidermal development and hair growth regulated by complex genetic interactions between $Tgf\alpha^{rs1}$, $Egrl^{rs2}$ and $Fgf5^{po}$. B.P. Davidson and D.L. Adelson. Presented at the First International Meeting of Hair Research Societies, June 11-14, 1997, St Vincent's Hospital, Melbourne.
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<thead>
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<th>Full Form</th>
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<tbody>
<tr>
<td>AER</td>
<td>apical ectodermal ridge</td>
</tr>
<tr>
<td>AP-2</td>
<td>activator protein 2</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>BMP</td>
<td>bone morphogenetic protein</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>cRNA</td>
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<tr>
<td>dCTP</td>
<td>deoxyadenosine-5'-triphosphate</td>
</tr>
<tr>
<td>dNTP</td>
<td>deoxynucleoside triphosphate</td>
</tr>
<tr>
<td>DEPC</td>
<td>diethylpyrocarbonate</td>
</tr>
<tr>
<td>DIG</td>
<td>digoxigenin</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal Growth Factor</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal Growth Factor Receptor</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast Growth Factor</td>
</tr>
<tr>
<td>FGFR</td>
<td>Fibroblast Growth Factor Receptor</td>
</tr>
<tr>
<td>gDNA</td>
<td>genomic DNA</td>
</tr>
<tr>
<td>HB-EGF</td>
<td>heparin-binding EGF</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloric acid</td>
</tr>
<tr>
<td>IRS</td>
<td>inner root sheath</td>
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<tr>
<td>kb</td>
<td>kilobase</td>
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<td>M</td>
<td>molar</td>
</tr>
<tr>
<td>mA</td>
<td>milliamperere</td>
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<tr>
<td>MAPK</td>
<td>mitogen activated protein kinase</td>
</tr>
<tr>
<td>min</td>
<td>minutes</td>
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<tr>
<td>MW</td>
<td>molecular weight</td>
</tr>
<tr>
<td>Neo</td>
<td>neomycin resistance gene</td>
</tr>
<tr>
<td>ORS</td>
<td>outer root sheath</td>
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<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
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<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PDGF</td>
<td>platelet derived growth factor</td>
</tr>
<tr>
<td>PGK</td>
<td>3-phosphoglycerate kinase</td>
</tr>
<tr>
<td>PKA</td>
<td>protein kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
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<td>RFLP</td>
<td>restriction fragment length polymorphism</td>
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<td>RNA</td>
<td>ribonucleic acid</td>
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<td>Abbreviation</td>
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<tr>
<td>RNase</td>
<td>ribonuclease</td>
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<tr>
<td>RPTK</td>
<td>Receptor Protein Tyrosine Kinase</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>sec</td>
<td>seconds</td>
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<tr>
<td>SHH</td>
<td>sonic hedgehog</td>
</tr>
<tr>
<td>SSC</td>
<td>standard saline citrate</td>
</tr>
<tr>
<td>TE</td>
<td>Tris-EDTA</td>
</tr>
<tr>
<td>TBE</td>
<td>Tris-borate-EDTA</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming Growth Factor</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>U</td>
<td>unit of enzyme activity</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>V</td>
<td>Volt</td>
</tr>
<tr>
<td>ZPA</td>
<td>zone of polarising activity</td>
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Summary

The widespread expression of polypeptide growth factors from the earliest stages of embryonic development through to mature tissues in the adult organism suggests an involvement in a reiterated developmental process affecting the underlying cellular growth and differentiation of many tissues. The hair follicle has taken on increased significance with the observation that many genetic mutations in these peptide growth factor genes affect its development. The targeted disruption of genes encoding members of the Epidermal Growth Factor (EGF) and Fibroblast Growth Factor (FGF) families in the mouse has revealed a functional role for these proteins in the regulation of hair follicle growth.

Experimental data from other studies has revealed that the Epidermal growth factor receptor (EGFR) ligands, EGF and TGF-α can activate the transcription of Fgf5 mRNA as an immediate-early response gene in vitro. A mutational analysis of the EGFR in the murine epidermis has indicated that the EGFR is involved in the transition to the catagen stage of the hair cycle. A targeted null mutation in the Fgf5 gene has revealed that this growth factor is also involved in the transition from anagen to catagen hair follicle growth. Furthermore, mutations in the mouse Tgfa genes suggest that this peptide growth factor is involved in the regulation of murine hair growth. Considering these mutant phenotypes and their potential coordinated regulation in vitro it is possible that TGF-α, the EGFR and FGF-5 constitute a signal transduction pathway regulating hair growth.

This thesis examines a potential interrelationship between these three members with regard to their role in mammalian epidermal development. Two experimental systems have been used to determine whether an interrelationship between these growth factors members exists in vivo. Initially a genetic analysis was undertaken to examine the phenotypic effects of combining mutations in this potential signalling cascade. Three mouse mutations were used; a null mutation in the epidermally expressed TGF-α gene, Tgfa<sup>−/−</sup>; a partially functional mutation in the EGFR, Egfr<sup>−/−</sup>; and a null mutation in the peptide growth factor FGF-5, Fgf5<sup>−/−</sup>. Genotyping assays for each of the three mutations were developed and an analysis of several phenotypes including epidermal development, viability and growth rate was conducted with regard to the genotype of each animal.

Mice doubly homozygous for the Tgfa<sup>−/−</sup> and Egfr<sup>−/−</sup> mutations revealed an additive effect between a mutation in a peptide growth factor and its receptor. A mild viability effect for viability when homozygous for the Egfr<sup>−/−</sup> mutation was also apparent. Mice which were doubly homozygous for the Tgfa<sup>−/−</sup> and Fgf5<sup>−/−</sup> or the Egfr<sup>−/−</sup> and Fgf5<sup>−/−</sup>
mutations expressed an essentially combined epidermal phenotype. However the mild viability phenotype associated with the Egfr<sup>−/−</sup> mutation was strongly enhanced in mice homozygous for both the Egfr<sup>−/−</sup> and Fgf5<sup>−/−</sup> mutations. A milder enhancement of this viability phenotype by Tgfa<sup>−/−</sup> was also suggested from the data. When individuals homozygous for all three recessive mutations were generated a retardation in epidermal development and a dramatic effect on viability was observed.

Overall, the genetic disruption of a functional relationship between an extracellular ligand, its receptor and a downstream immediate-early response gene resulted in an enhancement of the mild viability effect of Egfr<sup>−/−</sup> mutation. This combined enhancement could be considered as genetic evidence that these molecules are components of a common developmental pathway. Interestingly, one triple homozygote did not exhibit the enhanced retardation of epidermal development and viability effects noted in the other compound mutants generated during the course of this study. A 'suppression' of the Fgf5<sup>−/−</sup> hair phenotype occurred and no reduction in viability was observed. Therefore, the combined enhancement observed has confirmed that Fgf5<sup>−/−</sup> and Tgfa<sup>−/−</sup> act as genetic modifiers of the EGFR signalling pathway while other genetic or epigenetic effects can further modulate this compound mutant phenotype.

A second experimental system was used to determine if a functional relationship between the epidermally expressed peptide growth factors, TGF-α and FGF-5 was conserved in the Merino sheep. The EGF-treated Merino undergoes a synchronous change in the morphology of the wool fibre resembling the early stages of the catagen transition described in the mouse hair cycle. An in situ analysis of Fgf5 and Tgfa expression in the epithelium of an EGF-treated Merino revealed a temporally distinct induction of transcripts for both growth factors after EGF administration. Transcript for Fgf5 exhibited an induction consistent with that of an immediate-early response gene in the wool follicle bulb while a delayed up-regulation of Tgfa was apparent in the epithelium. The induction of a catagen-like state in the wool follicle and other epidermal changes associated with EGF treatment may be related to the transcriptional induction of these peptide growth factors.