ROLE OF THE BONE MORPHOGENETIC PROTEIN SIGNALLING IN SKIN CARCINOGENESIS

Effect of transgenic overexpression of BMP antagonist Noggin on skin tumour development; molecular mechanisms underlying tumour suppressive role of the BMP signalling in skin

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Abstract

Bone morphogenetic protein (BMP) signalling plays key roles in skin development and also possesses a potent anti-tumour activity in postnatal skin. To study mechanisms of the tumour-suppressive role of BMPs in the skin, a transgenic (TG) mouse model was utilized, in which a transgenic expression of the BMP antagonist Noggin was targeted to the epidermis and hair follicles (HFs) via Keratin 14 promoter. K14-Noggin mice developed spontaneous HF-derived tumours, which resembled human trichofolliculoma. Initiation of the tumours was associated with a marked increase in cell proliferation and an expansion of the hair follicle stem/early progenitor cells. In addition, the TG mice showed hyperplastic changes in the sebaceous glands and the interfollicular epidermis. The epidermal hyperplasia was associated with an increase in the susceptibility to chemically-induced carcinogenesis and earlier malignant transformation of chemically-induced papillomas.

Global gene expression profiling revealed that development of the trichofolliculomas was associated with an increase in the expression of the components of several pro-oncogenic signalling pathways (Wnt, Shh, PDGF, Ras, etc.). Specifically, expression of the Wnt ligands and (β-catenin/Lef1 markedly increased at the initiation stage of tumour formation. In contrast, expression of components of the Shh pathway was markedly increased in the fully developed tumours, compared to the tumour placodes. Pharmacological treatment of the TG mice with the Wnt and Shh antagonists resulted in the stage-dependent inhibition of the tumour initiation and progression, respectively.

Further studies revealed that BMP signalling antagonizes the activity of the Wnt and Shh pathways via distinct mechanisms, which include direct regulation of the expression of the tumour suppressor Wnt inhibitory factor 1 (Wif1) and indirect effects on the Shh expression.

Thus, tumour suppressor activity of the BMPs in skin epithelium depends on the local concentrations of Noggin and is mediated, at least in part, via stage-dependent antagonizing of the Wnt and Shh signalling pathways.

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Abbreviations

AB-Antibody

AP - Alkaline phosphotase

BCC- Basal Cell Carcinoma

BDNF - Brain derived neurotrophic factor

BMP - Bone Morphogenic Protein

BMPR - Bone Morphogenic Protein Receptor

BrdU - 5-Bromo-2-deoxyuridine

BSA - Bovine serum albumin

CK-Cytokeratin

CldU- Chlorodeoxyuridine

CTS - Connective Tissue Sheath

DEPC- Diethyl Pyrocarbonate

DIG - Digoxygenin

DNA - deoxyribonucleic acid

DP- Dermal Papilla

Eda - Ectodysplasin-A

EdaR - Ectodysplasin-A Reseptor

EGF - Epidermal Growth Factor

FGF - Fibroblast Growth Fcator

FITC-Fluorescein Isothiocyanate

H - Hematoxylin

H&E - Hematoxylin and Eosin

HGF - Hepatocyte Growth Factor

HF-Hair follicle

Hic2 - Hypermethylated in cancer 2

IdU - Iododeoxyuridine

IGF - Insulin Growth Factor

i/p - intraperitoneal

IRS-Inner Root Sheath

K-Keratin

KGF/FGF7 - Keratinocytes Growth Factor

Krt-Keratin

Lef-1 - Lymphoid enhancer binding factor 1

LRC - Label retaining cell

MAPK- Mitogen-activated protein kinase

mRNA - Messenger RNA

NCAM - Neural cell adhesion molecule

NT-3, NT-4 - neutrophins

ORS - Outer Root Sheath

NF-kB - Nuclear factor of kappa light chane gene enhancer in B-cells

PAGE - Polyacrilamid gel electrophoresis

PDGF - Platelet-derived growth factor

PFA - Paraphormaldehyde

Ptch - Patched

RNA- Ribonucleic Acid

RT- Room temperature

s/c -subcutaneous

SCC - Squamous Cell Carcinoma

SDS - Sodium dodecyl sulfate

SG - Sebaceous Gland

SHG - Secondary hair germ

Shh - Sonic Hedgehog

Smad - MAD (mother against decapentaplegic, Drosophila) homolog

Smo - Smoothened

Stat-3 - Signal transducer and activator of transcription 3

TCF-3 - Transcription factor 3

TG -Transgenic

TGFβ - Transforming growth factor β

TNFα - Tumour necrosis factor α

TRITC-Tetramethyl Isothiocyanate

Trp73-Transformation related protein 73

TUNEL - Terminal deoxynucleotidyl transferase dUTP nick end labelling

UTR - Untranslated Region

Wif1 - WNT inhibitory factor 1

Wfdc1 - WAP four-disulfide core domain 1

Wnt - Wingless-related MMTV integration site

WT-Wild type

I. INTRODUCTION

1.1. Functional morphology of the skin

The skin is one of the largest organs of the human body and provides a number of critical functions necessary for survival. Skin protects the body from water loss, temperature change, radiation, trauma, and infections, and it allows the body to perceive the environment through tactile sense. It also serves as important decoration for social and reproductive behavior (Chuong., 2002; Stenn and Paus, 2001). To fulfill such diversity of functions the skin shows structural complexity of its organization. The skin in humans and other mammals is composed of a variety of cellular structures of ectodermal and mesodermal origins that form a complex functional system and interplay between each other by means of numerous inductive and inhibitory signals [reviewed in (Fuchs, 1993; Millar, 2002; Odland, 1983; van Genderen, 1994). The epidermis and several appendages (hair follicles, sebaceous glands, sweat glands) represent the ectodermal compartment of the skin, while dermis and hypodermis (subcutaneous fat tissue) have mesodermal origin (Holbrook and Wolff, 1993).

1.1.1. Epidermis

The skin epithelium consists of cells that are both structurally and functionally different and form a multilayered stratified squamous epithelium (interfollicular epidermis, IFE), and patterned cutaneous appendages, such as the hair follicles (HFs) and sebaceous glands (Bowden, 1987). Central to this variegated cell population are keratinocytes. The keratinocytes are the epithelial cells producing keratins, which are the proteins that form the intermediate filament system in epithelial cells (Moll, 1982).

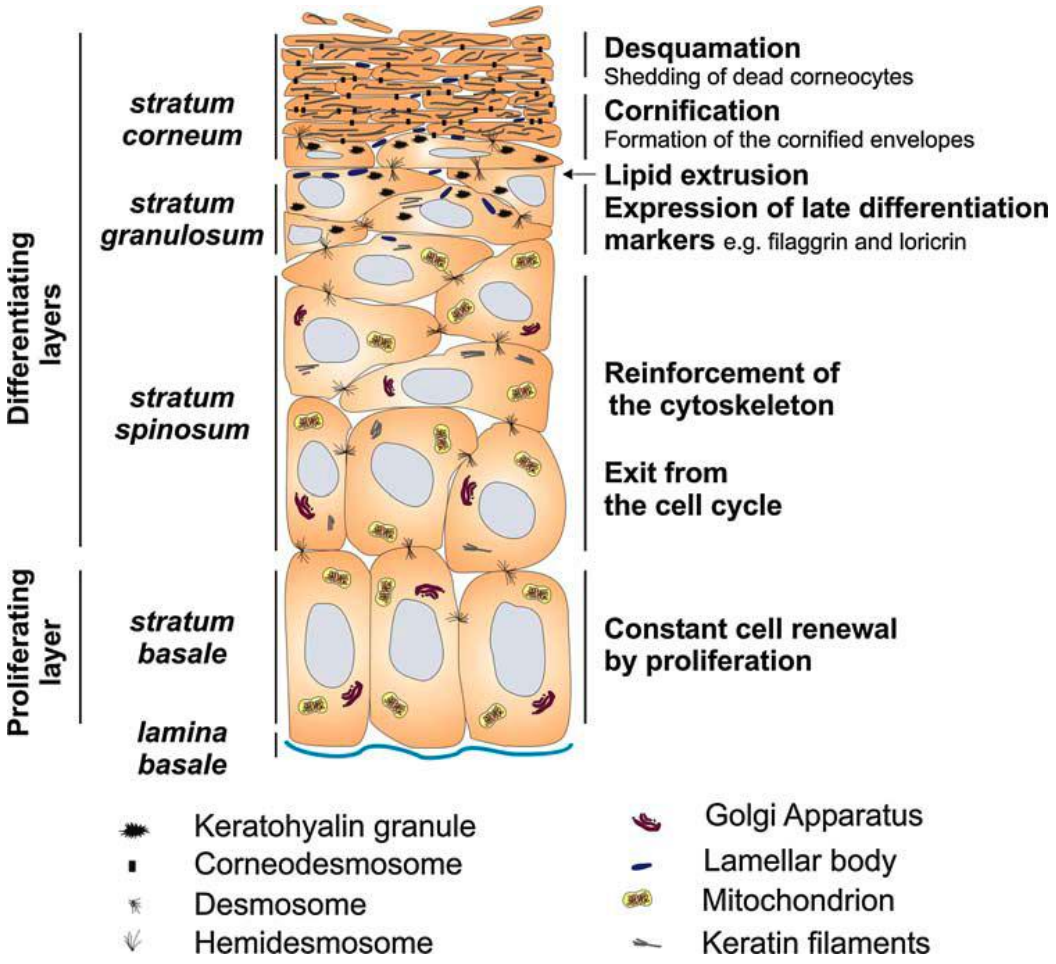

Figure 1

Fig. 1.1. The epidermis

Depending on the differentiation stage of the keratinocytes, the epidermis is subdivided into different layers or strata: basal, spinous, granular and horny layers (Lippens, 2009)

The protective function of the skin in general is a result of the cellular arrangements of the keratinocytes within the epidermis and the building up of an extensive keratin cytoskeletal network (Fuchs, 1993)**.**

In the IFE, keratinocytes are generated by mitosis in the innermost basal (**stratum basale**) or germinative layer. The basal layer is a monolayer of cuboidal cells attached to the basement membrane (lamina basalis) by hemidesmosomes. The basal keratinocytes are the only cells that are mitotically active and represent a reservoir of putative epidermal stem cells (SCs) and transiently amplifying (TA) progenitors, which give rise to terminally differentiated suprabasal layers (Alonso and Fuchs, 2003). This programme operates continually throughout the life of an organism; dead cells sloughed off from the skin surface are replenished by basal cells moving outward in a highly synchronized fashion. The self-renewing capacity of epidermal stem cells is enormous. Human epidermis turns over every 2 weeks, and each transiently amplifying basal cell divides only three to six times before it differentiates (Alonso and Fuchs, 2003). The basal cells are characterized by specific expression of keratin 5 (K5) and keratin 14 (K14) (Bowden*,* 1987; Sridhar Rao*,* 1996). As the basal cells divide and enter the first suprabasal layer, a downregulation of K5 and K14 expression occurs (Sridhar Rao*,* 1996) .

The next level of the epidermis is several cell layers thick the **stratum spinosum** (spinous layer). These cells contain a web-like system of interfilaments, mainly tension-regulating bundles of pre-keratin filaments, which span their cytosol to attach to a specialized type of cell junction (desmosomes) to resist physical trauma. These keratinocytes start to express keratin 1 (K1)

and keratin 10 (K10) (Bowden, 1987; Sridhar Rao, 1996). The K1 and K10 are only expressed in differentiating epidermal cells. In human skin, this synthesis continues up to 4-8 spinous cell layers and represents one of the earliest changes indicating the commitment of the cell to terminal differentiation (Sridhar Rao, 1996). Epidermal differentiation also involves induction of proteins such as involucrin, transglutaminase, fillagrin and loricrin, that form a cornified cell envelope as an important part of the epidermal barrier (Eckert and Rorke, 1989; Fuchs, 1980).

The **granular layer** (stratum granulosum) of the epidermis consists of three to five cell layers. These cells are characterized by flattened shape, disintegration of nuclei and organelles, and by accumulation of two types of granules: keratohyaline and lamellated granules. The keratohyaline granules represent a mixture of several smaller protein units containing keratohyaline, filaggrin and small proline-rich proteins (Matoltsy and Matoltsy, 1970). The lamellated granules (membrane-coating granules) are specialized secretory granules, which contain lipids (glucosylceramide, cholesterol and phospholipids) and their respective extracellular processing enzymes (Menon, 1992; Odland and Holbrook, 1981). The plasma membranes of these cells thicken as cytosol proteins bind to the inner membrane surface and lipids released by the lamellated granules coat their external surface (Lee, 2006). These processes are essential for the proper formation of the epidermal permeability barrier.

The outermost **stratum corneum** (horny layer) is a broad zone consisting of 20-30 cell layers that accounts for up to three-quarters of the human epidermal thickness. The stratum corneum forms a continuous sheet of protein-enriched cells (corneocytes) connected by corneodesmosomes and embedded

into the intercellular matrix enriched in non-polar lipids and organized as lamellar lipid layers (Madison, 2003). The final steps in keratinocyte differentiation are associated with profound changes in their structure, resulting in their transformation into the flat and anucleated corneocytes, which are loaded with keratin filaments and surrounded by a cell envelope composed of cross-linked proteins (cornified envelope proteins) as well as a covalently bound lipid envelope (Madison, 2003; Proksch, 2008). Gradually, these cells flake off and are replaced by underlying cells. The structure of the horny layer provides an effective physical barrier to most microorganisms, chemicals, and fluids. It also protects against uncontrolled loss of fluid from the body and is capable of withstanding mechanical forces (Madison, 2003; Proksch, 2008).

Other cells of different origin also populate the epidermis: melanocytes, Langerhans cells, Merkel cells. Melanocytes are specialized neural crest-derived cells in the epidermis that produce melanin, the pigment responsible for coloration and UV protection of the skin, hair, and the iris (Tobin and Kauser, 2005). Melanocytes account for only 1% of epidermal cells and occur at an approximate ratio of 1:10 among keratinocytes in the basal skin layer (Brenner and Hearing, 2008). Via their elongated dendrites, melanocytes transport their ovoid membrane-bound organelles (melanosomes), in which melanin is synthesized and stored, to neighbouring keratinocytes, where melanosomes form a critical barrier as supranuclear “caps” to shield DNA from ultraviolet radiation (Brenner and Hearing, 2008). Proliferating keratinocytes in the suprabasal epidermal layers gradually ascend towards the skin surface along with their ingested melanin to contribute to photoprotection.

Merkel cells are another type of neural crest-derived cells that are

responsible for transmission of touch sensation through the cutaneous nerves, among the other functions. Merkel cells are present only in the basal layer of the epidermis. They are innervated by unmyelinated nerve fibers and are the most abundant in areas of greatest tactile sensitivity, such as the fingertips (Moll, 2005).

Langerhans cells (LC) are epidermal antigen-presenting bone marrow-derived dendritic cells that are typically localized in the basal and suprabasal layers of the epidermis. These cells represent a critical outpost of the immune system at the interface to the external environment (Koch, 2006). The unique marker for LCs, langerin, is the major constituent of Birbeck granules, which are special phagosomes present in LCs. On capture of pathogens, LCs release inflammatory cytokines and chemokines, attracting other immune cells to the infection site (Koch, 2006). Mature LCs migrate from the epidermis to the regional lymph nodes for presentation of antigenic peptides to T cells and activation of an antigen-specific immune response (Koch, 2006).

1.1.2. Dermis

The dermis, the second major skin compartment, is a dense, irregular connective tissue that lies beneath the epidermis. The dermis is divided into two layers based on differences in the texture and arrangement of collagen fibrils. The superficial layer is the papillary dermis that is approximately 300-400 μm deep (Sorrell and Caplan, 2004). This depth is variable and depends upon such factors as age and anatomical location. Typically, the superficial portion of the papillary dermis is arranged into ridge-like structures, the dermal papillae, which contain microvascular and neural components that sustain the epidermis

(Cormack, 1987; Sorrell and Caplan, 2004). Dermal papillae greatly extend the surface area for epithelial-mesenchymal interactions and delivery of soluble molecules to the epidermis. A vascular plexus, the rete subpapillare, demarcates the lower limit of the papillary dermis.

The reticular layer of the dermis extends from this superficial vascular plexus to a deeper vascular plexus, the rete cutaneum, which serves as the boundary between the dermis and hypodermis (Cormack, 1987; Sorrell and Caplan, 2004). The deeper reticular layer, accounting for about 80% of the thickness of the dermis, is dense irregular connective tissue. Its extracellular matrix contains thick bundles of interacting collagen and elastin fibers that run in various planes; however, most run parallel to the skin surface (Kurt, 1991). The collagen fibers of the dermis give skin strength and resiliency that prevent most jabs and scrapes from penetrating the dermis. In addition, collagen binds water, helping to keep skin hydrated. Elastin fibers provide the stretch-recoil properties of skin (Kurt, 1991). Hair follicles and their associated dermal cells extend into and often through the reticular dermis to terminate in the hypodermis, a tissue rich in adipocytes.

1.1.3. Hair follicle as an epidermal derivative of the skin

The hair follicle (HF) is one of the most complex mini-organs in mammals. This is an exquisitely productive protein fiber factory, which doubles as a sensory organ and serves as an instrument of psychosocial communication, excretion, and protection. Importantly, the HF undergoes constant cyclic transformation over the total life-time of a mammal [reviewed in (Alonso and Fuchs, 2006; Panteleyev *et al.*, 2001; Stenn and Paus, 2001)]. The cyclic

nature of the HFs is supported by a signalling interplay between epithelial and mesenchymal compartments of the HF. However, the differentiation programme of the follicular keratinocytes is much more complex than in the epidermis and results in formation of the hair shaft (Hardy, 1992; Millar, 2002; Paus and Cotsarelis, 1999; Paus and Foitzik, 2004)

The mature HF is composed of a multicylindric stem that contains the hair shaft in its center and originates as an oval hair bulb proximally (**Fig. 1.2**) (Whiting, 2004). Embraced by the hair bulb lies the dermal papilla (DP) (sometimes referred to as the “follicular papilla” to avoid confusion with the most superficial region of the dermis). The dermal papilla cells are in close communication and exchange with another follicular fibroblast population, the cells of the dermal sheath, also called connective tissue sheath (CTS) (**Fig. 1.2**) (Tobin *et al.*, 2003). Both the DP and dermal sheath fibroblasts are different from others dermal cells, and they function as the “command center” to induce hair follicles and determine thickness, length of the hair, and likely the hair cycle itself (Jahoda and Reynolds, 1996; Jahoda *et al.*, 1984; Paus and Foitzik, 2004; Reynolds *et al.*, 1991).

The epithelial part is divided into an upper permanent region, distal to the arrector pili muscle (APM) and an inferior region (including the hair bulb), which dramatically remodels itself over the cycle. Within the hair bulb there is a population of cells with the highest proliferation rate in the human body: the keratinocytes of the hair matrix. These cells differentiate into trichocytes, or cells of the hair shaft and the inner root sheath (IRS), surrounding the hair shaft.

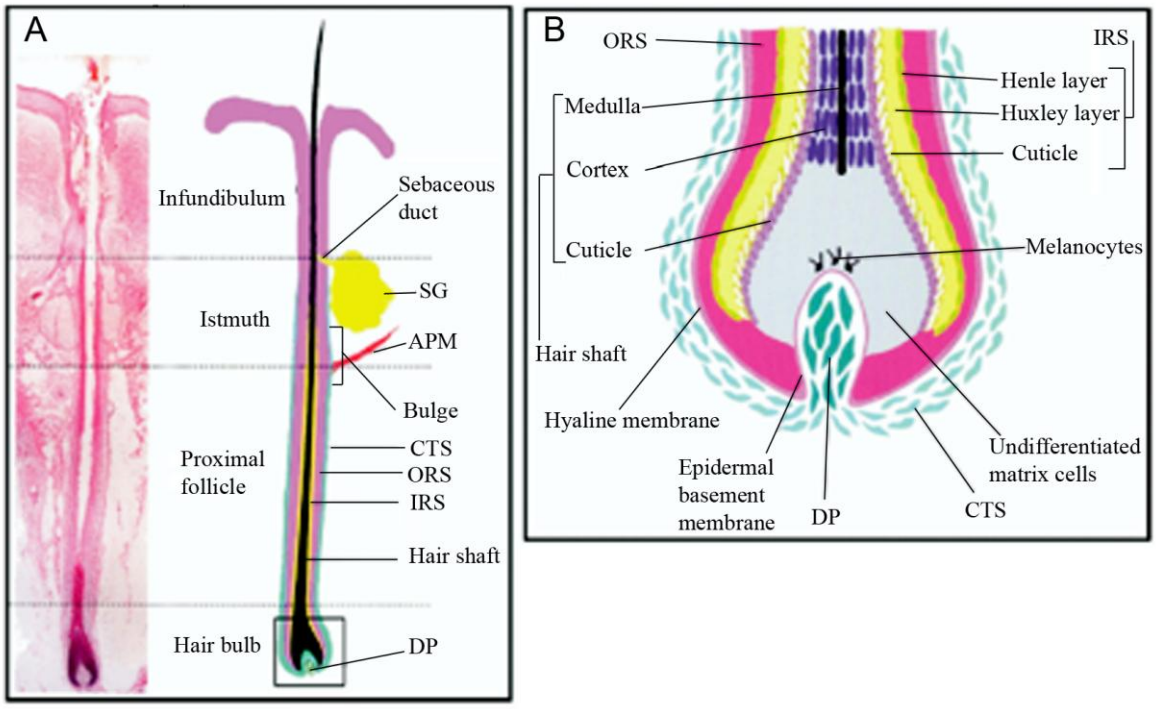

Fig. 1.2. The Hair follicle

(**A**) Anagen VI hair follicle. Histologic longitudinal section on the left hand side. Schematic drawing of an anagen VI follicle with anatomical details on the right hand side. (**B**) Anagen VI hair bulb in detail (enlargement of schematic drawing in A). APM, arrector pili muscle; CTS, connective tissue sheath; DP, dermal papilla; IRS, inner root sheath; ORS, outer root sheath; SG, sebaceous gland (modified after Whiting, 2004).

As hair shaft cells terminally differentiate, they extrude their organelles and become tightly packed with bundles of 10-nm filaments assembled from cysteine-rich hair keratins, which become physically cross-linked to give the hair shaft high tensile strength and flexibility (Alonso and Fuchs, 2006). The IRS also keratinizes in a way that it can rigidly support and guide the hair shaft during its differentiation process. The IRS cells degenerate as they reach the upper follicle, thereby releasing the hair shaft that emerges through the skin surface (Alonso and Fuchs, 2006). The outermost layer of hair follicle, the outer root sheath (ORS), is a non-keratinizing proliferative cell layer of the exterior HF, which is continuous with the epidermis (Tanaka *et al.*, 1998).

The ORS, hair matrix, IRS and hair haft derive from the epithelial cells in the bulge area, functioning as a pluripotent epithelial stem cell population for the skin (**Fig. 1.2**) (Cotsarelis *et al.*, 1990; Morris *et al.*, 2004). The bulge resides at the base of the permanent epithelial portion of the HF within the ORS; this is the deepest, most protected place in the contiguous epithelial compartment of the skin. Morphologically it is well defined as a region between sebaceous glands and the APM attachment site (Cotsarelis *et al.*, 1990; Lavker *et al.*, 2003).

1.1.4. Hair cycle

All mature HFs undergo a growth cycle consisting of the phases of growth (anagen), regression (catagen), rest (telogen), and shedding (exogen) [reviewed in (Botchkarev and Kishimoto, 2003; Cotsarelis and Millar, 2001; Fuchs *et al.*, 2001; Hardy, 1992; Panteleyev *et al.*, 2001; Paus and Cotsarelis, 1999; Paus and Foitzik, 2004; Stenn and Paus, 2001)] (**Fig. 1.3**).

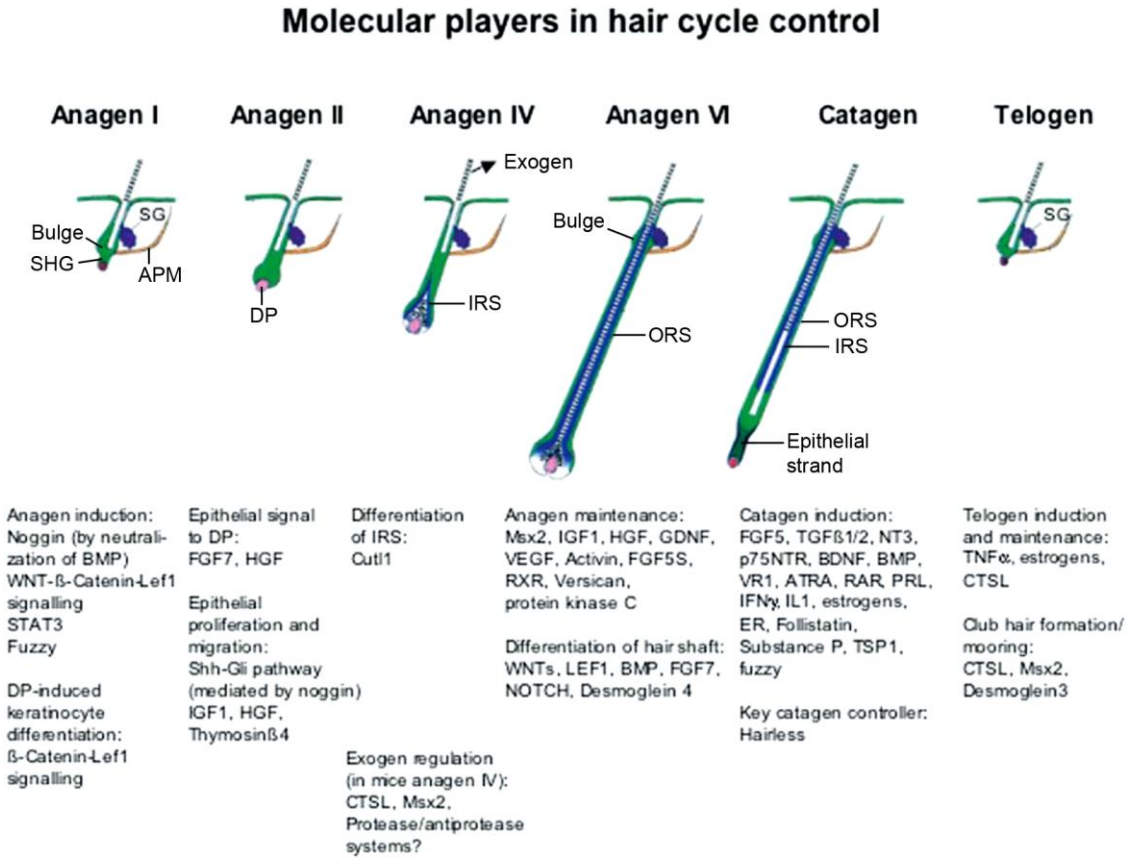

Fig. 1.3. Molecular players in hair cycle control

The figure shows key factors that drive the HF through the hair cycle stages or keep it in a given stage. BMP, bone morphogenetic protein; WNT, wingless; STAT3, signal transducer and activator of transcription 3; FGF7, fibroblast growth factor 7; HGF, hepatocyte growth factor; Shh, sonic hedgehog; IGF1, insulin like growth factor; CTSL, cathepsin L; cutl, transcriptional repressor; GDNF, glial cell line-derived neurotrophic factor; BDNF, brain-derived nerve growth factor; VEGF, vascular endothelial growth factor; ATRA, all-trans retinoid acid; RXR, retinoid x receptor; RAR, retinoid acid receptor; NGF, nerve growth factor; Lef1, lymphoid enhancer-binding protein; SHG, secondary hair germ; TGFβ, transforming growth factor β; p75NTR, low affinity neurotrophin receptor; PRL, prolactin; PRLR, prolactin receptor; IFNα, interferon α; ER, estrogen receptor; IL1, interleukin 1; VR1, vanilloid receptor 1; TNFα, tumour necrosis factor α; TSP1, thrombospondin 1 (modified after (Paus and Peker, 2003).

The HF is a regenerating system; the inferior part of HFs dramatically reforms over the cycle but the upper, permanent, part remains relatively unchanged (Alonso and Fuchs, 2006; Paus and Cotsarelis, 1999). It is well accepted that the ability of HFs for self-regeneration is a result of presence of HF stem cells in the bulge. In the classical experiments of Cotsarelis *et al.* (1990), stem cells were identified based primarily on their slow cycling nature, i.e. ability to retain the labeled DNA precursors for a long time. The bulge activation hypothesis that developed from this observation states that at the onset of anagen, slow-cycling multipotent stem cells in the bulge activate, giving rise to transiently amplifying (TA) cells.

Recently, the secondary hair germ (SHG) in telogen hair follicles, which appears as a small cell cluster between the bulge and DP, was identified as another pool of slow-cycling cells but more readily responds to stimulating signals (Blanpain *et al.*, 2004; Greco *et al.*, 2009). The TA cells derived from the bulge and SHG migrate to the base of the HF and become the matrix cells. The latter give rise to several lineages of terminally differentiated cells comprising different compartments of the HF (Blanpain *et al.*, 2004; Cotsarelis *et al.*, 1990; Ghazizadeh and Lome, 2001; Taylor *et al.*, 2000; Tumbar *et al.*, 2003).

In the mouse pelage, anagen lasts ~2 weeks, and the first three cycles are fairly synchronous, while anagen for human scalp hair can last for several years (Stenn and Paus, 2001). During anagen, matrix cells proliferate rapidly every approximately 18h (Lavker *et al.*, 2003). As they move upward, they exit the cell cycle and differentiate according to a strict spatial programmeme, reconstituting the concentric rings of the differentiated cell types of the IRS and hair shaft

(Alonso and Fuchs, 2006). The duration of anagen determines the length of the hair and is dependent upon continued proliferation and differentiation of matrix cells at the follicle base.

Following the anagen phase, HFs enter an apoptotic destructive phase known as catagen. During catagen, the lower (proximal) cycling portion of HFs is reduced to a thin epithelial strand surrounded by a basement membrane. As it retracts, the DP moves upward until it rests beneath the bulge (Fuchs *et al.*, 2001). In mice, the onset of the first catagen ranges from postnatal day 14 (P14) at the upper back near the head to P18 in the lower back near the tail and lasts three-four days (Alonso and Fuchs, 2006).

Following the catagen, HFs enter into a resting phase, telogen. In mice, the first telogen is short, lasting only one or two days, from approximately P19 to P21 in the mid back before the new hair growth emerges. Although the lengths of anagen and catagen phases are similar from one cycle to the next, telogen phase becomes progressively longer compared to the previous one. The second telogen lasts nearly four weeks, and the third telogen is even longer. These increasingly prolonged telogen phase result in progressive asynchrony in HF cycling with age (Alonso and Fuchs, 2006; Greco *et al.*, 2009).

1.1.5. Molecular control of the hair cycling

The majority of investigations on the molecular control of postnatal skin remodeling were performed using mouse models and many basic principles of the epidermal and HF biology were first established in the murine system (Hardy, 1992; Millar, 2002). An ever increasing number of novel mouse

mutants, where the genetically engineered knockout or overexpression of a defined gene product is associated with an epidermal and hair phenotype, has allowed instructive insights into the molecular controls of the HF cycling (Chen and Roop, 2008; Nakamura *et al.*, 2001). In fact, current understanding of the molecular controls of HF induction and morphogenesis is almost entirely based on the analysis of mutant mice. This is because the technology to create knockout and transgenic mice provides outstanding tools for studying the coordinate control of epithelial cell growth and differentiation during development (Chen and Roop, 2008).

It is now widely accepted that HF transformation during cycling is caused by alterations in the local signalling milieu. There are key regulators that build up local gradients with competing stimulating and inhibitory signals in the HF micro-environment (**Fig. 1.3**). Rhythmic changes of signal transducers in the key compartments of the follicle (bulge, secondary hair germ, dermal papilla) are thought to drive cyclic hair follicle transformation (Botchkarev and Paus, 2003).

Key factors inducing anagen include soluble proteins of the WNT family that activate the corresponding Wnt pathway via stabilization of β-catenin. Increased level of stabilized β-catenin in resting stem cells leads to the induction of a new hair (Lo Celso *et al.*, 2004; Lowry *et al.*, 2005; Van Mater *et al.*, 2003). As β-catenin becomes stabilized and localized to the nucleus in the activated hair germ (Lowry *et al.*, 2005; Merrill *et al.*, 2001), a number of transcriptional changes occur, which include those that regulate cell proliferation, extracellular matrix remodelling and HF fate specification (Lowry *et al.*, 2005).

Despite the importance of the Wnt signalling in the activation of hair follicle stem cells and their fate specification, ectopic levels of stabilized β-catenin do not cause bulge stem cells to lose their slow-cycling characteristics (Lowry *et al.*, 2005). The relative resistance of the bulge stem cells to elevated β-catenin signalling is also shown by the rarity of *de novo* follicle formation originating from bulge stem cells (Lo Celso *et al.*, 2004), as well as the ability of the bulge to maintain its constant cell number (Lowry *et al.*, 2005). These findings imply that additional factors are required to promote stem cell activation.

Increasing evidence has pointed onto the view that equilibrium between secreted Bone morphogenetic proteins (BMPs) and their soluble inhibitors, for example, noggin and gremlin, has an important role in controlling the SC activity during adult homeostasis. Gain- and loss-of-function studies in mice suggest that BMP signalling stimulates SC quiescence (Andl *et al.*, 2004; Blanpain *et al.*, 2004; Kobielak *et al.*, 2003; Kobielak *et al.*, 2007) and involved in the regulation of anagen initiation (Botchkarev, 2001). These data will be discussed in more detail below.

Other molecules can also be involved in the process of anagen initiation, such as the transcription factor STAT3. Deletion of STAT3 is associated with a prolongation of telogen (Sano *et al.*, 2000).

Once bulge stem cells have been activated, downstream signals are required to maintain the growth and differentiation phase of the hair cycle. Sonic hedgehog (SHH) has long been known to be a key signalling pathway that operates downstream of Wnts and is essential for maintaining the keratinocyte proliferative phase (Oro and Higgins, 2003; Silva-Vargas *et al.*, 2005; St-Jacques *et al.*, 1998). Hepatocyte growth factor (HGF) and Fibroblast growth

factor 7 (FGF7) support this process and stimulate the subsequent steps of anagen development (Danilenko *et al.*, 1995; Lindner *et al.*, 2000).

Fibroblast growth factor 5 (FGF5) is the most powerful regulator known so far to control anagen-catagen transition. Constitutive deletion of FGF5 in mice results in ‘‘angora-like’’ phenotype characterized by the extension of anagen for several days and dramatic increase in hair length (Hebert *et al.*, 1994). In addition to FGF5, neurotrophins and TGF-β1 induce a premature catagen onset. Transgenic over-expression of members of the neurotrophin family, BDNF and NT–3 show premature catagen development (Botchkarev *et al.*, 1999b). TGF-β1 induces premature catagen in isolated human hair follicle and in mouse skin in vivo, and TGF-β1 knockout mice display delay in the onset of catagen development (Foitzik *et al.*, 2000).

Catagen onset is also associated with down-regulation of molecules that promote proliferation and differentiation in hair matrix keratinocytes. In anagen hair follicles, dermal papilla fibroblasts secrete a variety of growth factors [HGF, IGF–1, keratinocyte growth factor (KGF), VEGF] that support proliferation and differentiation in hair matrix keratinocytes (Danilenko *et al.*, 1995; Guo *et al.*, 1996; Kozlowska *et al.*, 1998; Lindner *et al.*, 2000; Paus, 1996).

In addition to the growth factors mentioned above, onset of catagen is stimulated by several other molecules, such as insulin-like growth factor binding proteins–3/4/5 (IGF-BP3/4/5) (Batch *et al.*, 1996), interleukin-1α/β (Xiong and Harmon, 1997), vitamin D receptor (Reichrath *et al.*, 1994), or prolactin (Pearson *et al.*, 1999).

1.2 Molecular mechanisms of skin carcinogenesis

Skin cancer is the most common cancer in the world and the incidence continues to increase (Glick and Yuspa, 2005). During the last decade, considerable progress has been achieved in identification of molecular mechanisms underlying the development of the major cutaneous cancers, such as malignant melanoma and nonmelanoma (epidermal) cancer (Owens and Watt, 2003).

Epithelial tumours of the skin are usually classified into epidermal tumours, and neoplasms deriving from skin adnexae, such as hair follicle, sebaceous gland, apocrine and eccrine glands (Elder *et al.*, 1997; Headington, 1990). In fact, all skin cell types can undergo neoplastic transformation, giving rise to a vast and complex potential for benign and malignant development. Epidermal tumours [basal cell carcinoma (BCC) and squamous cell carcinoma (SCC)] account for over 90% of all skin cancers (Owens and Watt, 2003). BCC is more common with a ratio of 4:1 to SCC (Koh *et al.*, 2003). Although BCC and SCC can be lethal, they are not associated with significant mortality; nevertheless the associated morbidity and therapeutic costs are an increasing burden to the health care system.

Among the documented risk factors associated with nonmelanoma skin cancer development, skin colour and the response of the skin to sunlight (constitutional factors) are most important. This fact is obvious in Caucasians who have a combination of light skin and blue eyes, and red or blond hair; many of them get sunburn instead of a tan when they are exposed to direct sunlight (Gallagher *et al.*, 1995). In contrast to Caucasians, nonmelanoma skin cancer is uncommon in black populations, Asians and Hispanic (Koh *et al.*, 2003). The

major environmental cause of BCC and SCC is exposure to sunlight, in particular the UVB component of the sunlight (Situm *et al.*, 2008; Welsh *et al.*, 2008).

Several molecular regulatory systems were documented, perturbations in which were associated with skin tumourigenesis.

1.2.1. p53

The *p53* tumour suppressor gene is involved in cell cycle arrest and activation of programmemed cell death. Activation of p53 tumour suppressor protein occurs in response to a variety of cellular stresses including DNA damage, oncogenic stimulation, hypoxia, oxidative stress or telomere shortening and directs cells toward cell cycle arrest or apoptosis depending on the amount of DNA damage. This allows p53 to exerts its function as a "guardian of the genome" and tumour suppressor by blocking proliferative expansion of damaged cells (Lane, 1992). Mutations in the *p53* gene have been detected in 50% of all human cancers and in almost all skin carcinomas (Basset-Sequin *et al.*, 1994).

Analysis of mutations in p53 gene has established an unequivocal connection between UV exposure, DNA damage, and skin carcinogenesis. UVB and UVC radiation induces unique types of DNA damage, producing cyclobutane-type pyrimidine dimers (CPD) and pyrimidine (6-4) pyrimidone or (6-4) photoproducts. UV radiation induces predominantly C→T and CC→TT transitions at dipyrimidine sequences, which have become the "signature" of UV-induced mutagenesis (Brash, 1988). The mutations in *p53* gene appear to be an early genetic change in the development of UV-induced skin cancers.

Thousands of p53-mutant cell clones are found in normal-appearing sun-exposed skin (Jonason *et al.*, 1996). High frequency of p53 mutations has been reported in pre-malignant actinic keratosis lesions (66%), which are considered to be pre-SCCs, SCC (58%) and BCC (71%) (Bolshakov *et al.*, 2003; Moles *et al.*, 1993; Stern *et al.*, 2002).

A murine model of UV-induced carcinogenesis allowed a unique opportunity for investigating the fate of p53-mutant keratinocytes during various stages of skin cancer development. In skin of hairless mice, *p53* mutations induced by chronic UV exposure could be detected by allele-specific PCR as early as one week after initiation of the experiment, with 100% of the animals incurring *p53* mutations after eight weeks of UV treatment (Ouhtit *et al.*, 2000). As a tumour promoter, UV induces cell proliferation by stimulating the production of various growth factors and cytokines, as well as activation of their receptors (Bender *et al.*, 1997; Kuhn *et al.*, 1999; Peus *et al.*, 2000; Rosette and Karin, 1996). Repeated exposure of skin to UV radiation therefore results in clonal expansion of initiated *p53*-mutant cells (Zhang, 2001). Every successive UVB exposure allows *p53*-mutant keratinocytes to colonize adjacent epidermal stem-cell compartments without incurring additional mutations (Zhang, 2001). Two mechanisms are believed to contribute to selective expansion of p53-mutant cells: resistance to UV-induced apoptosis, and higher proliferative potential over normal keratinocytes in response to stimulation with UV. Indeed, single UV exposure was shown to stimulate the proliferation of p53-mutant cells while inducing apoptosis in normal keratinocytes in culture and in artificial skin models (Mudgil *et al.*, 2003; Ziegler *et al.*, 1994). However, chronic UV irradiation of skin quickly induces apoptosis-resistance and stimulates

hyperproliferation throughout the epidermis as an adaptive response (Ouhtit *et al.*, 2000). The mechanism of selective proliferative advantage of *p53*-mutant cells is yet unclear, but it may be a critical factor promoting a clonal expansion of initiated cells.

One mechanism that may contribute to expansion of initiated keratinocytes is the deregulation of the UV-induced Fas/Fas-Ligand mediated apoptosis in the skin. It was shown that accumulation of *p53* mutations in the epidermis of *FasL* deficient mice occurred at much higher frequency compared with wild-type mice after chronic UV irradiation (Hill *et al.*, 1999). Hill et al. (1999) concluded that *FasL*-mediated apoptosis is important for skin homeostasis, and that the dysreguration of Fas-FasL interactions may be central to the development of skin cancer. Ouhtit et al. (2000) further found that in skin of chronically-irradiated SKH-hrl mice, the progressive decrease of FasL expression was accompanied by accumulation of *p53* mutations and the decrease in a number of apoptotic cells. These findings suggest that chronic UV exposure would induce a loss of FasL expression and a gain in *p53* mutations, leading to dysregulation of apoptosis, expansion of mutated keratinocytes, and initiation of skin cancer.

1.2.2. Hedgehog signalling pathway

There is increasing evidence that genes critically involved in the regulation of epidermal stem cell proliferation and differentiation are also important in skin carcinogenesis (Owens and Watt, 2003). This relationship is not surprising because the cellular processes disrupted in cancer, e.g., proliferation, differentiation, apoptosis, motility and senescence, are also crucial for the

normal growth and development of tissues and organs.

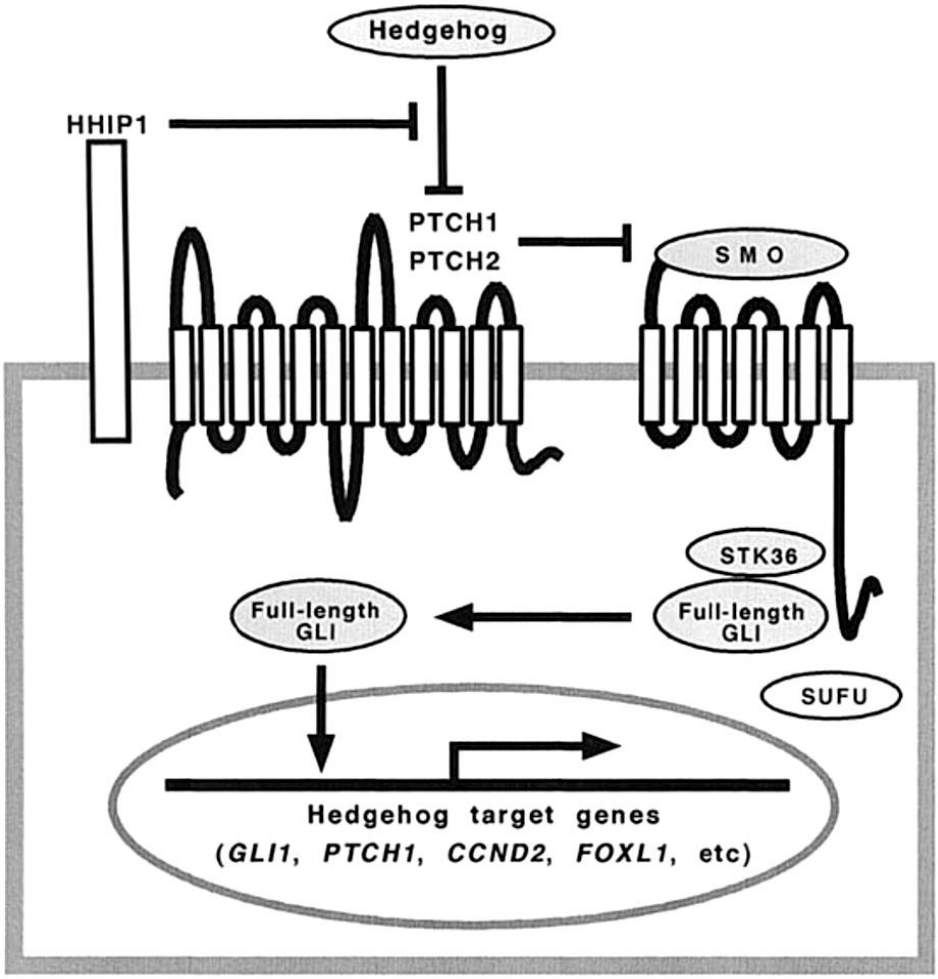

Fig. 1.4. Hedgehog signalling pathway

Hedgehog (SHH, IHH or DHH) binds to Patched family receptors (PTCH1 and PTCH2) to release Smoothened (SMO) signal transducer from Patched-dependent suppression. SMO activates STK36 serine/threonine kinase to stabilize GLI family members (GLI1, GLI2 and GLI3) for nuclear translocation. Hedgehog signalling activates GLI-dependent transcription of target genes, such as *GLI1, PTCH1, CCND2,* and *FOXM1*. Positive regulators of Hedgehog signalling are shown in gray. Adopted from (Katoh and Katoh, 2006,, 2008).

The hedgehog pathway is a fundamental signal transduction pathway in embryogenesis that is responsible for the correct development of various organs, including skin and hair (Bitgood and McMahon, 1995; Chiang *et al.*, 1999; Dlugosz, 1999). Its ability to regulate cell differentiation and renewal in a dose-dependent manner implies that deregulation of the hedgehog pathway can result in uncontrolled cell proliferation and tumourigenesis.

Sonic hedgehog (Shh), Indian hedgehog (Ihh) and Desert hedgehog (Dhh) are the three mammalian homologues of the *Drosophila* Hh protein. The available evidence suggests that all Hh ligands trigger the same signalling pathway, with specificity arising from the cellular context (Ingham and McMahon, 2001).

The activity of the Hh pathway involves several steps (**Fig. 1.4**). Two transmembrane proteins mediate the Hh signal: Patched (PTCH1 and PTCH2 in vertebrates) and Smoothened (SMO). In the absence of Hedgehog signalling, PTCH1 and PTCH2 inhibit the SMO signal transducer [reviewed in (Ingham and McMahon, 2001; Ogden *et al.*, 2004)]. SMO inactivation leads to formation of the cytoplasmic GLI degradation complex, in which GLI family members (GLI1, GLI2 and GLI3) are phosphorylated by casein kinase I (CK), glycogen synthase kinase-3β (GSK3β) and protein kinase A (PKA) (Price, 2006). Phosphorylated GLI is recognized by FBXW1/β and mediated for ubiquitination; ubiquitinated GLI is partially degraded to release its intact N-terminal half functioning as a transcriptional repressor (Bhatia, 2006; Ruiz *et al.*, 2007). Hedgehog-binding to Patched family receptors releases the SMO signal transducer from Patched-dependent suppression. SMO then activates STK36 serine/threonine kinase [also known as Fused (Fu)] to inhibit the assembly of GLI degradation complex

for the stabilization of full-length GLI (Osterlund *et al.*, 2004). Hedgehog signalling activation leads to GLI-dependent transcriptional activation of target genes, such as *GLI1, PTCH1, CCND2, FOXL1* and *JAG2* (Kasper *et al.*, 2006)

There is a number of positive or negative modulators; some of them being target genes of Hh signalling are involved in negative feedback, such as Hipl, Gasl and Ptchl itself, thus the activity of this pathway is tightly controlled in time and space [reviewed in (Katoh and Katoh, 2008; Mullor *et al.*, 2002)].

In the skin, aberrant sonic hedgehog expression results in the formation of basal cell carcinoma, which arises from the outer root sheath of the HF (Owens and Watt, 2003). Proliferation and differentiation of the ORS cells as well as the hair growth cycle are physiologically regulated by Shh (Dlugosz, 1999). Thus, aberrant activation of Shh signalling may disturb the physiologic homeostasis in this compartment and initiate tumour growth (Pasca and Hebrok, 2003).

Initially, germline mutation in *PTCH1* gene was found in nevoid basal cell carcinoma syndrome (NBCCS, Gorlin syndrome). A major manifestation of this rare syndrome is multiple BCC of the skin (Hahn *et al.*, 1996). Subsequently, somatic *PTCH1* mutation and/or deletion were identified in the majority of cases of sporadic BCC (more than 50%), thus implicating *PTCH1* inactivation as the most common genetic alteration in these tumours, resulting in continuous activation of the Hh pathway (Reifenberger *et al.*, 2005). Interestingly, UV as a major factor responsible for development of nonmelanoma cancer causes *PTCH1* mutations in sporadic BCC in less than 50%, while in BCCs of xeroderma pigmentosum patients UV is responsible for the majority (80%) of *PTCH1* mutations (Daya-Grosjean and Sarasin, 2000).

Sporadic basal cell carcinomas may carry mutations in other Shh pathway

genes, but these appear to be far less common. For example, somatic mutations in the *SMO* gene have been identified in about 10% of sporadic basal cell carcinomas. The majority of the cases carry a particular missense mutation (c. 1604G>T: W535L) resulting in constitutive Smo activation that can transform cells in vitro and is able to induce basal cell carcinoma-like tumours in transgenic mice. Somatic mutations in yet other Shh pathway genes, such as *SHH* itself and *SUFUH,* are rarely seen in individual cases of basal cell carcinoma (Dlugosz *et al.*, 2002; Reifenberger *et al.*, 2005).

Further support for the role of aberrant Shh signalling in basal cell carcinoma pathogenesis is provided by the finding of increased expression of Shh target genes, such as *PTCH, HIP, GLI1* and *GLI2,* in virtually all cases. *In vitro* experiments indicate that GLI1 and GlI2 are part of a positive feed-back loop in keratinocytes, in which GLI1 induces the expression of the transcriptional activator isoforms GLI2α and GLI2β which in turn up regulate GLI1 expression by binding directly to the *GLI1* gene promoter. In basal cell carcinomas, the activity of GLI1 is further enhanced by the expression of transcripts containing alternative 5'-untranslated sequences that enhance translational efficiency of GLI1 proteins. Activation of the GLI1 and GLI2 transcription factors in basal cell carcinomas results in enhanced transcription of several growth promoting and anti-apoptotic genes (Pasca and Hebrok, 2003). These include certain cell cycle regulatory genes, such as the forkhead/winged-helix domain transcription factor genes *FOXM1* and *FOXE1,* as well as the cyclin genes *CCND1* and *CCND2* (Eichberger *et al.*, 2004; Liang, 2000; Teh, 2002). GLI1 also induces transcription of the growth factor receptor gene *PDGFRA,* which in turn may activate signalling via the ras/ERK-pathway in

basal cell carcinomas (Xie *et al.*, 2001). An additional SHH target gene upregulated in basal cell carcinomas and directly regulated by GLI2 is *BCL2,* whose gene product inhibits apoptosis (Regl *et al.*, 2004).

The relevance of aberrant hedgehog signalling for the development of BCCs has been experimentally substantiated in a number of transgenic and knockout mouse models (Dlugosz *et al.*, 2002; Grachtchouk *et al.*, 2003; Hutchin *et al.*, 2005; Pasca and Hebrok, 2003). Overexpression of Shh using a keratin 14 (KI4) promoter resulted in upregulation of Shh target genes and development of basal cell carcinoma-like proliferations in the skin of newborn mice (Oro *et al.*, 1997). Furthermore, overexpression of mutant Smo isolated from human BCC can induce “basal cell-like” proliferations in newborn mouse skin when driven by a keratin 5 (K5) promoter (Xie *et al.*, 1998). Mouse models with defective *Ptch* have also been developed. Interestingly, the *Ptch*+/- mice develop BCC-like tumours of the skin only after ionizing radiation or repetitive UVB exposure (Calzada-Wack *et al.*, 2002).

Other transgenic mouse models have focused on the Gli proteins as effectors of hedgehog signalling. Interestingly, overexpression of Gli1 or Gli2 under a K5 promoter yielded different results. K5-Gli1 transgenic mice developed a variety of hair follicle-derived tumours, including a small fraction of basal cell carcinoma-like tumours (Nilsson, 200). In contrast, K5-Gli2 mice selectively developed skin tumours with basal cell carcinoma-like morphology (Grachtchouk *et al.*, 2000).

Taken together, the findings clearly underline the importance of aberrant Hh signalling in basal cell carcinomas. In contrast to BCC, SCC development is not Shh signalling dependent, which is in the line with its distinct origin from

transient amplifying cells in the interfollicular epidermis.

1.2.3. Wnt/β-catenin signalling

Wnt signalling is one of powerful signalling pathways that play crucial roles in controlling the genetic programmes of embryonic development and adult homeostasis in mammals. Deregulation in this signalling pathway causes developmental defects, or diseases, among them cancer.

The canonical Wnt/β-catenin signal transduction pathway is schematically illustrated in **Fig. 1.5** and reviewed elsewhere (Clevers, 2006; MacDonald *et al.*, 2009; Nelson and Nusse, 2004; Willert and Jones, 2006). In the absence of Wnt ligands, intracellular levels of β-catenin are regulated by a multiprotein complex encompassing kinases, such as GSK3β (glycogen synthase kinase-3β) and CK1 (casein kinase 1), the scaffolding proteins APC (adenomatous polyposis coli), Axin. This "destruction complex" binds and phosphorylates β-catenin at serine and threonine residues in the N-terminus. Phosphorylation of β-catenin creates a binding site for the E3 ubiquitin ligase β-Trcp, leading to β-catenin ubiquitination and degradation. When Wnt signalling is inactive, TCF/LEF family of transcription factors are bound to Wnt-target gene promoters in a complex with transcriptional co-repressors, such as Tle/Groucho, to keep target genes silent. Presence of Wnt ligands triggers formation of a complex between co-receptors LPR5/6 (low-density lipoprotein receptor-related proteins) and Frizzled (Fz) at cell membrane.

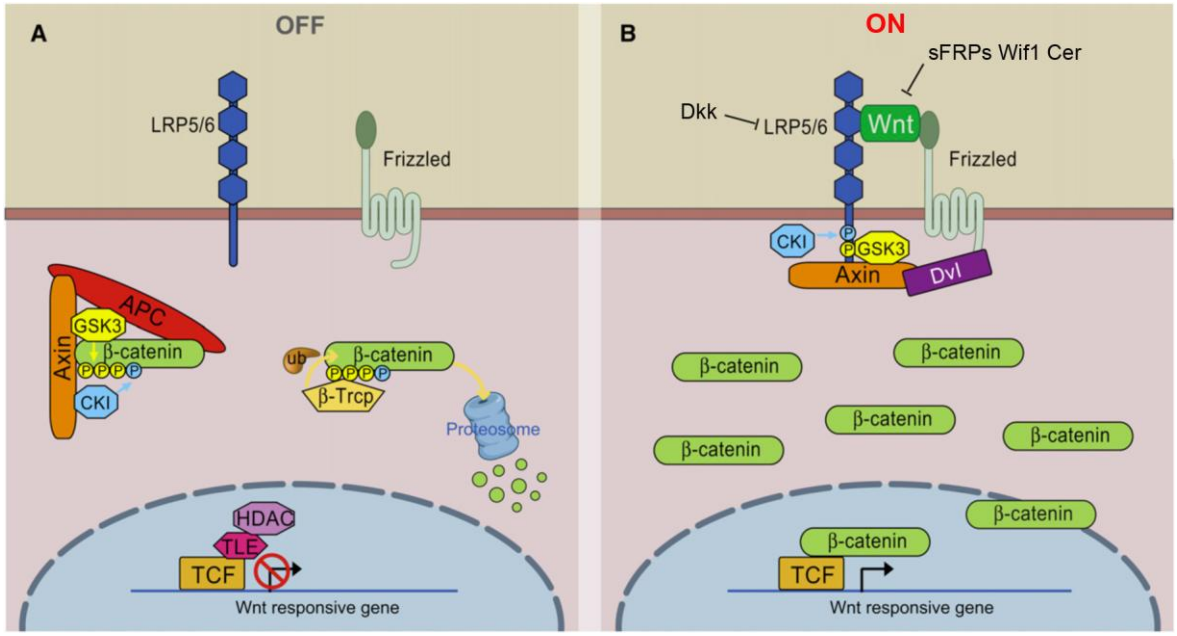

Fig. 1.5. Scheme of the Wnt/β-catenin signalling pathway

(**A**) In the absence of Wnt, cytoplasmic β-catenin forms a complex with Axin, APC, GSK3, and CK1, and is phosphorylated by CK1 (blue) and subsequently by GSK3 (yellow). Phosphorylated β-catenin is recognized by the E3 ubiquitin ligase β-Trcp, which targets β-catenin for proteosomal degradation. Wnt target genes are repressed by TCF-TLE/Groucho and histone deacetylases (HDAC).

(**B**) In the presence of Wnt ligand, a receptor complex forms between Frizzled and LRP5/6. Dishevelled (Dvl) recruitment by Frizzled leads to LRP5/6 phosphorylation and Axin recruitment. This disrupts Axin-mediated phosphorylation/degradation of β-catenin, allowing β-catenin to accumulate in the nucleus where it serves as a coactivator for TCF/Lef to activate Wnt-responsive genes. Modified after (MacDonald *et al.*, 2009).

Upon Wnt stimulation, LRP6 is phosphorylated by Dishevelled (Dvl) and recruits Axin-GSK3β to the plasma membrane, coincident with the inhibition of the β-catenin disruption complex and the consequent stabilization of β-catenin.

Cytosolic β-catenin accumulation eventually results in its nuclear translocation. In the nucleus, β-catenin binds to members of the TCF/LEF transcription factors, replacing the transcriptional inhibitor Groucho and recruiting co-activators, such as the histone acetyltransferase CBP/p300, to stimulate transcription of a broad range of Wnt target genes, such as *c-MYC*, *CCND1*, *c-JUN*, *AXIN2*, *FGF9, Eda* etc. (current list of known target genes are available on Wnt genes homepage http://www.stanford.edu/~rnusse/wntwindow.html).

Several extra-cellular inhibitors tightly regulate spatial-temporal activity of the pathway. Secreted factors like secreted Frizzled-related proteins (sFRPs), Wif-1 and Cer bind to Wnts and block the interaction with Frizzled receptors, while Dickkopf antagonizes Wnt signalling by blocking LRP receptors [reviewed in (Kawano and Kypta, 2003; MacDonald *et al.*, 2009)].

Aberrant activation of Wnt/β-catenin signalling pathway has been shown to be an important factor in the development of many malignancies including colorectal, breast, ovarian, prostate, and many other cancers [reviewed in (Fodde and Brabletz, 2007; Morin, 1999). In these types of cancer, an abnormal Wnt pathway activation and stabilization of the cytoplasmic β-catenin are due to an impairment of the β-catenin N-terminal phosphorylation, which could be a result of: (1) oncogenic mutations in its phospho-acceptor sites; (2) failure to recruit GSK3β because of APC mutations; (3) inhibition of the GSK3β activity PI3K/Akt signalling [reviewed in (Morin, 1999; Polakis, 1999)].

However, little is known about the role of Wnt/β-catenin signalling in non-

melanoma skin cancers. Studies utilizing transgenic approaches showed that canonical Wnt signalling plays a crucial role in development of the skin and skin appendages primarily by promoting the hair placode and hair shaft fate of the epithilium at the expense of epidermal differentiation (Andl *et al.*, 2002; van Genderen *et al.*, 1994; Zhang *et al.*, 2008) and stimulating hair follicle regeneration in postnatal life (Lo Celso *et al.*, 2004; Lowry *et al.*, 2005; Van Mater *et al.*, 2003). Importantly, these studies also showed that an aberrant activation of the Wnt signalling might be involved in the development of some skin tumours. It was reported that transgenic mice expressing an activated β-catenin developed two types of hair follicle-derived tumours, pilomatricoma and trichoepithelioma, which were also found in humans (Gat *et al.*, 1998; Lo Celso *et al.*, 2004). These results were confirmed by genetic studies, which revealed activating mutations of the β-catenin gene in human pilomatricoma in at least 75% of the tumours (Chan *et al.*, 1999; Durand and Moles, 1999; Kajino *et al.*, 2001; Xia *et al.*, 2006). However, β-catenin mutations have not been detected in other skin tumours (Kajino *et al.*, 2001). To date, mutations in other components of the Wnt/β-catenin pathway, such as adenomatous polyposis coli (APC) and AXIN2, have been not reported (Doglioni *et al.*, 2003); they are unlikely involved in skin tumourigenesis.

Despite no activating mutations in the β-catenin gene in most of the skin tumours, abnormal expression and nuclear localization of β-catenin was observed in a subset of human non-melanoma skin cancers. It was reported that about 50-60% of basal cell carcinomas showed strong nuclear β-catenin expression predominantly at the peripheral part of the tumours (Doglioni *et al.*, 2003; El-Bahrawy *et al.*, 2003; Yamazaki *et al.*, 2001). The nuclear localization

was most notable in the infiltrative and morphoec variants, suggesting that this may be associated with more aggressive types of invasion (El-Bahrawy *et al.*, 2003). Thus, nuclear localization of β-catenin is a characteristic feature of BCC, however, a causal link between Hh pathway deregulation, nuclear β-catenin accumulation and increased proliferation in BCC development remains to be confirmed.

Moderate increase of β-catenin nuclear staining was also detected in a significant proportion of Bowen disease, spiroadenomas, and squamous cell carcinomas (Doglioni *et al.*, 2003; Malanchi *et al.*, 2008).

The important role of Wnt/β-catenin in epidermal carcinogenesis was demonstrated in an experimental study using the two-stage chemical carcinogenesis protocol. Mouse skin tumours induced by DMBA/TPA treatment showed an increase of β-catenin protein expression accompanied with the dramatic increase in nuclear β-catenin levels throughout papillomas and SCC, suggesting that β-catenin was stabilized during skin carcinogenesis (Bhatia and Spiegelman, 2005). In addition, expression of well characterized target genes of the Wnt/β-catenin signalling pathway, c-*myc* and c-*jun*, was up-regulated in all chemically induced tumours (Bhatia and Spiegelman, 2005). Furthermore, papillomas and SCCs were characterized by high expression levels of skin-specific Wnt proteins (Wnt3, Wnt4 and Wnt10b), suggesting that stabilization of β-catenin may be a result of such increased expression of Wnt ligands (Bhatia and Spiegelman, 2005). Interestingly, a transcriptional co-partner of β-catenin, Tcf4, was highly expressed in all papillomas and carcinomas, while immunoblot analysis of Lef1, Tcf1 and Tcf3 proteins revealed very low levels of their expression during mouse skin carcinogenesis (Bhatia and Spiegelman, 2005).

Furthermore, stable transfection of SCC cells (Ca8/29) with a dominant negative Tcf4 mutant exhibited about 30% decrease in cell growth in vitro, thus emphasized the significance of Wnt/β-catenin/Tcf-dependent transcription in the proliferative potential of SCC cells (Bhatia and Spiegelman, 2005).

Taken together, these data suggest that unlike to carcinogenesis in other organs activation of canonical Wnt/β-catenin signalling pathway in skin tumours occurs in the absence of mutations in genes coding components of the pathway. This is well in line with a current concept of the role of cancer stem cells (CSCs) in the epidermal tumour development, which postulates that a small proportion of tumour cells have capacity to self-renew and form new tumours [reviewed in (Ambler and Määttä, 2009; Owens and Watt, 2003)]. These CSCs do not reflect a simple expansion of stem cells; rather, tumour cells bypass the homeostatic controls that operate in normal stem cells, eliminating those that maintain stem cell quiescence (Jensen *et al.*, 2008). Wnt/β-catenin signalling is essential for activation of normal epidermal stem cells (Lo Celso *et al.*, 2004; Lowry *et al.*, 2005; Van Mater *et al.*, 2003), and, if abnormally activated, likely involved in the acquisition of the cancer stem cell capacities by normal epidermal stem cells. Findings from Malanchi *et al.* (2008) study support the idea that cutaneous cancer stem cell maintenance is dependent on β-catenin signalling. Using two-stage skin chemical carcinogenesis as a model, Malanchi *et al.* (2008) identified a population of CD34+ cells in early epidermal tumours characterized by phenotypic and functional similarities to normal bulge skin stem cells. Isolated CD34+ cells were the only cells with tumour initiation properties when orthotopically transplanted in a skin reconstitution assay (Malanchi *et al.*, 2008). Moreover, nuclear β-

catenin was enriched in CD34+ versus CD34- tumour cells, suggesting a potential functional relevance to this pathway. Conditional ablation of the β-catenin gene resulted in complete regression of established, chemically induced tumours. Following deletion of β-catenin, CD34 expression was lost, and tumour cells lose their ability to initiate secondary tumour formation (Malanchi *et al.*, 2008). Finally, authors also provide an evidence that β-catenin signalling may be functionally relevant in human SCCs. Knockdown of the β-catenin expression by short hairpin RNA reduces tumour growth of human SCC13 cell lines in xenografts, suggesting that β-catenin signalling is required for the tumourigenesis (Malanchi *et al.*, 2008).

1.2.4. Transforming Growth Factor β (TGF-β) signalling

TGF-βs (TGF-β1, TGF-β2, TGF-β3) regulate many fundamental processes during embryonic development and in adult tissues, such as cell growth, differentiation, remodeling of the extracellular matrix, cell migration and adhesion, angiogenesis and the immune response (Massague, 1998).

TGF-β was early recognized as a potent inhibitor of epithelial cell growth. Subsequent studies showed that some carcinoma cell lines escape from TGF-β growth inhibition, and reported a direct role for TGF-β as an autocrine stimulator of tumour cell invasion and metastasis (Newman, 1993; Wright *et al.*, 1993). In the past decade, a large body of experimental evidence has accumulated suggesting a dual role for TGF-β in cancer (Derynck *et al.*, 2001; Siegel and Massague, 2003). Thus, it is now widely accepted that TGF-β can act as a tumour suppressor at early stages of tumourigenesis, and, also, as a potent

driver of malignant progression, invasion and metastasis at later stages. Of the three classical members of the TGF-β family, TGF-β1 is most frequently up-regulated in tumours (Derynck *et al.*, 2001) and is the focus of most studies on the role of TGF-β in carcinogenesis.

In vitro studies have documented that TGF-β1 is a potent growth inhibitor for primary and immortalized keratinocytes, whereas some carcinoma cell lines are less sensitive to the inhibitory effect of the growth factor (Glick *et al.*, 1993). A complete loss of the anti-proliferative response appears to occur late during malignant progression, associated with the spindle stage (Haddow *et al.*, 1991). The mechanism for TGF-β1-induced growth arrest in keratinocytes involves down-regulation of c-myc (Pietenpol *et al.*, 1990) and induction of the Cdk inhibitors p21 and p15 (Li *et al.*, 1995; Reynisdottir *et al.*, 1995). Carcinoma cells do not only exhibit an attenuated response to growth inhibition, but they are also stimulated to malignancy by the growth factor. Chronic exposure of transformed keratinocytes to TGF-β1 induced a reversible epithelial-mesenchymal transition (EMT) (Caulfn *et al.*, 1995). Further reports demonstrated that the phenotypic changes induced by TGF-β1 in transformed keratinocytes were associated with increased invasive and metastatic abilities (Frontelo *et al.*, 1998), and with up-regulation of extracellular matrix degrading proteases, such as uPA and MMP-9 (Santibanez *et al.*, 1999; Santibanez *et al.*, 2002).

Deeper understanding of the role for TGF-β in epithelial skin cancer has been made using mouse chemical carcinogenesis model system. Multistage mouse skin chemical carcinogenesis has provided a paradigm to study the genetic and epigenetic events that contribute to the development of squamous

cell carcinomas (SCCs) (Yuspa, 1994). The most common chemical carcinogenesis protocol is two-stage induction, which involves the topical application of a single dose of a carcinogen initiator; i.e., the polycyclic aromatic hydrocarbon 7,12-dimethylbenz-(a)anthracene (DMBA), followed by once or twice weekly treatment with the tumour promoter TPA. This protocol results in the development of multiple benign papillomas, most of which are promoter-dependent and regress rapidly after the end of TPA treatment (Glick *et al.*, 2007). However, a small proportion of papillomas (about 5-10%) do not regress in the absence of the promoter and spontaneously progress to malignant SCCs (Glick *et al.*, 2007). SCCs can vary in their histological grade, from well differentiated, with clear basal and suprabasal layers, to poorly differentiated, with little structural organization. The latest stage of tumour progression is the development of spindle cell carcinomas (SpCCs), a highly malignant type of tumour formed by cells that have lost the epithelial phenotype and acquired mesenchymal characteristics (Klein-Szanto *et al.*, 1989). Spindle carcinoma cells have down-regulated the expression of keratinocyte specific proteins, such as cytoskeletal keratins and the cell-cell adhesion molecules E- and P-cadherin, and are characterized by the expression of fibroblastic cell markers, such as vimentin (Navarro *et al.*, 1991). Initiation involves a specific oncogenic mutation in the H-Ras gene (To *et al.*, 2005), and additional changes in the H-Ras mutated allele leading to overexpression of H-Ras oncogenic protein are associated with malignant progression (Rodriguez-Puebla *et al.*, 1999a; To *et al.*, 2005). Other alterations associated with tumour progression lead to deregulation of the cell cycle machinery and promote cell growth and, possibly, genetic instability. Among these genetic alterations, the most relevant are Ras-

dependent overexpression of cyclin D1 (Rodriguez-Puebla *et al.*, 1999b), inactivation of the p53 gene (Yuspa *et al.*, 1994), and deletion or altered regulation of the INK4 locus encoding the cyclin-dependent kinase (cdk) inhibitors p15, p16 and p19, the latter associated with the late spindle stage (Linardopoulos *et al.*, 1995).

The combination of the two-stage chemical carcinogenesis protocol and transgenic/knock-out approaches has provided the experimental framework to demonstrate a double and paradoxical role of TGF-β signalling in skin carcinogenesis in vivo. Disruption of TGF-β signalling by expression of a dominant-negative type-II TGF-β receptor in the epidermis of transgenic mice (L- or K5-ΔTGFβRII mice) resulted in increased sensitivity to DMBA/TPA carcinogenesis with respect to control mice. These mice showed a higher frequency of malignant conversion from papillomas to SCCs (Amendt *et al.*, 1998; Go *et al.*, 1999). Furthermore, TPA promotion alone induced papilloma formation in L-ΔTGF/3RII mice, and most of TPA-induced papillomas did not exhibit H-Ras mutations, suggesting that loss of TGF-β signalling can serve as an initiating event in skin carcinogenesis. Nevertheless, the most striking result on the role of TGF-β in skin carcinogenesis was obtained by Cui and co-workers (1996) utilizing two-stage chemical carcinogenesis in transgenic mice with TGFβ1 expression targeted to the epidermis. Overexpression of TGF-β1, in epidermal keratinocytes inhibited benign tumour formation, but enhanced conversion of papillomas to SCCs, as well as the squamous to spindle cell transition (Cui *et al.*, 1996). Taken together, these studies support a direct role for TGF-β, as a tumour suppressor at early stages of carcinogenesis, yet it also behaves as a promoter of malignancy at later stages.

In addition to its direct role on tumour cells by inhibiting cell growth or promoting an EMT associated with development of highly aggressive spindle tumours, TGF-β profoundly affects the tumour microenvironment. TGF-β acts on several cell types that are in the proximity to the tumour, including fibroblast, endothelial and immune cells. Thus, TGF-β has potent extracellular matrix remodeling, immunosuppressor and angiogenic effects, creating an environment that facilitates tumour growth, invasion and metastasis (Siegel and Massague, 2003). It is likely that the complex and paradoxical effects of TGF-β on tumour development implicates an intricate network of autocrine and paracrine responses on keratinocytes and stromal cells involving TGF-β as well as other cytokines. Thus, overexpression of the TGF-β1 transgene in the epidermis enhances angiogenesis (Wang *et al.*, 1999), apparently not by a paracrine mechanism involving diffusion of the growth factor into the dermis, but rather by stimulating vascular endothelial growth factor (VEGF) expression in the keratinocytes (Wang et al.).

1.2.5. Platelet-derived Growth Factor (PDGF) pathway

In the last few years, compelling evidence supports the essential role of PDGF signalling for cancer cell proliferation and tumour angiogenesis in several types of human cancers, including nonmelanoma skin cancers.

PDGFs belong to PDGF/VEGF family of growth factors. PDGFs (PDGF-A, PDGF-B, PDGF-C, PDGF-D) expression is observed in a variety of cell types, including fibroblasts, keratinocytes, neurons, endothelial and epithelial cells, and functions as a potent mitogen. In addition, PDGF regulates cell morphology and cell movement, such as chemotaxis (Hoch and Soriano, 2003).

PDGF subunits are synthesized as precursor molecules, which undergo proteolytic processing. A variety of PDGF dimers can be formed from these subunits, which then activate the PDGF receptor tyrosine kinases. PDGF receptors (PDGFRα, PDGFRβ) function in the cell through activating downstream effectors, such as PLC-γ, Grb2/SOS, PI3K, GAP and Stat (Hoch and Soriano, 2003).

Development of skin and hair requires the PDGF-A/PDGFRα signalling axis. PDGF-A is expressed in the hair follicle epithelium, whereas PDGFRα is found in the dermal papillae. PDGFRα null embryos show severe dermal defects, with an absence of dermal mesenchymal cells and formation of epidermal blisters (Soriano, 1997). In contrast, the phenotype of PDGF-A null mutant embryos is quite mild (Karlsson *et al.*, 1999), suggesting a substitutive role of other PDGFs in skin development. Hair follicles of *PDGF-A*$^{-/-}$ mice are misshaped and smaller (Karlsson *et al.*, 1999).

PDGF pathways are altered in several physiological human conditions including carcinogenesis. The role of PDGFs in carcinogenesis is initially demonstrated by the fact that *v-sis* oncogene encodes a PDGF-B-like protein. Both *v-sis* and its cellular counterpart *c-sis* transform cultured cells through an autocrine mechanism. Studies in last two decades clearly indicate that PDGFs and their receptors are involved in human cancers through autocrine stimulation of tumour cell growth (Ostman and Heldin, 2001; Yu *et al.*, 2003). In addition to the autocrinal regulation of tumour growth, PDGF signalling exerts paracrinal stimulation on stroma cells. Such an example is shown in tumour angiogenesis, which has been observed in breast cancer, colorecral cancer, melanoma and small cell lung cancer (Yu *et al.*, 2003). The level of PDGFs is correlated with

the density of blood vessel in the tumours.

Several experiments demonstrated that PDGF pathway served as functional downstream of SHH signalling. PDGF-A and PDGFRα expression was elevated in BCCs derived from *Ptch1*$^{+/-}$ mice where the hedgehog pathway is constitutively activated (Xie *et al.*, 2001). In most human BCCs, PDGF-A and PDGFRα expression was also up-regulated, and accompanied by an increased levels of phosphorylated Erk, an effector of PDGF signalling, indicating an activation of the Ras/Erk pathway in the tumour cells (Xie *et al.*, 2001). Xie et al. (2001) also demonstrated that PGFRα expression was regulated by Gli1 in vitro. Moreover, perturbation of PDGFRα in mouse BCC cell line ASZ001, whether directly by neutralizing antibodies or indirectly by Ptch1, leads to decreased cell proliferation (Xie *et al.*, 2001). Therefore, up-regulation of PDGFRα appears to be an important mechanism by which hedgehog signalling induces BCCs. Consistent with these findings, an inhibition of Hh signalling by Smo antagonist cyclopamine decrease the levels of PDGFRα and phosph-Erk resulting in apoptosis in BCC cells (Athar *et al.*, 2004).

The role of PDGF for tumour development of SCC is not well studied. However, there is a good correlation of angiogenic cytokine secretion with the microvessel density in the primary tumours of SCC (Ninck *et al.*, 2003). In head and neck SCCs, VEGF and PDGF-AB are secreted in high amounts (Gleich *et al.*, 1996; Ninck *et al.*, 2003). Keratinocytes are a major source of cutaneous PDGF, whereas human dermal fibroblasts do not produce any detectable PDGF (Zhang *et al.*, 1995). It appears, PDGF regulates stromal cells through a paracrine mechanism in skin SCCs. Subcutaneous injection of PDGF-B transfected HaCaT cells leads to marked mesenchymal cell proliferation and

angiogenesis during early SCC development, yet this effect vanished at later stages concomitantly with increased epithelial cell proliferation and enhanced tumour growth (Mueller, 2002). These results demonstrate that an activated stromal environment can promote tumourigenic conversion of the keratinocytes by inducing sustained epithelial hyperproliferation. Thus, PDGF-B appears to promote tumour growth by inducing angiogenesis and stromal formation, and PDGF-activated stromal cells maintain elevated keratinocyte proliferation via a paracrine mechanism.

1.2.6. STAT3

Signal transducer and activator of transcription 3 (Stat3) is one of a family of cytoplasmic proteins that participate in normal cellular responses to cytokines and growth factors, including IL-6, IFN, EGF, PDGF, as transcription factors (Darnell, 1997). Upon activation by a wide variety of cell surface receptors and nonreceptor tyrosine kinases, STAT3 is phosphorylated and translocates into the nucleus (Darnell, 1997). After translocation, STAT3 modulates the expression of target genes that are involved in various physiological functions including apoptosis (Survivin, Bcl-xL, and HSP27), cell-cycle regulation (Cyclin D1, c-fos, and c-myc), and angiogenesis (vascular endothelial growth factor (VEGF)) (Kisseleva *et al.*, 2002; Levy and Darnell, 2002).

Constitutive activation of STAT3 is found in human epithelial tumours and cancer cell lines (Bromberg, 2001; Buettner *et al.*, 2002; Jing and Tweardy, 2005). Furthermore, inhibition of STAT3 can suppress growth of cancer cells, implying its critical role in the maintenance of proliferation and survival of various cell types (Gritsko *et al.*, 2006; Liu, 2007).

Recent studies with skin-specific gain and loss of Stat3 function in

transgenic mice have shown that Stat3 plays critical roles in skin carcinogenesis. Keratinocyte-specific Stat3-deficient mice were viable and the epidermis and hair follicles developed normally. However, these mice showed abnormal hair cycle with impaired progression of the second and subsequent hair cycles. Wound healing was also impaired in Stat3-deficient mice due to a defect in keratinocyte migration (Sano *et al.*, 1999). These observations suggested that Stat3 is not required for skin morphogenesis, but that it is essential for skin remodeling including hair cycle and wound healing.

In mouse chemical skin carcinogenesis model, Stat3 is activated following the treatment with different classes of tumour promoters, including TPA, okadaic acid, and chrysarobin (Chan *et al.* et al.). Stat3 was also found to be constitutively activated in both papillomas and SCC generated by the two-stage skin carcinogenesis regimen with DMBA as initiator and TPA as promoter (Chan *et al.*, 2004a). Furthermore, recent studies using Stat3-deficient mice have provided evidence that Stat3 is required for both the initiation and promotion stages of epithelial carcinogenesis (Chan *et al.*, 2004b). Stat3-deficient mice were found to be completely resistant to skin tumour development with a standard DMBA/TPA skin carcinogenesis bioassay. In addition, Stat3-deficient mice showed a marked increase in keratinocyte apoptosis in the bulge region of HFs after treatment with the tumour initiator DMBA. Furthermore, treatment with the tumour promoter TPA resulted in a significantly reduced epidermal hyperproliferative response associated with downregulation of cell cycle regulatory proteins (cyclin D1, cyclin E) and c-myc expression in the epidermis of Stat3-deficient mice (Chan *et al.*, 2004b). These results suggest that Stat3 plays a critical role in mediating G1-to-S cell-cycle

progression and proliferation in murine keratinocytes following treatment with TPA (Chan *et al.*, 2004b).

The important role of Stat3 in epithelial carcinogenesis was demonstrated in a transgenic mouse model in which the expression of a constitutively active form of Stat3 (Stat3C) was driven in the basal layer of epidermis by the keratin 5 promoter (referred to as K5.Stat3C transgenic mice) (Chan *et al.*, 2008). In two-stage (DMBA/TPA) chemical carcinogenesis experiments, K5.Stat3C mice developed skin tumours with shortened latency compared to nontransgenic littermates (Chan *et al.*, 2008). Remarkably, 100% of the skin tumours from K5.Stat3C transgenic mice bypassed the premalignant stage and initially developed as carcinoma in situ. These tumours were highly vascularized and poorly differentiated; invasion into surrounding dermal/mesenchymal tissue was observed at a very early stage (Chan *et al.*, 2008). Further studies showed that overexpression of Stat3C led to enhanced cell migration and enhanced invasion in vitro (Chan *et al.*, 2008).

Stat3 also plays a crucial role in UVB-induced skin carcinogenesis (Enk *et al.*, 2006). Constitutive activation of Stat3 was observed in both human and mouse UVB-induced SCCs (Sano *et al.*, 2005). Studies on Stat3 during UVB exposure-mediated responses showed that both protein levels of Stat3 and active phosphorylated Stat3 were increased in skin of hairless mice upon UVB irradiation (Sano *et al.*, 2005). In addition, Stat3-deficient mice are resistant to UVB-induced skin carcinogenesis and show a high incidence of apoptosis upon UVB-irradiation (Sano *et al.*, 2005). Thus, Stat3 promotes UVB-induced proliferation of follicular keratinocytes and epidermal thickening.

In summary, aberrant growth factor signalling pathways, which are

frequently found in human epithelial cancers, may play an important role in the constitutive activation of Stat3 (Chan *et al.*, 2004a). Activation of Stat3 appears to be critical in maintaining the malignant phenotype of these cancers.

1.3 BMPs as members of TGF-β superfamily of multifunctional cytokines. Bone morphogenetic protein (BMP) signalling pathway

1.3.1. Bone morphogenetic proteins

Bone morphogenetic protein (BMP) signalling is a one of the key pathways involved in regulation of cell proliferation, differentiation, and apoptosis. Therefore, this pathway plays essential roles during embryonic development and postnatal tissue remodeling and regeneration in various organs, including the skin (Botchkarev and Paus, 2003; Li *et al.*, 2003; Massague, 1998; Mishina, 2003). BMPs are members of a large group of secreted polypeptide growth factors, the transforming growth factor-β (TGF-β) superfamily, which includes TGFβs, activins/inhibins, Nodal, myostatin and anti-Mullerian hormone (AMH) (Miyazawa *et al.*, 2002; Miyazono *et al.*, 2005). There are more than 20 BMP-related proteins known that can be subgrouped according to the homology in their sequence and functions (**Table 2**) (Miyazono *et al.*, 2005). The first group includes BMP-2 and BMP-4 (BMP-2/4 group). BMP-5, BMP-6 and BMP-7 (also termed osteogenic protein-1, OP-1), BMP-8 (OP-2) form another subgroup (OP-1 group). Growth-differentiation factor-5 (GDF-5), GDF-6 and GDF-7 (BMP-12) form a third group (GDF-5 group). Members of the BMP family have distinct

spatiotemporal expression profiles. Moreover, the biological activities of BMPs are not identical but overlapped among members, since they bind to their receptors with different affinities (Miyazono *et al.*, 2005). All BMPs are secreted as precursor proteins with a hydrophobic stretch of about 50-100 amino acids. The mature BMP derives from the carboxyterminal region and obtained by proteolytical cleavage (Constam and Robertson, 1999). Every BMP monomer contains seven cysteins, in which six of the cysteins form a cystin knot and the seventh is used for dimerization with a second monomer (Scheufler *et al.*, 1999). BMPs function as homo or hetrodimers through binding to the transmembrane BMP receptor complex (Miyazono *et al.*, 2005). Interestingly, the heterodimers of BMP4/7, BMP2/6, BMP2/7 and BMP7/GDF7 are more effective than when they form homodimers (Butler and Dodd, 2003; Israel *et al.*, 1996).

1.3.2. BMP receptors and signal transduction pathways

BMP signals are mediated by receptors which are dedicated to TGF-ß signalling, and include type I and type II serine/threonine kinase receptors (**Fig. 1.6**) (Massague, 2003; Miyazono *et al.*, 2001). Seven type I receptors and five type II have been identified in humans. Six of the type I receptors and three of the type II receptors are responsible for BMP signalling (**Table 1)**.

Table 1. Transmembrane serine/threonine kinase receptors (Ye *et al.*, 2007)

Type I receptor	Type II receptor
ACVRL1 (ALK-1, ACVRLK1, ALK1, SKR3)	TGFBR2 (TGFR-2, TGFbeta-RII)
ACVR1 (ALK2, ACTRI, ACVRLK2, FOP, SKR1)	TGFBR3
BMPR1A (ALK3, ACVRLK3, CD292)	***BMPR2*** (BMPR-II, BMPR3, BMR2, BRK-3, T-ALK)
ACVR1B (ALK4, ACTRIB, ACVRLK4, SKR2)	***ACVR2B*** (ActR-IIB)
TGFBR1 (ALK-5, ACVRLK4, SKR4, TGFR-1)	***ACVR2A*** (ACTRII, ACVR2)
BMPR1B (ALK-6, ALK6, CDw293)	
ACVR1C (ALK7, ACVRLK7)	

There are seven Type I and five Type II transmembrane serine/threonine kinase receptors identified in humans. Six Type I receptors and three Type II receptors that have been found involved in the signal transduction of BMPs, which are bold italic in the table. ACVRL1, activin A receptor type II-like 1; ACVR1, activin A receptor, type I; BMPR1A, bone morphogenetic protein receptor, type IA; ACVR1B, activin A receptor, type IB; TGFBR1, transforming growth factor, beta receptor I; BMPR1B, bone morphogenetic protein receptor, type IB; ACVR1C, activin A receptor, type IC; TGFBR2, transforming growth factor, beta receptor II; TGFBR3, transforming growth factor, beta receptor III; BMPR2, bone morphogenetic protein receptor, type II; ACVR2B, activin A receptor, type IIB; ACVR2A, activin A receptor, type IIA.

Different members of BMP family show different affinity to several type I and type II receptors (**Table 2**). However, BMPR1A, BMPR1B and BMPR2 are specific for the BMPs. Both types of the BMP serine/threonine kinase receptors consist of an N-terminal extracellular ligand binding domain, a transmembrane region and a C-terminal serine/threonine kinase domain.

Ligand binding to the BMP receptor complex results in phosphorylation of the intracellular domain of the type I receptor by the type II receptor kinases and leads to the transmission of an intracellular signal through BMP–Smad and/or BMP–MAPK pathways (**Fig. 1.6**) (Derynck and Zhang, 2003; Nohe *et al.*, 2004; von Bubnoff and Cho, 2001).

Table 2. Receptors and R-Smads involved in BMP signalling (Ye *et al.*, 2007)

Official Symbol	Type II receptor	Type I receptor	R-Smad
BMP2	BMPRII ActRIIA	ALK 3/6	Smad 1/5/8
BMP3	ActRIIA	ALK 4	Smad 2/3
BMP4	BMPRII ActRIIA	ALK 3/6	Smad 1/5/8
BMP5		ALK 3	Smad 1/8
BMP6	ActRIIA ActRIIB BMPRII	ALK 1/2/3/6	Smad 1/5
BMP7	BMPRII ActRIIA	ALK 2/3/6	Smad 1/5/8
BMP8A			
BMP8B			
BMP10	BMPRII ActRIIA	ALK 3/6	Smad 1/5/8
BMP15	BMPRII	ALK 6	Smad 1/5/8
GDF1	ActRIIB	ALK 4	Smad 2/3
GDF2	ActRIIA ActRIIB BMPRII	ALK 1	Smad 1/5
GDF3			
GDF5	BMPRII ActRIIA	ALK 3/6	Smad 1/5/8
GDF6	BMPRII ActRIIA	ALK 3/6	
GDF7	BMPRII ActRIIA	ALK 3/6	
GDF8	ActRIIB	ALK4/5	Smad 2/3
GDF9	BMPRII	ALK5	Smad 2/3
GDF10			
GDF11	ActRIIA ActRIIB	ALK4	Smad 2/3
GDF15			Smad 2/3

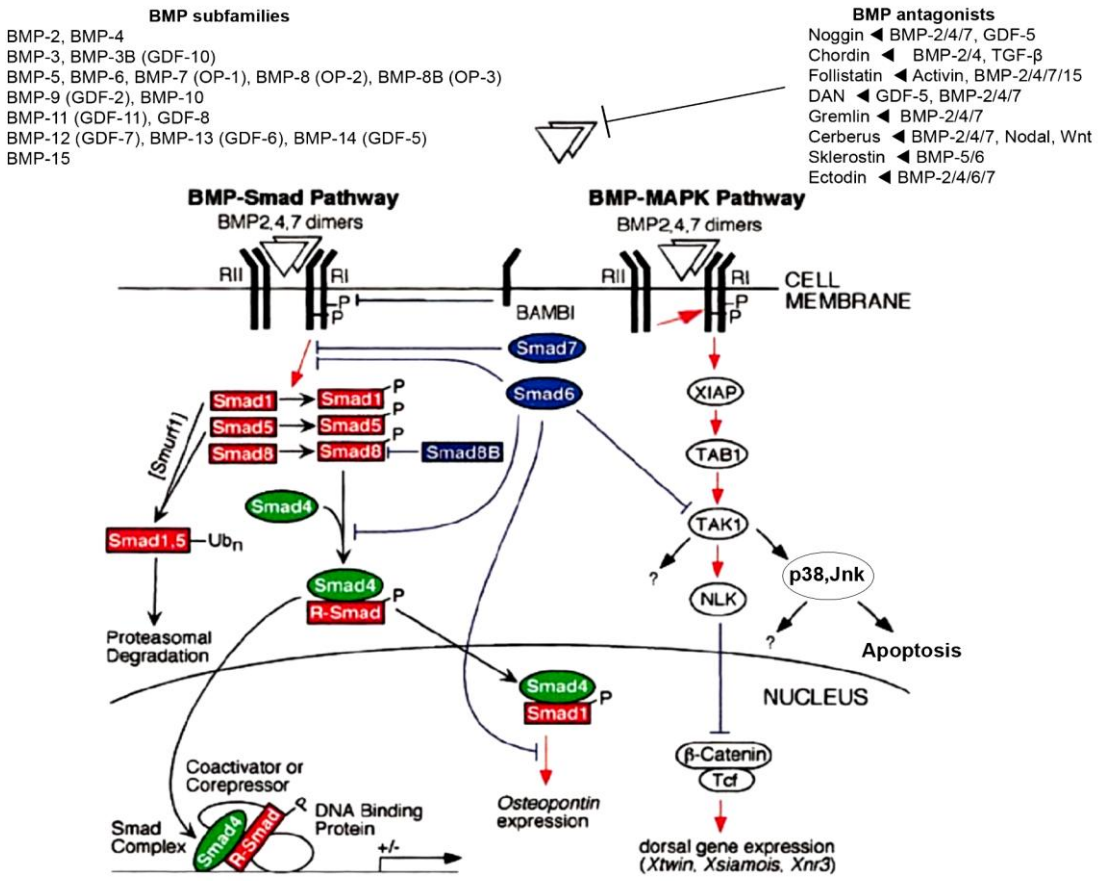

Fig. 1.6. Molecular composition of the BMP signalling pathway

BMP interactions with BMP receptor complex are modulated by diffusible BMP antagonists that prevent BMP binding to BMP receptors. BMP binding to preformed BMPR complex activtes BMP_sma signal transduction. BMP binding to BMP-IA/B folofed by recruitment of the BMP-II leads to activation of the BMP-MAPK pathway that links BMPRs with TAK1 kinase, which via NLK antagonizes Wnt/β-catenin, as well as may activate apoptosis via p38/jnk pathway. Modified from (von Bubnoff and Cho, 2001).

Distinct activation of these two pathways depends on the mode of ligand binding to the BMP receptors: binding to preformed receptor complexes induces signal transduction via the BMP–Smad pathway, while binding to the BMPR-I with subsequent recruitment of BMPR-II activates the BMP–MAPK pathway (Nohe *et al.*, 2002; Nohe *et al.*, 2004).

The BMP–Smad pathway is activated following BMP interaction with the preformed BMPR complex and includes BMPRII-dependent phosphorylation of the type I receptors, which in turn phosphorylate intracellular Smad1, Smad5, or Smad8 proteins (or receptor-activated Smads, R-Smads) at their C-terminal domain (**Table 2**) (Tamaki *et al.*, 1998; ten Dijke, 2002,, 1994). These R-Smads then form heteromeric complexes with Smad4 (or common-partner Smad, Co-Smad) and translocate into the nucleus to regulate the transcription of BMP-responsive genes (**Fig. 1.6**). Smad2 and Smad3, which also belong to the R-Smad subfamily, are activated by ActRI and TGF-βRI kinases and do not mediate the BMP signalling pathway (ten Dijke and Hill, 2004).

Both R-Smads and Smad 4 possess domains with similar amino acid sequences at the N- and C-terminals, called MH1 and MH2, respectively (Massaque and Chen, 2000; Miyazono *et al.*, 2001; Shi and Massague, 2003). Also, there is a linker region between the MH1 and MH2 domains that is responsible for binding of R-Smads to Smad4 (Kretzschmar *et al.*, 1997; Massague, 2003). After translocating into the nucleus, the complex of Smad4 and one of the R-Smads (Smad1 or Smad5) bind DNA through its MH1 domains, which contain specific DNA-binding sequences (GCCG for Smad1 and TGTGC for Smad5) (Kusanagi *et al.*, 2000; Li *et al.*, 2001). The MH2

domain of the R-Smads is responsible for binding to cytoplasmic co-regulators, as well as to a number of co-activators and co-repressors in the nucleus (Massague, 2003).

The BMP–MAPK pathway is activated when BMPs bind to one of the type I BMPRs followed by the BMPRII recruitment into the BMPR complex (Nohe *et al.*, 2002; Nohe *et al.*, 2004). The activated BMPR complex may interact with intracellular adaptor proteins XIAP and/or BRAM1, which link BMP receptors with TAB1 (TAK1 binding protein) that, in turn, activates TAK1 (**Fig. 1.6**) (TGF-β-activated kinase 1) (Morita *et al.*, 2001; Yamaguchi *et al.*, 1999). TAK1 is a member of the MAPK kinase family, whose activity is also stimulated by TGF-β1 (Fig. 1). TAK1 activates the p38 and JNK pathways, which are involved in BMP-induced apoptosis (Kimura *et al.*, 2000; Zhang *et al.*, 2000).

1.3.3. Regulation of BMP signalling.

Activity and specificity of the BMP signalling are regulated on several levels: (i) at the cell surface, by modulating binding of the BMPs to the BMPR receptors; (ii) in the cytoplasm, by inhibitory Smads and Smad binding proteins, and (iii) in the nucleus, by controlling the transcription of BMP target genes (Massague, 2003; Miyazono *et al.*, 2001; ten Dijke and Hill, 2004).

1.3.3.1. *Regulation of BMP signalling by extracellular events.*

BMP antagonists. The activity of BMPs at the cell surface is modulated by a number of secreted BMP antagonists. More than 10 BMP antagonists (noggin, chordin, follistatin, cerberus/DAN family of proteins, ectodin, etc.) have been identified to date (**Table 3)**, which belong to structurally distinct protein

families (Balemans and Van Hul, 2002; Laurikkala *et al.*, 2003). However, all these proteins selectively bind to distinct members of the BMP family with higher affinity than the BMPR complex, thus restricting BMP activity to the tissue compartments that are free of BMP antagonists. For instance, noggin binds BMP-2 and BMP-4 with a 10–15 times higher affinity than BMP receptors, and also neutralizes the activity of BMP-7 and GDF-5 with lower affinity. On the other hand, noggin expression can be induced by BMP2,4 and 6. Therefore, the BMP are able to modulate their effect via a negative feed back loop by upregulation of the expression of their antagonists (Gazzerro *et al.*, 1998).

In addition to the secreted BMP antagonists, there are other possible mechanisms by which BMP signalling is regulated extracellularly. One of these extracellular mechanisms is the expression of co-receptors or dominant negative non-signalling pseudoreceptors in a cell. The pseudoreceptor, BMP and activin membrane bound inhibitor (BAMBI), is a membrane protein, which has an extracellular domain similar to that of the type I BMP receptor. However, the pseudoreceptor lacks the intracellular serine/threonine domain (Onichtchouk *et al.*, 1999).

BMP signalling stimulators. Recent evidence suggest that, like other members of the TGF-β family, there are co-receptors for the BMP ligand, which enhance the signalling of BMPs (**Table 3)**. DRAGON is the first co-receptor reported for BMP, which is a glycosylphosphatidylinositol-anchored member of the repulsive guidance molecule family. DRAGON binds directly to BMP2 and BMP4, but not BMP7 or other TGF-β ligands.

Table 3. Regulatory factors of BMP signalling (Ye *et al.*, 2007)

Location	Category	Official Symbol	Target
Extracellular	Antagonist	Noggin	BMP2, 4, 6 and 7; GDF5 and GDF6
		Chordin	BMP4, 7
		Chordin like 2(CHL2)	BMP2, 4, 5, 6, 7 and GDF5
		Follistatin	BMP6, 7, 11 and 15, GDF8 and 9
		Ventroptin	BMP4
		FLRG	BMP2
		Twisted gastrulation(Tsg)	BMP2, 4
		Gremlin(DRM)	BMP2, 4 and 7
		Dan	BMP2, 4 and GDF5
		Cerberus	BMP2, 4
		(PRDC)	BMP2, 4
		Sclerostin(SOST)	BMP6 and 7
		Caronate	BMP2, 4 and 7
		(DAND5)	BMP4
	Enhancer	Kielin/Chordin like	BMP7
Membrane	Pseudoreceptor	BAMBI	BMP4
	Co-receptor	Dragon	BMP2, 4
		RGMa	BMP2, 4
Intracellular	Inhibitory Smads	Smad6 and 7	R-Smad, Co-Smad
	Smad binding protein	Ski	Smad 2, 3 and 4
		SKIL/SnoN	Smad 2, 4
		Tob	Smad 1, 5 and 8
		AMSH	Smad6
	Ubiquitination and degradation of Smad	Smurf1 and 2	Smad 1, 5, 6 and 7
		NEDD4-2	Smad 2
	Deubiquitination of Smad	UCH37	Smad7

The interaction between DRAGON and BMPs enhances the signalling and ultimately leads to a stronger biological response from the cell. Interestingly, this enhanced effect due to the DRAGON/BMP interaction can be reduced by the BMP2/4 antagonist, Noggin (Samad *et al.*, 2005). A homologue of the Dragon, repulsive guidance molecule (RGMa), has been identified as another co-receptor for BMPs (Babitt *et al.*, 2005).

1.3.3.2. *Regulation of the intracellular signalling*

Binding of BMP ligands to the BMP receptors may results in activation of inhibitory Smads (Smad 6 and 7), Smad binding proteins (Ski and Tob) and Smad ubiquitin regulatory factors (Smurf 1 and 2), which can also regulate the intracellular signal transduction of BMPs.

Inhibitory Smads. Smad 6 and 7 inhibit signal transduction of BMPs, by interference with the activation of Smad 1 and 5, which are phosphorylated by the BMP Type I receptor (**Table 3** and **Fig. 1.4**). In addition, Smad6 may inhibit BMP signalling by competing with Smad4 for binding to Smad1 (Hata *et al.*, 1996). However, Smad1/Smad5 and Smad3 can induce the expression of Smad6 and Smad7, respectively, suggesting a negative feedback loop in the BMP–Smad signalling pathway (Ishida *et al.*, 2000; Nagarajan *et al.*, 1999).

Smad binding proteins*.* Smad binding proteins suppress BMP signalling by associating with the MH2 binding domain of Smads. Sloan-Kettering retrovirus (Ski) binds Smad 1, 2, 3, 5 and 4 and inhibits BMP signalling (Luo, 2003; Wang *et al.*, 2000). The transducer of ErbB-2 (Tob) is probably associated with the MH2domain of Smad 1, 5, 6, 7 and 8 (Yoshida *et al.*, 2000; Yoshida *et al.*, 2003).

Molecules that facilitate degradation of the Smads, Smurf 1 and 2, modulate TGF-β/BMP signalling by selectively targeting the receptors and Smad proteins for degradation and ubiquitination (**Table 3** and **Fig. 1.4**) (Zhu *et al.*, 1999). Smurf 1 can directly interact with Smad 1/5, and facilitate their degradation (Zhu *et al.*, 1999). It can also indirectly interact with the BMP Type I receptor through I- Smad 6 and 7, and induce ubiquitination and degradation of the receptors (Murakami *et al.*, 2003).

NEDD4-2 (neural precursor cell expressed, developmentally down-regulated 4-2) was recently found to be a direct binding partner of Smad7 (Kuratomi *et al.*, 2005). NEDD4-2 is structurally similar to Smurfs 1 and 2 (Smad ubiquitin regulatory factors). It can interact with Type I receptor via Smad 7, and induce its degradation. It can also bind to Smad 2 and 3 in the ligand-dependent manner, and degrade Smad 2, but not Smad 3. Overexpression of NEDD4-2 inhibits the transcriptional activity induced by TGF-β and BMPs. Wicks and Haros et al. recently reported a novel ubiquitin: C-terminal hydrolase (UCH37). UCH37 is a deubiquitinating enzyme that can potentially reverse Smurf-mediated ubiquitination. It forms a stable complex with Smad 7, which deubiquitinates and stabilizes the type I TGF-β receptor (Wicks *et al.*, 2005). However, its role in BMP signalling remains unclear.

The associated molecule with the SH3 domain of STAM (AMSH) is a direct binding partner for Smad6 and has been found to inhibit the interaction between Smad6 and the activated BMP type I receptor, thereby allowing more efficient BMP receptor-induced phosphorylation of R-Smads. In addition, AMSH was found to interfere with the interaction between Smad6 and the activated R-Smad. Thus, AMSH promotes BMP signalling by negatively regulating the function of I-Smads (Itoh *et al.*, 2001).

In summary, the spatial and temporal specificity of BMP effects on distinct groups of cells is dependent on the bioavailability of distinct BMP ligands for BMP receptors, on the differential recruitment of intracellular Smad1/5 proteins or components of the MAPK pathway to signal transduction mechanism, as well as on the presence of distinct co-activators or co-repressors of BMP-dependent transcription in the nucleus. However, cellular responses to BMPs also show

striking dependence on a variety of other factors, such as the stage of cell differentiation, the activity of other growth stimulatory or inhibitory factors, stage of embryogenesis, etc.

1.3.4. BMP signalling in skin development and remodelling

1.3.4.1. *BMPs and epidermal development*

BMP ligands are differentially expressed in the skin during epidermal development. High levels of BMP-6 transcripts and protein are present in the suprabasal layers of the epidermis of mouse embryos starting from E15.5, while BMP-7 mRNA is seen in the basal epidermal layer during the last stages of embryonic development (Lyons *et al.*, 1989; Takahashi and Ikeda, 1996; Wall *et al.*, 1993). The expression of BMP-2 and BMP-4 transcripts in the developing murine skin is more restricted to HF epithelium and mesenchyme, respectively (Bitgood and McMahon, 1995; Lyons *et al.*, 1989,, 1990). BMPR-IA is expressed in the basal layer of murine epidermis at E16.5, while BMPR-IB expression is restricted to suprabasal keratinocytes (Botchkarev *et al.*, 1999a). Smad1, Smad5, Smad6, and Smad7 are also abundantly expressed in the developing murine epidermis (Dick *et al.*, 1998; Flanders *et al.*, 2001; He *et al.*, 2001).

Data obtained from mice overexpressing BMP-6 via the K10 promoter show that the effects of BMP-6 on epidermal development strongly depend on the levels of transgene expression. High expression inhibits epidermal proliferation, while moderate BMP-6 expression stimulates proliferation and leads to the aberrant appearance of proliferating cells and K6, K14, and K16 expression in the suprabasal epidermis (Blessing *et al.*, 1996). Excessive BMP

activity in Noggin-deficient mice also results in increased epidermal proliferation, downregulation of K10, and appearance of ectopic proliferating cells that express K14 in the suprabasal layers (Botchkarev *et al.*, 2002; Botchkarev *et al.*, 1999a).

A unique role for BMP during mouse postnatal development is the regulation of eyelid opening. During eye development the skin forms eyelids that first grow and fuse, and then reopen at a specific developmental stage. The process of eyelid separation requires a high degree of coordination between cell proliferation, apoptosis, and differentiation in the eyelid epithelium. In wild-type mice, eyelids are open at postnatal day 13.5 (P13.5). Eyelid separation is strongly inhibited in transgenic mice that overexpress the BMP antagonist Noggin under the control of the K5 promoter (Sharov *et al.*, 2003a). Noggin overexpression leads to a reduction of apoptosis and the retardation of cell differentiation in the eyelid epithelium, which are associated with decreased expression of the apoptotic receptors and differentiation markers. This suggests that in eyelid skin epithelium, BMPs are important for regulating a genetic differentiation programme that incorporates traditional apoptotic pathways.

In vitro data demonstrated that BMP-2 and BMP-6 transcripts are also expressed in primary mouse keratinocytes (Drosdoff *et al.*, 1994; Park and Morasso, 2002), and that similar to the embryonic skin, BMP signalling is involved in the control of keratinocyte proliferation and differentiation in postnatal skin. In primary mouse keratinocytes, BMPs inhibit cell proliferation and promote differentiation (D'Souza *et al.*, 2001; Drosdoff *et al.*, 1994; McDonnell *et al.*, 2001; Park and Morasso, 2002). In differentiating epidermal keratinocytes, BMPs stimulate expression of K1, involucrin, and Dlx-3

transcription factor, suggesting that these molecules may be targets for BMP signalling differentiation (D'Souza *et al.*, 2001; Drosdoff *et al.*, 1994; McDonnell *et al.*, 2001; Park and Morasso, 2002). Thus, BMPs stimulate the differentiation of epidermal keratinocytes, while their effects on epidermal proliferation during skin development are strikingly dose-dependent.

1.3.4.2. *BMP pathway in regulation of hair follicle development*

BMP signalling plays a pivotal role in the control of epidermal appendage development. Genetically engineered mice with loss or gain of BMP signalling show severe alterations in the development of skin appendages. Noggin knock-out mice lack approximately 90% of their HFs (Botchkarev *et al.*, 2002; Botchkarev *et al.*, 1999a), while transgenic mice overexpressing noggin via K14 promoter are characterized by increased HF density, formation of compound vibrissa HFs, smaller eyelids, ectopic cilia, hyperpigmentation of claws, interdigital webbing, trans differentiation of sweat glands into HFs, and increased size of external genitalia (Plikus *et al.*, 2004).

During the induction of all HF types, both BMP-2 and BMPR-IA are expressed in the hair placode, while BMP-4 and noggin expression is seen in cells of mesenchymal condensation (Botchkarev *et al.*, 1999a; Lyons *et al.*, 1989). The importance of noggin as a BMP neutralizing protein for proper HF induction is evident from the data showing lack of induction of all secondary (non-tylotrich) HFs in noggin knock-out mice (Botchkarev *et al.*, 2002; Botchkarev *et al.*, 1999a). Conversely, noggin overexpression in basal epidermal keratinocytes results in increased HF density (Plikus *et al.*, 2004). These data are consistent with other models, in which increased BMP signalling

inhibits the initiation phase of tooth and feather development (Jung *et al.*, 1998; Neubuser *et al.*, 1997; Noramly and Morgan, 1998).

Interestingly, noggin knock-out mice show a phenotype somewhat reciprocal to that seen in Tabby and Downless mice (with mutated genes for Ectodysplasin (Eda) and its receptor Edar, respectively). These mice are characterized by lack of guard and zig-zag HFs (Mikkola and Thesleff, 2003). Similarly, the induction of guard, awl, and auchene HFs is altered in transgenic mice overexpressing Shh under the control of K1 promoter (Ellis *et al.*, 2003). Although several findings suggest that Edar signalling may positively regulate BMP-4 expression (Mikkola and Thesleff, 2003), while noggin stimulates Shh in adult HFs (Botchkarev, 2001), the significance of these data and the molecular mechanisms underlying the cross-talk between BMP, Edar, and Shh signalling pathways during the initiation of distinct HF types remain to be carefully dissected.

It was also found that expression of the Lef-1 transcription factor, a critical downstream component of the Wnt/β–catenin signalling pathway, and the neural cell adhesion molecule (N-CAM) are strongly decreased in hair placodes of noggin knock-out mice (Botchkarev *et al.*, 1999a). A stimulatory effect of noggin on Lef-1 expression was also shown in primary epidermal keratinocytes (Jamora *et al.*, 2003). Lef-1 plays critical roles in HF development (Fuchs, 2007; Millar, 2002). Lef-1 knock-out mice are characterized by a greatly reduced number of secondary HFs (Jamora *et al.*, 2003; van Genderen *et al.*, 1994). In addition, conditional disruption of β-catenin in the epidermis or K14-driven overexpression of the Wnt antagonist Dickkopf-1 lead to induction failure in primary and secondary HFs (Andl *et al.*, 2002; Huelsken *et al.*, 2001). Since

Lef-1 stimulates N-CAM promoter activity (Boras and Hamel, 2002) and negatively regulates E-cadherin expression (Jamora *et al.*, 2003), the inhibitory action of BMP on hair placode formation may also be mediated by changes in the profile of adhesion molecules expressed in placode keratinocytes.

Adhesion molecules play important roles in the control of HF development (Kljuic *et al.*, 2003; Muller-Rover *et al.*, 1998). However, they may not be the only targets regulated by BMPs during HF initiation. BMP signalling may also be involved in extracellular matrix remodeling during the invasion of the developing hair placode into the dermis. It was previously shown that BMP-2 inhibits the expression of matrix metalloproteinase-13 (MMP-13, collagenase-3), thus suppressing extracellular matrix degradation (Varghese and Canalis, 1997). Although the expression of different MMPs and their inhibitors (TIMPs) in the skin of genetically engineered mice with a gain or loss of BMP signalling remains to be elucidated, it was demonstrated that one of the metalloproteinases, namely MMP-7 or matrilysin, was expressed in hair placode keratinocytes and showed co-localization with Smad1 (Botchkarev and Sharov, 2004). Interestingly, MMP-7 is also defined as a direct target of Wnt/β–catenin signalling (Crawford *et al.*, 2001), which raises the possibility that BMP and Wnt signalling pathways may also be involved in the regulation of the extracellular matrix remodeling during HF initiation.

1.3.4.3. *BMPs in cell lineage commitment and differentiation in the developing HF*.

In the developing HF, the inductive interactions between keratinocytes of the hair placode and fibroblasts of the dermal papilla lead to the differentiation

of progenitor cells into distinct cell populations, including sebocytes, outer root sheath keratinocytes, the cuticle, the Huxley, and Henle layers of the inner root sheath, as well as the medulla, the cortex, and the cuticle of the hair shaft (Fuchs *et al.*, 2001; Hardy, 1992; Philpott and Paus, 1998). Accumulating evidence suggests that BMP signalling plays essential roles in controlling cell lineage commitment and cell differentiation during HF development.

BMP-2, BMP-4, noggin, and BMPR-IA are broadly expressed in the epithelial and mesenchymal cells of the developing hair bulb, while expression of BMPR-IB is restricted to the dermal papilla, follicular connective tissue-sheath cells, and melanocytes (Botchkarev, 2001; Kulessa *et al.*, 2000). Data obtained from a number of genetic models indicate that BMP signalling is essential for the proper differentiation of the inner root sheath and hair shaft. Transgenic mice overexpressing noggin in hair matrix keratinocytes via Msx-2 promoter are characterized by impaired hair growth and show lack of external hairs due to severe alterations in the proliferation/differentiation transition of matrix keratinocytes and hair fiber synthesis (Kulessa *et al.*, 2000). Other transgenic models, in which K5 or NSE promoters drive noggin expression, show lack of zig-zag hairs, which are replaced by awl and auchene hairs (Guha *et al.*, 2004; Sharov *et al.*, 2003b). Furthemore, in noggin-null skin grafts transplanted onto SCID mice, long-term excess of BMPs leads to the developmental arrest of primary HFs prior to the onset of hair shaft formation (Botchkarev *et al.*, 2002). In addition, conditional deletion of BMPR-IA in epithelial cells of the HF leads to profound alterations in the inner root sheath and hair shaft formation (Andl *et al.*, 2004; Kobielak *et al.*, 2003; Yuhki *et al.*, 2004).

The essential requirement of BMPR-IA for the differentiation of hair shaft progenitors into hair shaft keratinocytes is evident from the fact that K14-Cre:BMPR-IAfl/fl mice are characterized by an absence of all three layers of hair shaft keratinocytes, while Msx2-Noggin and NSE-Noggin transgenic mice show relatively milder alterations in hair shaft structure (Andl *et al.*, 2004; Guha *et al.*, 2004; Kobielak *et al.*, 2003; Kulessa *et al.*, 2000). The partial decrease of BMP signalling in Msx2-Noggin mice is associated with upregulation and ectopic expression of Lef-1 in the hair shaft, while complete deletion of BMPR-IA results in a decrease in Lef-1 expression (Andl *et al.*, 2004; Guha *et al.*, 2004; Kobielak *et al.*, 2003; Kulessa *et al.*, 2000). In addition, Msx2-Noggin and K14-Cre:BMPR-IAfl/fl mice show decreased expression of the Foxn1 transcription factor. Given that Lef-1 and Foxn1 transcription factors bind selected hair keratin gene promoters (Prowse, 1999; Zhou *et al.*, 1995), these data suggest that BMP signalling can be involved in the control of hair shaft-specific differentiation.

In addition, the BMP signalling is involved in sebocyte-specific differentiation. NSE-Noggin mice showed the ectopic presence of sebocytes in the mid-portion of the outer root sheath (Guha *et al.*, 2004). This finding is consistent with an observation of BMPR-IA expression in the sebaceous gland of postnatal HFs (Botchkarev, 2001). Sebocyte development is severely affected in transgenic mice expressing the dominant-negative form of Lef-1 under the control of K14 promoter (Merrill *et al.*, 2001; Niemann *et al.*, 2003a). Interestingly, overexpression of dominant-negative Lef1 up-regulates Indian Hedgehog and stimulates the proliferation of undifferentiated sebocytes, suggesting a cross-talk between Wnt and Hedgehog pathways in the control of

sebaceous gland development (Niemann *et al.*, 2003b). Genetic studies suggest that Hedgehog signalling is intimately involved in the control of sebocyte differentiation: inhibition of the Hedgehog pathway selectively suppressed sebocyte development, while Hedgehog pathway activation led to a striking increase in the size and number of sebaceous glands (Allen *et al.*, 2003). Remarkably, ectopic Hedgehog signalling also triggered the formation of sebocytes in footpad epidermis, a region normally devoid of HFs (Allen *et al.*, 2003). However, it remains to be determined whether the ectopic sebocyte differentiation observed in NSE-Noggin mice (Guha *et al.*, 2004) is associated with increase of Hedgehog signalling in the HF, or other signalling pathways are involved in this phenomenon.

Thus, cell lineage commitment and the differentiation of keratinocyte progenitors into the hair shaft, inner root sheath, and sebaceous gland lineages are tightly controlled by the local balance of BMPs and their antagonists. An excess or limitation of BMP signalling may affect lineage-specific differentiation in HFs. However, additional efforts are required to define the specific BMP targets for each cell population in the HFs, as well as the molecules that underlie a cross-talk between the BMP and other signalling pathways (Wnt, Shh, Edar) involved in the control of lineage-specific differentiation during HF development.

1.3.4.4. *BMP signalling and hair cycle regulation*

HF morphogenesis and the hair cycle have many similar aspects. They both are characterized by activation of cell differentiation programmes that lead to the construction of the fiber-producing hair bulb. They are also controlled by

similar signalling networks within and between the follicular epithelium and mesenchyme that use molecules belonging to the Wnt, TGF-β/BMP, Hedgehog, FGF, Notch, EGF, TNF, and neurotrophin families (Fuchs, 2001; Stenn and Paus, 2001; Millar, 2002; Botchkarev and Kishimoto, 2003; Botchkarev and Paus, 2003). Several recent investigations showed an essential role for BMP signalling in the control of HF cycling.

BMP signalling, hair follicle stem cells, and hair cycle initiation. Accumulating evidences suggest that BMP signalling plays an important role in controlling stem cell behavior. BMP-4 supports the self-renewal of embryonic stem cells via inducing Id proteins through the BMP–Smad pathway and via inhibiting the MAPK pathway (Qi *et al.*, 2004; Ying *et al.*, 2003). Furthermore, BMPR-IA signalling controls the number of hematopoietic stem cells by regulating the size of the stem cell niche (Zhang *et al.*, 2003). Microarray analyses of isolated HF stem cells reveal markedly reduced expression of BMP-4 and strongly increased levels of the BMP antagonist gremlin and the BMP targets Id2 and Id4 proteins (Morris *et al.*, 2004; Tumbar *et al.*, 2003). Furthermore, the existence of two distinct stem cell populations was shown in the bulge, one of which is characterized by an elevated expression of BMP-6 (Blanpain *et al.*, 2004). Moreover, BMP-6 is capable to inhibit the growth of HF stem cells in vitro without inducing their differentiation (Blanpain *et al.*, 2004). Furthermore, conditional ablation of BMPR-IA expression in the epidermis leads to activation of stem cells proliferation, causing an expansion of the niche and loss of slow-cycling cells (Gosselet *et al.*, 2007; Kobielak *et al.*, 2007; Zhang *et al.*, 2006). Therefore, BMP signalling plays an important role in maintaining of

HF stem cells quiescence by inhibiting their differentiation into transient amplifying daughter cells. Recent studies suggest that bulge SC quiescence that is induced by BMP signalling is governed in part by its ability to regulate the transcriptional repressor nuclear factor of activated T cells C1 (nFATC1) (Horsley *et al.*, 2008).

Consistent with ability of BMPs to regulate SC activity, gain- and loss-of-function studies showed that BMP signalling was involved in the control of hair cycle initiation in telogen HFs. In telogen skin, BMP4 is expressed in the dermal papilla and secondary germ, whereas BMPR-IA is selectively expressed in the secondary germ keratinocytes (Botchkarev, 2001). Growth phase activation in the telogen HF is associated with up-regulation of noggin in the follicular epithelium and mesenchyme, and is also accompanied by down-regulation of BMPR-IA in the proliferating cells of the secondary hair germ (Botchkarev, 2001), suggesting down-regulation of the BMP pathway as a important event for anagen initiation. Indeed, noggin administration induces anagen in telogen mouse skin in vivo, while BMP4 treatment blocks anagen development (Botchkarev, 2001). Similar data were obtained from K14-Noggin mice and NSE-Noggin mice that showed accelerated entry of the telogen HFs into anagen (Guha *et al.*, 2004; Plikus *et al.*, 2004). Interestingly, Plikus *et al.* (2008) recently showed a periodic expression of BMP2 and BMP4 in the dermis of telogen skin, thus dividing the conventional telogen into two new functional phases: the refractory phase and the competent for hair regeneration phase, characterized by high and low BMP signalling, respectively (Plikus *et al.*, 2008). Interestingly, cyclic BMP2 and BMP4 expression in the dermis seems to dictate the competence of bulge stem cells for activation and HF regeneration (Plikus

et al., 2008). Importantly, this BMP cycle is out of phase with the WNT/beta-catenin cycle (Plikus *et al.*, 2008); the later is associated with bulge stem cell activation and anagen initiation (Fuchs *et al.*, 2004; Huelsken *et al.*, 2001). Thus, there is competitive equilibrium between BMP and WNT signalling that controls HF stem cells activity during hair cycle.

BMP signalling in the control of catagen development. Catagen is a tightly regulated programme of HF transition from active growth to the resting stage. During catagen, vast majority of keratinocytes in the cyclic portion of the HF eliminates by undergoing apoptosis. However, a small population of keratinocytes and melanocytes manages to survive and forms the secondary hair germ in the telogen HF (Alonso and Fuchs, 2006; Botchkarev and Paus, 2003; Stenn and Paus, 2001).

Several mouse genetic models developed recently suggested a role for BMP signalling in the control of catagen development. Deletion of BMPR-IA in follicular keratinocytes was accompanied by a markedly delayed entry of the HFs into first catagen (Andl *et al.*, 2004), suggesting an involvement of BMPR-IA in the control of the anagen–catagen transition. In contrast, over-expression of Noggin in NSE-Noggin mice lead to acceleration of catagen entry in secondary HFs, while the catagen onset in primary HFs was delayed (Guha *et al.*, 2004). Also, catagen development in the secondary HFs of NSE-Noggin mice was abnormal and resulted in hair loss due to alterations in club hair formation (Guha *et al.*, 2004). However, K14-Noggin mice showed lack of abnormalities in catagen development (Plikus *et al.*, 2004). These data are not necessarily contradictory, but instead suggest that the distinct magnitude of

signalling through BMPR-IA may be critical for the proper dynamics of catagen development. Clearly, careful systematic studies are required to fully understand the mechanisms underlying the involvement of BMP signalling in the control of apoptosis and survival in distinct populations of HF cells during catagen.

1.3.5. BMP signalling and skin carcinogenesis

Evidences obtained during last decade show that perturbation of BMP pathways can result in tumourigenesis. These have brought together two fields, developmental biology and cancer research, that had advanced independently until recently. The most striking indication that BMP signalling pathways contribute to carcinogenesis comes from genetic studies of familial cancer syndromes. Mutation of Smad4 and BMPR-IA (Alk3) is genetically responsible for familial juvenile polyposis (De Bosscher *et al.*, 2004). Germ line mutations in BMPRIA (Alk3) have also been identified in a subset of families with Cowden syndrome, an inherited breast cancer syndrome (Zhou *et al.*, 2001). Moreover, majority of sporadic human epithelial cancers (>85%) including pancreatic, colon, breast, prostate, and lung, have aberrations in components of the TGFβ/BMP signalling pathway (**Table 4**). The role of BMP signalling in carcinogenesis is quite complex, i.e., both stimulating and inhibiting effects on tumour growth have been described (Hsu *et al.*, 2005; Sun, 2004).

Table 4. Aberrations of BMP signalling pathways in human cancer, the functional relevance and clinical correlations (Hsu *et al.*, 2005)

Tumor type	Aberrations	Functional implications	Clinical outcome
Colorectal	BMP2↓	BMP2 serves as a tumor suppressor	
	Smad4↓		advanced disease, ↑lymph node metastasis, and ↓ survival
	Smad7↑		↓survival
	Smad8↓		
Breast	BMP2↓	BMP2 serves as a tumor suppressor	
	BMP7↑		Tumor differentiation
	Smad8↓		
Non-small cell lung	BMP2↑	↑migration, invasion, and growth	
Pancreatic	BMP2↑	BMP2 is mitogenic	
	Smad4 mutation		
	Alk3 and BMPRII↑		
Gastric	BMP4↑		Tumor dedifferentiation
Oral papilloma /Squamous cell carcinoma	BMP2, 4, and 5, and Alk3↑		High risk for aggressive disease
Esophageal squamous cell carcinoma	BMP6↑		Tumor dedifferentiation and poor prognosis
	Smad4↓		↑tumor depth and lymph node metastasis
	Smurf2↑		Poor prognosis
Prostate	BMP6↑		↑recurrence; ↓survival
	BMP7↑		Bone metastasis
	BMPR↓		Tumor dedifferentiation
Nephroblastoma	BMP7↓		
Malignant glioma	BMP7↑ and Alk3↑	BMP7 is growth-inhibitory	Tumor dedifferntiation
Cervical	Smad4↓		
Hepatocellular	Smad4↓		
	Glypican3↑		
Endometrial	Smad4↓		
Leukemia	Smad5↓		
Thyroid follicular	Smad7↑		
Melanoma	Ski↑	Ski acts as an oncogene	
Renal cell carcinoma	BMPRII↓	resistance to growth inhibition by BMP6	

Despite the tremendous progress achieved in delineating the functional significance of BMP pathways in carcinogenesis during the last decade, little is known about molecular mechanisms implicating BMP signalling in skin carcinogenesis.

For skin cancer, solar ultraviolet (UV) irradiation is a major etiological

factor. Quan et al. (2005) showed an increased expression of Smad7 mRNA in human sun-protected skin areas irradiated with an experimental ultraviolet B (UVB) light. However, no changes in the protein levels of Smad2, Smad3, and Smad4 were detected (Quan *et al.*, 2005). In addition, sun-exposed forearm and sun-protected upper inner-arm skin samples from human volunteers were compared. Increased expression of Smad7 mRNA and protein was observed in the sun-exposed epidermis compared to the sun-protected epidermis (Quan *et al.*, 2005).

During two-stage skin chemical carcinogenesis, Smad1, Smad2, Smad3, Smad4, and Smad5 proteins were significantly downregulated in chemically induced SCCs and their expression was completely lost in spindle cell carcinomas (SPCCs) (He *et al.*, 2001). In contrast to R-Smad loss in SCCs, Smad7 mRNA was upregulated in chemically induced papillomas and SCCs (He *et al.*, 2001). Thus, these data suggest that R-Smads exert tumour suppressive effects, whilst inhibitory Smad7 promotes skin tumour formation. Upregulation of Smad7 not only directly blocks BMP/Smad signalling, but could also be responsible for reduced R-Smad proteins by recruiting Smurf2 (Massague and Gomis, 2006).

To further dissect BMP/Smad deregulation during skin carcinogenesis, several components of the pathway were genetically modified in the epidermis. Transgenic overexpression of BMP4 under the control of the regulatory elements of the cytokeratin IV* gene prevented papilloma and SCC development in the skin carcinogenesis protocol using carcinogen N-methyl-N'-nitrosoguanidine (MNNG) and TPA (Blessing *et al.*, 1995). In addition, BMP4 transgene blocks TPA-induced proliferation and inflammation in the skin

(Blessing *et al.*, 1995). Similarly, a delayed development and strong suppression of the benign and malignant skin tumour formation was observed in mice with K10 promoter controlled BMP6 transgene expression (Wach *et al.*, 2001). In this model, expression of BMP-6 stimulated apoptosis and downregulated the transcription of AP-1 family members thereby establishing tumour resistance (Wach *et al.*, 2001). AP-1 is composed of members of the Jun- (c-Jun, Jun B, Jun D) and Fos-families (c-Fos, Fos B, Fra 1, Fra 2) and acts as homo- or heterodimer (Angel and Karin, 1991). In vivo experiments showed the requirement of c-Jun for the development of papillomas (Young *et al.*, 1999), whereas c-Fos is not essential for papilloma formation but is required for malignant transformation (Saez *et al.*, 1995).

Somatic inactivation of Smad4 has been documented in multiple tumour types including pancreatic, colon, breast, and prostate cancer (Bierie and Moses, 2006). Epidermal-specific Smad4 deletion blocks the growth inhibitory effect of TGFβ/BMP, resulting in hyperproliferation associated with downregulation of p21 and p27, and upregulation of c-Myc and cyclin D1 (Qiao *et al.*, 2006; Yang *et al.*, 2005). Further, all Smad4-/- mice developed spontaneous skin tumours including primarily SCCs, as well as sebaceous adenomas, basal cell carcinomas, and trichoepitheliomas (Qiao *et al.*, 2006; Yang *et al.*, 2005). Smad4-/- SCCs demonstrated inactivated Pten and activated Akt, representing activation of the key cell survival pathway (Qiao *et al.*, 2006; Yang *et al.*, 2005). Mice with epidermal-specific deletion of both Smad4 and Pten showed accelerated tumour formation, suggesting the cross-talk between the TGFβ/BMP and Pten signalling in the control of epidermal proliferation and survival (Yang *et al.*, 2005).

The HF phenotype in Smad4-/- mice was similar to the one in epidermal-specific BMPR-IA (ALK-3) knockout mice (Andl *et al.*, 2004; Kobielak *et al.*, 2003; Ming Kwan *et al.*, 2004; Yuhki *et al.*, 2004), which illustrates a possible dependence of Smad-4 on BMP signalling but not on the Activin or TGFβ signalling. Interestingly, in addition to the defects of hair shaft and IRS formation, epidermal-specific BMPR-IA (ALK-3) knockout mice, are also characterized by hyperplastic HFs and spontaneous development of hair follicle-derived tumours, pilomatricomas, in postnatal life (Ming Kwan *et al.*, 2004). However, development of malignant skin tumours was not observed in these mice.

Smad7 is expressed at very low levels in normal epithelia, but its expression is elevated in certain cancers (Bierie and Moses, 2006). To further investigate the role of Smad7 in the skin, keratinocyte-specific Smad7 transgenic mice were generated. Smad7 transgenic mice (K5 promoter) displayed severe epithelial hyperplasia, potentially because of negative regulation of the TGFβ/BMP induced growth inhibition and apoptosis. Further characterization of Smad7 transgenic epithelia revealed downregulation of TGFβ, BMP, and activin receptors, and decreased levels of activated Smad1 and Smad2 proteins, which is consistent with the known role of Smad7 in inhibiting TGFβ/BMP signalling (He *et al.*, 2002). A report from Liu et al. (2003) demonstrated that Smad7 cooperates with oncogenic ras to cause malignant conversion in a mouse model for SCC. Overexpression of Smad7 in H-ras-transduced keratinocytes resulted in a marked increase in cell proliferation, reduced H-ras-induced senescence, and upregulation of epidermal growth factor superfamily members. To further investigate the role of Smad7 in a

xenograft model, primary keratinocytes were coinfected with Smad7 and H-ras, mixed with dermal fibroblasts, and grafted onto nude mice. H-ras/Smad7, but not H-ras grafts progressed to SCCs (Liu *et al.*, 2003a). Characterization of H-ras/Smad7 SCCs revealed increased proliferation and invasive growth, upregulation of Keratin 8 expression, and lack of nuclear Smad2, Smad3, or Smad5 (Liu *et al.*, 2003a). Recently, it was shown that Smad7 binds a major mediator of Wnt signalling, β-catenin, and induces β-catenin degradation by recruiting the Smurf2 ubiquitin ligase to the Smad7/β-catenin complex (Han *et al.*, 2006). Since reduced Wnt signalling results in spontaneous skin cancer in mice (Niemann *et al.*, 2003a), it would be interesting to examine whether Smad7-mediated β-catenin degradation contributes to the oncogenic role of Smad7 in skin cancer. On the other hand, enhanced Wnt signalling contributes to many types of cancer and also results in spontaneous skin cancer formation in mice (Gat *et al.*, 1998). Therefore, Smad7 may play a dual role in carcinogenesis and future studies directly examining the role of Smad7 in cancer are required.

1.4 Aims of the project

Majority of human malignancies arises from epithelial tissues including the skin epithelium or epidermis. The skin undergoes constant remodeling and renewal; it is a tightly regulated system that has been shown to involve a hierarchy of cells with differing proliferative and self-renewal capacities (Miller *et al.*, 2005). The fine-tuning of the cell renewal process is based on coordinated action of several regulatory molecules that tightly control the key steps of cell

proliferation and differentiation. However, a combination of inherited and constitutional factors, with exposure to ubiquitously presented environmental factors may trigger molecular mechanisms resulting in dysregulation of the cell renewal processes and leading to carcinogenesis within the skin cell population (Miller *et al.*, 2005).

Despite the fact that considerable progress has been made in identification of molecular mechanisms underlying the development of the major cutaneous cancers, mechanisms suppressing tumour growth in keratinocytes remain to be further clarified. It was shown that mechanisms controlling skin development and carcinogenesis appear to be very similar, and key signalling pathways (Wnt, Hedgehog, TGFβ/BMP, etc.) that regulate skin development are also implicated in the pathobiology of cutaneous neoplasias (Athar, 2006; Bornstein *et al.*, 2007; Malanchi *et al.*, 2008; Owens and Watt, 2003).

Several indications suggest that BMP signalling operates as an evolutionary conserved potent tumour suppressor in the skin and other organs, while secreted BMP antagonists function as promoters of tumour growth (Blessing *et al.*, 1995; He *et al.*, 2001; Qiao *et al.*, 2006; Sneddon *et al.*, 2006). However, mechanisms and downstream targets that mediate tumour suppressor function of the BMP signalling pathway in skin remain to be explored. To address this issue we will:

i) Investigate effects of the BMP antagonist Noggin on the BMP anti-tumour activity in a transgenic mouse model with overexpression of Noggin in keratinocytes under control of the keratin 14 promoter. Dynamic changes in the development of skin tumours, analysis of cell proliferation and epithelial stem cells activity will be compared between K14-Noggin and wild-type mice.

ii) Determine genome-wide changes in the keratinocyte gene expression programmeme upon Noggin-induced BMP inhibition. Genome-wide microarray and qRT-PCR analysis, as well as in situ hybridization and immuno-fluorescent approaches will be employed to compare changes in gene expression in the skin of K14-Noggin and wild-type mice.

iii) Define BMP target genes mediating the BMP tumour suppressive functions in the skin. Chromatin immunoprecipitation (ChIP) and transient transfection promoter assay will be used to identify BMP targets in keratinocytes; experimental studies on modulating gene expression in tumour cells isolated from the skin of K14-Noggin mice will be performed.

II. Materials and Methods

2.1. Animals, tissue collection, chemical carcinogenesis, pharmacological experiments and morphometric analyses

All animal works were performed under the approval of PPL 40/2989 license at the University of Bradford and the Boston University IACUC protocol. Mice had free access to food (standard rodent diet) and tap water. The animal room was maintained under continuous 12-hr light and dark cycles with temperature and humidity at 21 ± 1^{o}C and 40-60%, respectively.

Transgenic (TG) *K14-Noggin* mice used in this study were provided by Dr. P Overbeek (Baylor College of Medicine, USA). These TG mice were generated on FVB background using transgenic construct, which contains human K14 promoter, mouse *Noggin* cDNA and human growth hormone poly-A sequence, as described previously (Tucker *et al.*, 2004). For control, FVB mice were purchased from Charles River. Skin samples were collected from neonatal (P0.5-P40) and adult mice (12-24 week-old, n=5-7 per time points for each mouse strain), frozen in liquid nitrogen and embedded into Tissue-Tek (Sakura, USA). Skin samples stored at -80^{0}C until use.

For chemical carcinogenesis, skin tumours were nduced using a carcinogen 7,12-dimethylbenz[a]antracen (DMBA) (Sigma-Aldrih) and a tumour promoter 12-tetradecanoil-phorbol-13-acetate (TPA) (Sigma-Aldrih). Back skin of 8 week-old female TG and WT mice (n=5 for each mouse strain) was shaved and treated with a single dose of DMBA (250 μg/ml) followed by twice per week application of the TPA (40 μg/ml) during 15 weeks. Tumour progression was observed up to 25 weeks.

For pharmacological experiment, the Wnt antagonist Aptosyn (4 mg/kg; OSI Pharmaceuticals, USA) and Shh inhibitor Cyclopamine (100 μg/kg; kindly

provided by Dr. W. Niu, Infinity Pharmaceuticals, Inc., USA) or vehicle control were administrated to neonatal TG mice (n=24) by daily subcutaneous (s/c) injections in the dorsal area from day P10 to P28. Skin was collected (P19, P21 and P28) and processed for histological and morphometric analyses, which were performed using a bright-field microscope (Nikon, USA), SPOT digital camera and image analysis software (Diagnostic Instruments, USA). Based on the morphology, HF-derived tumours were divided into several groups: stage 1 – small tumours (30-60 μm in diameter) arising from the HF outer root sheath, stage 2 – medium-sized tumours (60-120 μm in diameter), stage 3 – single large tumours (over 120 μm in diameter), stage 4 – multiple large tumours with epithelioid cyst containing keratinized substance in the center. Percentage of the HFs with tumours at distinct stage of the development was assessed in the Aptosyn (P19) and Cyclopamine-treated (P21 and P28) groups versus control; data were pooled, mean ± SEM was calculated, and statistical analysis was performed using unpaired Student's t-test.

2.2. Laser Capture Microdissection and microarray analyses

The hair matrix of anagen HFs of WT mice and tumour epithelium of *K14-Noggin* mice were dissected by Laser Capture Microdissection (LCM) system (Arcturus, USA), as described before (Sharov *et al.*, 2006). Briefly, 8 μm thick frozen sections were extensively dehydrated to preserve RNA integrity and stained with the Histological Reagent (Arcturus, USA) according to the manufacture protocol. After locating the cells of interest under the control of microscope, a CapSure Cap was placed over the target area. Pulsing the laser

beam (30-60 μm in diameter) through the cap caused the thermoplastic film to form a thin protrusion that bridged the gap between the cap and tissue and adhered to the target cell. By lifting of the cap, the target cells attached to the cap were removed. Laser capture was performed from 50-70 HFs of WT and TG mice, respectively. Total RNAs were isolated using PicoPure® RNA Isolation Kit (Arcturus, USA), following by two rounds of linear RNA amplification using RiboAmp® RNA Amplification Kit (Arcturus, USA).

Universal Mouse Reference RNA (Stratagene, USA) was used after one round of linear amplification in all analyses as a control. All microarray analyses were performed by Mogene Co. (USA) using 41K Whole Mouse Genome 60-mer oligo-microarray (manufactured by Agilent Technologies).

Real-time PCR of un-amplified reference RNA and reference RNA obtained after two rounds of amplification was employed for validation of possible alterations in gene expression caused by amplification procedure (see below). All microarray data on gene expression were normalized to the corresponding data obtained from the reference RNA. Two independent data sets were obtained from WT and TG mice, and p-values were calculated by the Agilent feature Extraction software (version 7.5) using distribution of the background intensity values to signal intensity and employing Student's t-test. Fold changes were determined as a ratio of normalized expression values. Functional annotation of the overrepresented and underrepresented genes was performed by the NIA Array Analysis software (http://lgsun.grc.nia.nih.gov/ANOVA/, 2007) according to the recommendations published previously (Sharov *et al.*, 2005a).

2.3. Isolation and treatment of tumour cells with rhBMP4

Tumours from *the* TG mice were dissected and minced with scissors in growth medium (William's medium, 10% FBS), followed by treatment with Collagenase/Dispase (Roche, 1mg/ml) for 1 h at 37^0 C. Disintegrated tumour cells were passed through a 70 μm nylon filter (Becton Dickinson) and spun down for 3 min at 100x g, followed by resuspension in the fresh growth medium and seeding onto collagen-coated P60 plates. The tumour cells cultured at 33°C with 8% CO_2 until they were about 80-90% confluent.

The tumour cells were treated either with i) 200 ng/ml BMP4, ii) 500 ng/ml BMP4, iii) 200 ng/ml BMP4 and 500 ng/ml Noggin (R&D Systems) or diluent control. Cells were harvested after 24 hours of treatment and processed for total RNA and protein isolation. Total RNA was isolated with TRIZOL reagent (Invitrogen) according to the manufacture protocol. 1μg of the total RNA used for cDNA synthesis using SuperScript III First-Strand Synthesis System (Invitrogen). 0,5 μl of the synthesized cDNA used for *Wif1* and *Shh* expression analysis by qRT-PCR (see section 2.7). Proteins extraction is described in section 2.6

2.4. Chromatin Immunoprecipitation (ChIP)

Skin from newborn FVB mice was incubated in 0.25% Trypsin (Invitrogen) overnight at $+4^0$C. After separation from the dermis, epidermis minced and stirred for 30 min at $+4^0$C to release keratinocytes. The cells were platted onto collagen-coated P100 plates (1 skin per P100) in growth medium [EMEM (Lonzo) supplemented with chelated 4%FBS, 0.05 mM $CaCl_2$, 0.4 μg/ml

hydrocortisone (Sigma), 5μg/ml insulin (Sigma), 10^{-10} M cholera toxin (ICN), 10 ng/ml epidermal growth factor (Invitrogen), $2x10^{-9}$ M T3 (3,3',5-triiodo-L-thyronine; Sigma), 1x Antibiotic/Antimicotic (Sigma)] and incubated at 33^0C with 8% CO_2.

Confluent cells were cross-linked with 2mM Di(N-succinimidyl) glutarate (Sigma) for 45 min at RT, then with 1% PFA for 15 min at RT. After lysing in Buffer I [50 nM HEPES (pH7,5), 0.14 M NaCl, 2.5% Glycerol, 1mM EDTA (pH8.0), 0.5% IGEPAL CA-630, 0.25% Triton X-100, 1x Protease and phosphatase inhibitor cocktail (Roche)], cross-linked chromatin sonicated for 20 min using Branson Sonifier 450CE equipped with cup horn. After preclearing treatment, cell extracts were incubated with 5 μg phospho-Smad1/5/8 antibodies (Chemicon) overnight at 4°C followed by precipitation with ProteinA sepharose (Invitrogen) for 1 hours. Sepharose beads with precipitated antibody-chromatin complex were washed with Wash buffer I [50 mM Tris-HCl (pH8), 0.15 M NaCl, 2 mM EDTA, 0.1% SDS, 1% Triton X-100)], then with Wash Buffer II (same as Wash Buffer I with 0.5 M NaCl) and Wash Buffer III [25 mM Tris-HCl (pH8), 0.5 M LiCl, 1 mM EDTA, 0.1% SDS, 1% IGEPAL CA-630]. Following to the extensive washing, DNA is eluted with TE buffer/1% SDS and, after adding 0.2 M NaCl to the eluted solution, decrosslinked overnight at 65^oC. After Proteinase K treatment (100 μg/ml) for 1 hour at 50^oC, DNA was extracted with phenol-chlorophorm method, followed by ethanol precipitation. Purified DNA analyzed for presence putative Smad1/5/8 binding sites by PCR using primers (see Tab. 1) amplifying different genomic regions within *Wif1* promoter. Comparison of promoter sequences among different species was performed using the whole genome mVISTA tool (http://genome.lbl.gov/vista/index.shtml),

which evaluates conservation of genomes between pairs of species (specified as mouse and humans).

2.5. Cell transfection

Following plasmid were used for transient transfection of HaCaT keratinocytes: i) pWif1-Luc and pShh-Luc reporter plasmids containing human *Wif1* and *Shh* promoter regions (nucleotides -1512 to +6 bp of human *Wif1* gene and nucleotides -3347 to -1512 of human *Shh* promoter sequence, respectively); ii) plasmids containing constitutively active BMP receptors (BMPR-IA or Alk3QD and BMPR-IB or Alk6QD) (kindly provided by Prof. K. Funa); iii) Smad1 and Smad5 expressing plasmids under pCMV promoter (kindly provided by Prof. K. Funa); iv) BMP-responsive plasmid 3GC2-Lux (contains three repeats of GC-rich sequence derived from the proximal region of Smad6 promoter) as a positive control; v) pGVB2L-Luc plasmid as a negative control; vi) pRL-*null* as an internal control plasmid to compensate variable transfection efficiencies.

HaCaT keratinocytes were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum until 60-70% confluent. Transfecton was performed on twellve-well plates for 24 hours using Lipofectamine 2000 reagent (Invitrogen) according to the manufacture protocol. Luciferase activity was assessed using Dual-Luciferase Reporter Assay System (Promega). All assays were performed in triplicates. Data were pooled; mean ± SEM was calculated. Statistical analysis was performed using unpaired Student's *t*-test.

2.6. Western Blotting

Western blot analysis of total tissue proteins obtained from the extracts of full thickness skin of TG and WT mice was performed, as described before (Sharov *et al.*, 2005b; Sharov *et al.*, 2003a).

Proteins were extracted from snap frozen skin samples or cultured cells with lysis buffer (0.05 M Tris-HCl (pH7.5), 0.15 M NaCl, 1% Sodium deoxycholate, 1% Triton X100, 0.1% SDS) supplemented with Complete Mini (EDTA free) Protease Inhibitor Cocktail (Roche). 20 μg of protein was used for SDS-PAGE at a constant voltage of 125 V for 1.5hr, followed by transblotting into nitrocellulose membrane with transblott buffer (0.02 M Tris-HCl (pH10.4), 0.15 M glicine, 20% Methanol) at 300 mA for 40 min. After washing in 1xTBS/0.05% Tween 20, the membrane blocked in 5% non-fat milk for 1hr at RT, followed by incubation with primary antibodies overnight at +4^{o}C. After washing in 1xTBS/0.05% Tween-20, secondary antibody (peroxidase-conjugated, 1:2000, Jackson ImmunoResearch) was applied for 1hr. The membrane developed using Western Lightning Chemiluminoscence Reagent (PerkinElmer). Immunoblotting against β-catenin served as a loading control.

2.7. Quantitative Real-time PCR (qRT-PCR) analyses

LCM isolated RNA samples after two rounds of amplification (see section 2.2) were further processed for cDNA synthesis. Equal amount of amplified RNA (2 μg) from control and TG samples was reverse transcribed with random primers using Superscript III First Strand Synthesis System kit (Invitrogen) according to the manufacture protocol. qRT-PCR was performed using MyiQ

Single-Color Real-Time PCR Detection System (Bio-Rad Corp.) PCR primers were designed with Beacon Designer software (Premier Biosoft International; **Table 5**). Reaction mixture contained 1x iQ SYBR Green Supermix (Bio-Rad Corp.), 1 uM primers and 10 ng cDNA. Amplification was done in following conditions: initial denaturation (95^{o}C for 5 mins), followed by 40 cycles of denaturation (95^{o}C for 15 sec), annealing (for 30 sec at temperature experimentally determined for each primer pairs) and elongation (72^{o}C for 15 sec). Following the amplification, melting curve was created by gradual heating the PCR products (from 60^{o}C to 90^{o}C). For each gene of interest, qRT-PCR was performed in triplicates. Expression of a housekeeping gene (*Gapdh*) was used for normalization. Differences in expression between samples and controls were calculated using Gene Expression Macro programme (Bio-Rad Corp.) based on the $\Delta\Delta$Ct equitation method, data were pooled, mean ± SEM was calculated, and statistical analysis was performed using unpaired Student's t test.

Table 5. List of PCR primers

Accession Number	**Sequence Definition**	**Sense/Anti-sense Primers**
qRT-PCR primers		
NM_009744	B-cell leukemia/lymphoma 6 (BCL6)	CATACCTGTAATGTGTCCTCAC ACAAGCATGACGCAGAATG
NM_00761	Caspase 7 (Casp7)	TGTAAGAGGACTTCGGTTC GACTCAGTTCTGGCTTGG
NM_009829	Cyclin D2 (Ccnd2)	TACCTTAGACAGTCCAACCTTG GCTGTTGACCACCACCTG
NM_007634	Cyclin F (Ccnf)	AGGAGAGCGAAGGCGAGAAG GTTCAGGTAGACCACAGTGACATC
BC052714	Cyclin M3 (Ccnm3)	CTGCTGGAGAATACTAACC

		GTCTATGGAACAGTCTATGG
NM_010100	Ectodysplasin-A receptor (Edar)	CCCACCGAGTTGCCGTTT, CCAATCTCATCCCTCTTCA
NM_010464	Homeo box C13 (Hoxc13)	CTCAGTTCTTGCCTCTTC ACCTTGCCTATGGAGTTC
NM_010496	Inhibitor of DNA binding 2 (Id2)	GACTCGCATCCCACTATC ATGCTGATGTCCGTGTTC
NM_010591	Jun oncogene (Jun)	CTGATTTGTAGGAATAGATACCC CACAGCACATGCCACTTC
NM_010659	Keratin 1-1 (Krt1-1)	GTCTCCAATCCCTGTGTC TGTCCTTGCTCTGTTGAC
NM_008470	Keratin 1-16 (Krt1-16)	AATATCCACTCCTCCTCAC GTTGAACCTTGCTCCTTG
NM_010666	Keratin 1-c29 (Krt1-c29)	TCGTGGAAGAGTTAGACC TTAGAGGCGGAGTTCAAG
NM_010663	Keratin 1-17 (Krt1-17)	ACCTGACTCAGTACAAGCC CCTTAACGGGTGGTCTGG
BC006780	Keratin 2-5 (Krt2-5)	AATGTAAGCCACCAAAGCAGAACC GGAGGAAGTCAGAACCAGGACAG
AY028606	Keratin 2-20 (Krt2-20)	GAACCACTGTGACAACCTACG CTGCCTCAATGTCCTGCTG
NM_010703	Lymphoid enhancer binding factor 1 (Lef1)	GCCAGCCACCGCCGATTC GGCGGCGTTGGACAGATC
NM_008808	Platelet-derived growth factor alpha (Pdgfa)	AGACAGATGTGAGGTGAG ACGGAGGAGAACAAAGAC
NM_176996	Smoothened homolog (Smo)	AAAGTGTTTATTGTGTCATTTGTC GGAACTGAGATGTGAATGTAGG
NM_009170	Sonic hedgehog (Shh)	CATTCCTCTCCTGCTATGCTCCTG ATGACAAAGTGGCGGTTACAAAGC
NM_011718	Wingless related MMTV integration site 10b (Wnt10b)	AGCGTCTTCTCTACCTACAG ACACAATGCCTGCTATTATCC
NM_009524	Wingless-related MMTV integration site 5A (Wnt5a)	CCACGAATACCAGGAAGCAAGC CCCACAAAGAACACCAAAGAGAGG
NM_011915	Wnt inhibitory factor 1 (Wif1)	CCACCTGAATCCAATTACATC TGAACAGCATTTGAACATCC
ChIP primers		
Gene	**Genomic region**	**Sense/Anti-sense Primers**
Wif1	-326 to +37	AGACAGGCACACAGAGATG AAAGGCGAGCACTGAGAG
	-2830 to -2463	GTGTGTGAGTGTGTATAAGTG CGAGGACCAGAGTTCATATC

2.8. Immunohistochemistry

Cryosections (9 μm) were fixed in acetone (10 mins at -20^{o}C) or 4% PFA (10 mins at RT). For each primary AB (see **Table 6**), the fixation method was determined experimentally. Sections were initially preincubated in 5% BSA/0.1% Triton X100 to block non-specific binding, following by overnight incubation with primary AB at +4^{o}C. Sections were then incubated with corresponding FITC-, TRITC- or biotin-labeled secondary AB (Jackson ImmunoResearch) for 1hr at +37^{o}C. In case of using the biotin-labeled secondary AB, the reaction product was visualized with a tyramide amplification kit (Perkin Elmer/ NEN). Cell nuclei were visualized with 4'6'-Diamidino-2-phenylindol (DAPI).

Image preparation and analyses were performed by using bright-field and fluorescent microscope (Nikon), in combination with SPOT digital camera and image analysis software (Diagnostic Instruments).

2.9. TUNEL assay

To visualize apoptotic cells, freshly cut cryosections (9 μm) were fixed in 4% PFA (10 mins at RT), followed by postfixation in acetone/acetic acid (in a ratio 2:1) (10 mins at -20^{o}C). The reaction was performed using ApopTag® Fluorescein *In Situ* Apoptosis Detection Kit (Chemicon, S7110) according the manufacture protocol. Briefly, sections incubated in a reaction mixture (70% Reaction buffer, 30% TdT enzyme) for 1hr at +37^{o}C, followed by an application of FITC-labeled anti-DIG antiserum.

Cell nuclei counterstained with DAPI. Image preparation and analyses

were performed by using fluorescent microscope (Nikon), in combination with SPOT digital camera and image analysis software (Diagnostic Instruments)

Table 6. List of primary antibodies

Anigen	Host	Dilution	Manufacturer
BrdU	Mouse	1:50	BD Pharmingen,
β-Catenin	Mouse	1:100	Sigma
CldU	Rabbit	1:50	BD Pharmingen
Ki-67	Rat	1:1000	Dako Denmark
Lef1	Rabbit	1:100	R&D Systems Inc
Lhx2	Goat	1:250	Santa-Cruz Biotechnology
Loricrin	Rabbit	1:150	R&D Systems Inc.
pSmad1/5/8	Rabbit	1:100	Chemicon International Inc.
Shh	Rabbit	1:100	R&D Systems Inc.
Sox9	Rabbit	1:200	Santa-Cruz Biotechnology
Wif1	Goat	1:1000 (Tyramide amplification)	R&D Systems Inc.
Wnt 10b	Goat	1:100	R&D Systems Inc.

2.10. Label retaining cell detection

Pulse-chase experiment for simultaneous detection of proliferating and Label Retaining Cells (LRCs) based on the differential antigen properties of BrdU analogues CldU and IdU (Sigma-Aldrih). Three-day old mice were injected with IdU (50 μg/g, i/p) during 4 days. After two weeks of chasing, pulse injection of CldU (50 μg/g, i/p) was performed; skin samples were collected in 2 hours. Cryosections (9 μm) were fixed in 4% PFA for 10 mins; DNA hydrolyzed by incubation in 1N HCl for 15 min at RT. Further, the sections were treated with

0.1M Sodium Tetraborate (pH 8.5) for 5 mins at RT, followed by washing in PBT and blocking in 10% Goat Normal Serum. Sections were then stained with rat anti-BrdU antibody 1:100 (Novus, clone BU1/75 (ICR1); reacts with CldU) for 16-18 hours at RT, followed by TRITC-conjugated Goat anti-Rat serum. Subsequently, sections were incubated with FITC-conjugated mouse anti-BrdU 1:50 (Caltag, clone 3D4; reacts with IdU). Finally, the sections were washed in high salt Tris buffer (pH 8.0) (0.5 M NaCl, 28 mM Tris-HCl, 21.8 mM Tris Base, 0.5% Tween 20) and counterstained with DAPI.

2.11. RNA in situ hybridization

For mRNA detection on tissue sections, cryosections (9 µm) were fixed in 4% PFA for 10 mins at room temperature. After acetylation in triethanolamine buffer (4.5 mM Triethanolamine, 6N NCl, 3mM Acetic Anhydride) for 10 mins and pre-mobilisation (1% Triton X100/1x DEPC-treated PBS) for 30 mins, sections incubated in hybridization buffer (50% Formamide DI, 2x SCC, 1% Dextran sulphate, 10 mg/ml t-RNA, 100 ng DIG-labeled riboprobes) for 16-18 hours at 50^{o}C. Slides subsequently washed in 2x SCC (10 mins, 4 times, 57^{o}C), 0.1x SCC (60 mins, 57^{o}C), 0.2x SCC (10 min, RT). Immunodetection of hybridized probes performed with sheep anti-DIG antibody (Roche, 1:5000) diluted in Buffer B1 (0.1 M Maleic acid, 0.15 M NaCl, pH7.5) for 3hrs at RT. To visualize the immunoreaction products, sections were incubated in Buffer B2 (0,1 M Tris-HCl, 0.1 M NaCl, 5 mM $MgCl_2$, pH9.5) with 5 µl/ml NBT (Roche) and 3.75 µl/ml BCIP (Roche) for 16-18 hrs at RT.

DIG-labeled RNA probes for detection *Noggin* and *Krt15* transcripts were produced by in vitro transcription according to the instructions of the DIG RNA

Labelling kit (Roche). To detect transgenic *Noggin* expression, *K14-Noggin* plasmid served as a template to make probes that target human growth hormone poly-A sequence of the transgenic construct. Template for *Krt15* probes was synthesized by PCR with primers introducing the T7 promoter into antisense strand as described (Liu *et al.*, 2003b):

Forward primer, mK15F

(GCAGTAGCAGCAGCAGCAATTTC)

Reverse primer, mK15R-T7

(GTAATACGACTCACTATAGGGCCACTCAGAAGGAAGCCGAGAAAGC)

This pair of primers generates the DNA template for the antisense riboprobe spanning 263BP of the 3' end of the mouse K15 cDNA (Liu *et al.*, 2003b).

DIG-labeled RNA probes for detection *Shh, Ptch1, Ptch2, Gli1, Gli2, CcnD1* and *CcnD2* were provided by Prof. A. Dlugosz (Department of Dermatology and Comprehensive Cancer Center, University of Michigan, USA).

2.12. Alkaline Phosphatase staining

For morphological analysis of skin sections histochemical detection of alkaline phosphatase (AP) activity was performed. In skin, AP is strongly expressed by fibroblasts of dermal papilla; this makes the AP staining as an useful method in staging HF development and cycling (Handjiski *et al.*, 1994).

To perform the staining, cryosections (9 µm) were fixed in 4% PFA (10 mins at RT), and then incubated in developing solution (100 mM NaCl, 100 mM Tris, pH 9.5, 50 mM $MgCl_2$, 0.005% Naphtol ASBI phosphate, 0,5% DMF) for 15 mins. Sections counterstained with haematoxylin.

2.13. Oil Red-O staining

Histochemical method of lipids detection by Oil Red-O dye is based on the physical properties of the dye being more soluble in lipids. Cryosections (9 μm) fixed in 10% buffered formalin for 10 mins, and then stained in Oil Red-O solution (0.5% Oil Red-O/ isopropanol diluted in water, ratio 2:5) for 15 mins. Sections counterstained with haematoxylin.

2.14. Quantitative histomorphometry

To measure epidermal thickness, AP-stained skin sections were analyzed by "SPOT Advantages" (Diagnostic Instruments) image analysis software. Measure of the epidermal thickness in TG dorsal skin (8 wks old female, n=3) was performed every 100 μm within 1 cm and compared to that of sex- and age-matched WT controls (n=3). All sections were analyzed at x200 magnification. Means and standard error of means (SEMs) were calculated from pooled data and statistical analysis performed using unpaired Student's *t*-test; differences were judged as significant if $p<0.05$.

Skin sections stained with Ki67 antiserum were analyzed to evaluate proliferating cells. Under x400 magnification, the number of epidermal Ki67-positive cells was counted in 30 microscopic fields derived from sex- and age-matched TG and WT skin sections (n=3), respectively; percent of proliferating cells was calculated from the total number of analyzed cells. Means and standard error of means (SEMs) were calculated from pooled data and

statistical analysis performed using unpaired Student's *t*-test; differences were judged as significant if $p<0.05$.

III. RESULTS

3.1. Phenotypic characterization of K14-Noggin mice

3.1.1. K14-Noggin mice: genotype and its effect on BMP signalling

To elucidate a role for BMP signalling in skin development and carcinogenesis, K14-Noggin transgenic (TG) mice over-expressing BMP antagonist Noggin in keratinocytes were used in this study. TG mice were generated on FVB/NJ background, which is more susceptible to experimentally induced carcinogenesis compared to other mouse strains (Hennings *et al.*, 1993). The transgenic construct drives high-copy expression of the mouse *Noggin* cDNA in the Keratin 14 (K14) promoter-active cells (**Fig 3.1A**). K14 promoter becomes fully active by day 14.5 of mouse embryonic (E14.5) development in the basal layer of the epidermis and subsequently in the outer root sheath (ORS) and sebaceous glands of the developing hair follicles, maintaining the high active level throughout life (Byrne *et al.*, 1994; Wang, 1997). Western blot analysis of the protein extracts from dorsal TG skin confirmed the increased levels of the 64-kDa Noggin protein compared to wild type (WT) skin **(Fig 3.1B)**. By *in situ* hybridisation with probes specific to the human growth hormone polyadenylation sequence, cells expressing TG construct were detected in the basal layer of epidermis, HF outer root sheath and sebaceous gland **(Fig 3.1D**, arrows**)**; whereas lack of the expression was seen in the skin of WT mice (**Fig. 3.1C**).

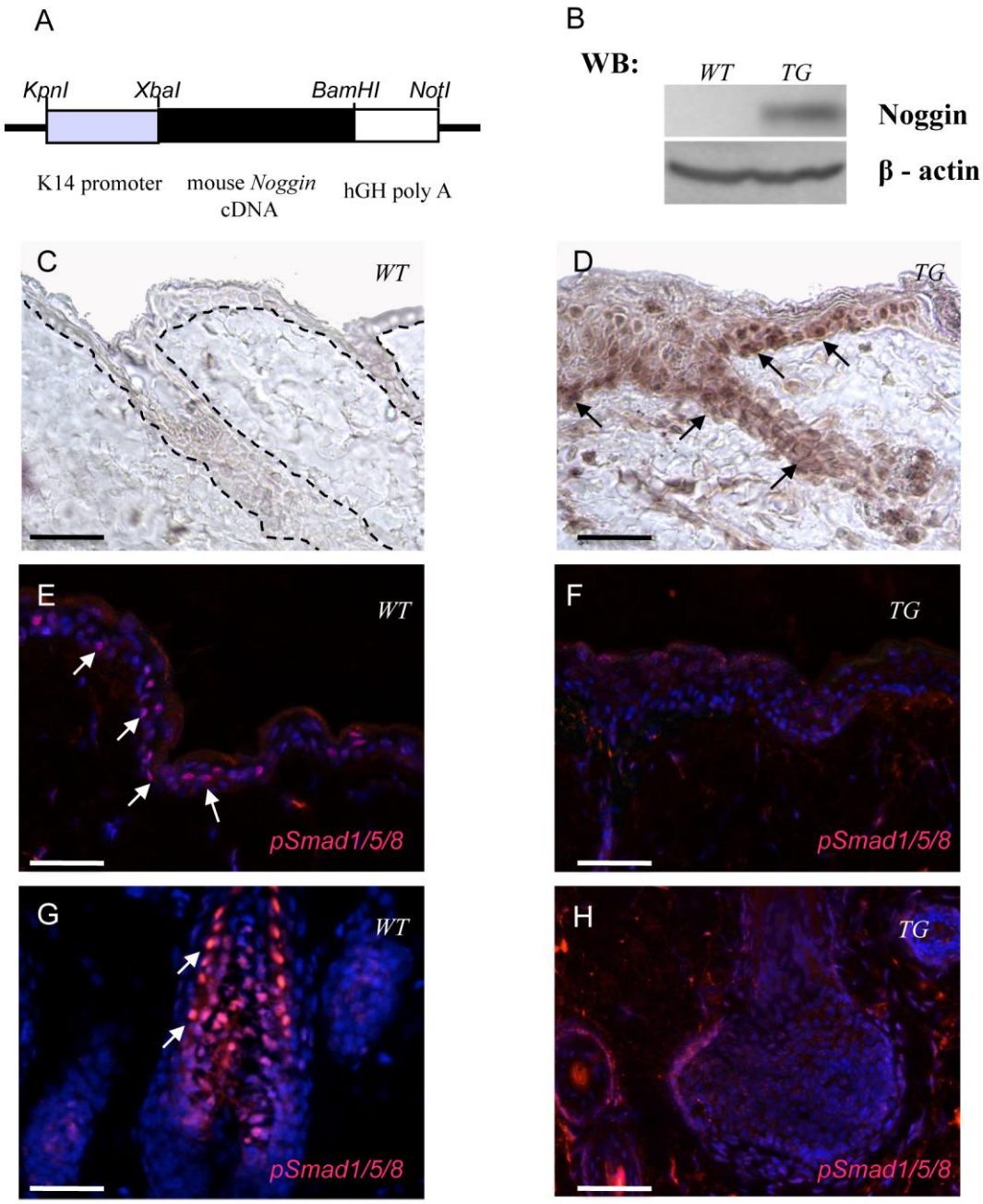

Fig 3.1. K14-Noggin construct and pSmad1/5/8 expression in WT and TG

(A) TG construct consists of human K14 promoter, mouse *Noggin* cDNA and poly-A sequence of human growth hormone; (**B**) Noggin protein expression in WT and TG skin by Western blotting; (**C, D)** Expression of the transgenic noggin mRNA in WT (C) and TG (D) skin (arrows indicate sites of noggin expression; dashed line separate epidermis and HF from the dermis); **(E-H**) pSmad 1/5/8 in the epidermis and HF in WT (E,G) and TG (F,H) skin (arrows indicate pSmad1/5/8-positive cells). Scale bars, 50 μm.

To determine whether Noggin over-expression in the TG mice leads to the alterations in BMP signalling, immunofluorescent detection of phospho-Smad1/5/8 (pSmad1/5/8) proteins, which are the markers of the active BMP-Smad signalling, was carried out.

In WT skin, pSmad1/5/8 were expressed in the basal and suprabasal cells of the interfollicular epidermis (IFE) and in the differentiating cells of the inner root sheath (IRS) and hair shaft (**Fig 3.1E** and **3.1G**, arrows). In contrast, expression of the pSmad1/5/8 was markedly decreased in the TG skin compared to WT (**Fig 3.1F** and **3.1H**). Therefore, transgenic expression of Noggin indeed leads to inhibition of the canonical BMP pathway in epidermal and HF keratinocytes.

3.1.2. Macroscopic phenotype of the *K14-Noggin* mice.

Despite strong activity of the K14 promoter in embryonic skin by E14.5, an overt phenotype did not emerge until the third week of postnatal life when TG mice began to display first signs of hair loss on back skin (**Fig 3.2A**). Over 60 % of the TG mice became almost completely bald by 8-10 weeks. In addition, overexpression of Noggin resulted in the development of hyperplastic disorders of nails (**Fig 3.2B**). However, the most profound feature of K14-Noggin mice was the spontaneous development of multiple skin tumours by 3-6 months after birth (**Fig 3.2C**, arrows). The tumours distributed randomly over the skin surface with predominant localisation on the tail, back, and ventral skin. Usually, the first signs of the tumour growth appeared on the tail as an increase of its thickness. The tail gradually became thicker and looked swollen (**Fig 3.2D**, arrows).

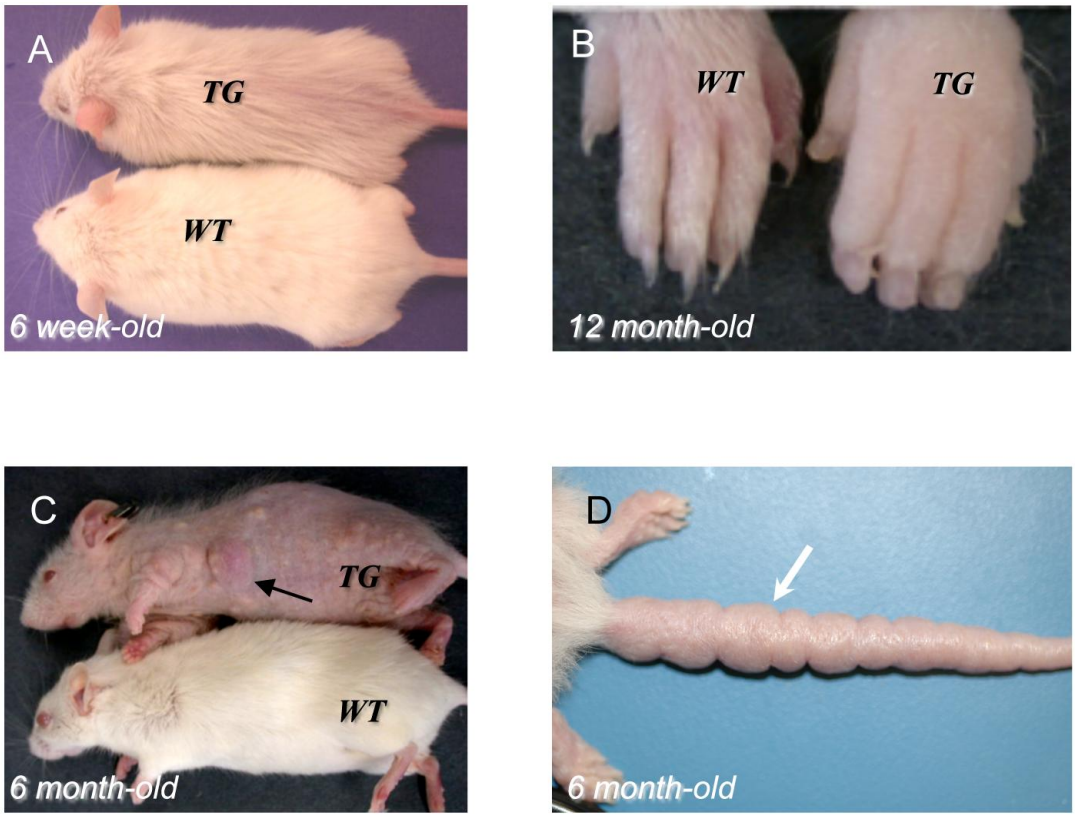

Fig 3.2 Macroscopic phenotype of *K14-Noggin* mice

(**A**) Hair loss on 1 month-old back skin; (**B**) Hyperplastic nails of TG mice; (**C**) Multiple skin tumours of different locations (arrows indicate tumours); (**D**) Tumour growth in the tail.

On other parts of the skin, the tumours grew as hemispheres of different sizes with an ability to growth up to 2 cm in diameter (**Fig 3.2C**). Despite the development of multiple tumours, TG did not show any obvious signs of distress.

However, the large tumours had a high risk for haemorrhage and necrosis that were potentially dangerous for animal wellbeing and health. Due to that, the tumours in 1 cm and bigger were surgically excised or the animals were sacrificed. Despite the extravasations into the tumours, no visible signs of invasion into surrounding tissues and metastases into other organs were observed (data not shown).

3.1.3. Microscopic study of the tumour development in K14-Noggin mice

Histological analysis of 4 days old (P4) skin sections did not reveal any differences between TG and WT littermates, suggesting that epidermal development and HF morphogenesis were not affected by Noggin over-expression (**Fig. 3.3A** and **3.3B**). However, TG mice showed markedly larger HFs compared to WT in two weeks after birth (P14) (**Fig. 3.3C and 3.3D**). Moreover, the TG mice developed epithelial buds growing from the upper and middle portions of the HF outer root sheath (**Fig. 3D** insert, arrow).

Histological analysis of TG skin during first catagen development (P16-P18) revealed that the HFs failed to regress and remained in the anagen-like growth phase (**Fig. 3.3F**), whereas in WT skin all HFs, as expected, entered into catagen (**Fig. 3E**).

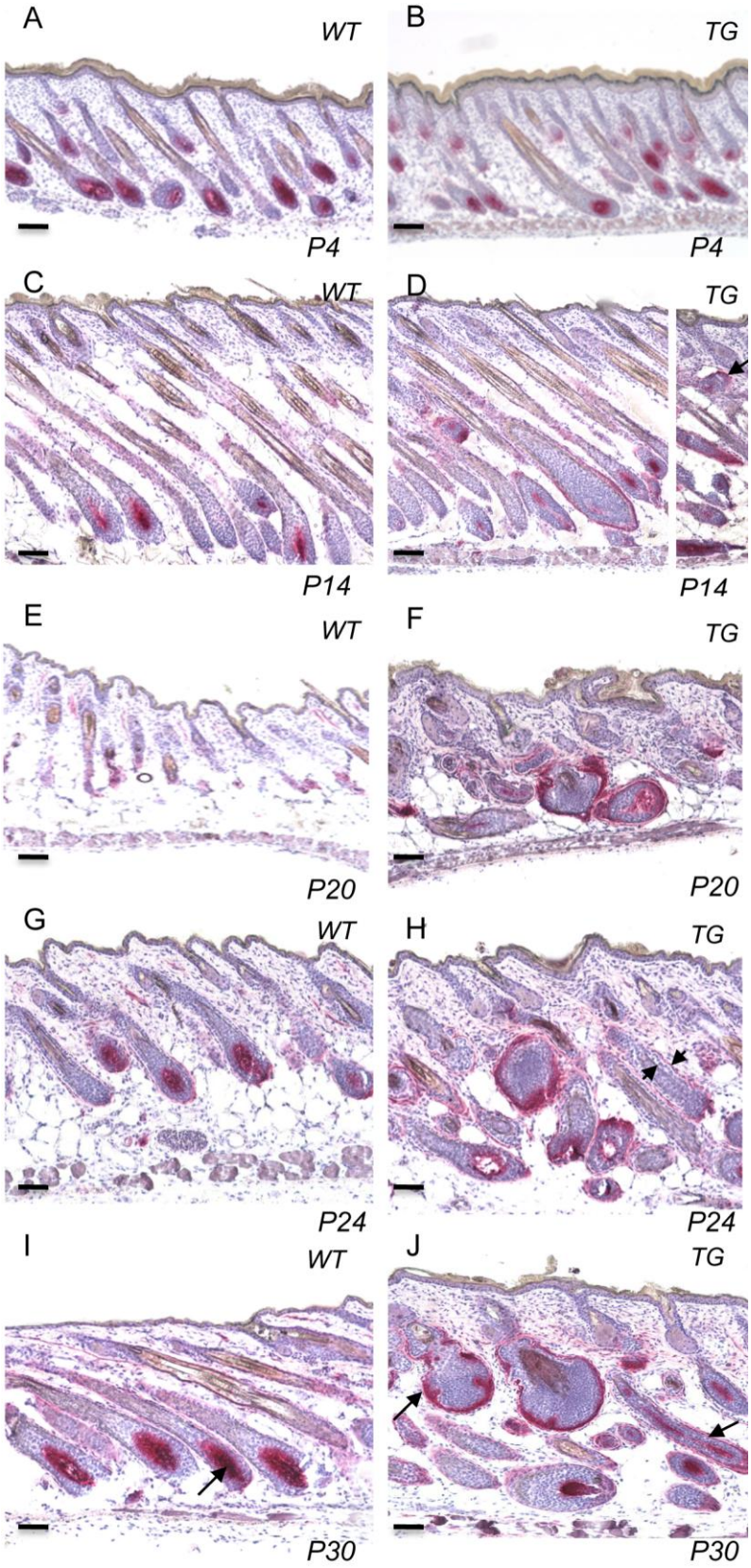

Fig 3.3. Development of the HF-derived tumours in K14-Noggin mice

Skin sections at different days of postnatal life stained for AP (**A-M, O-P**), H&E (**N**), and Oil Red-O (**Q-R**). In WT skin, hair follicles cycle normally: anagen (**A, G, I**), catagen (**C**), telogen (**K**) (continued on next page)

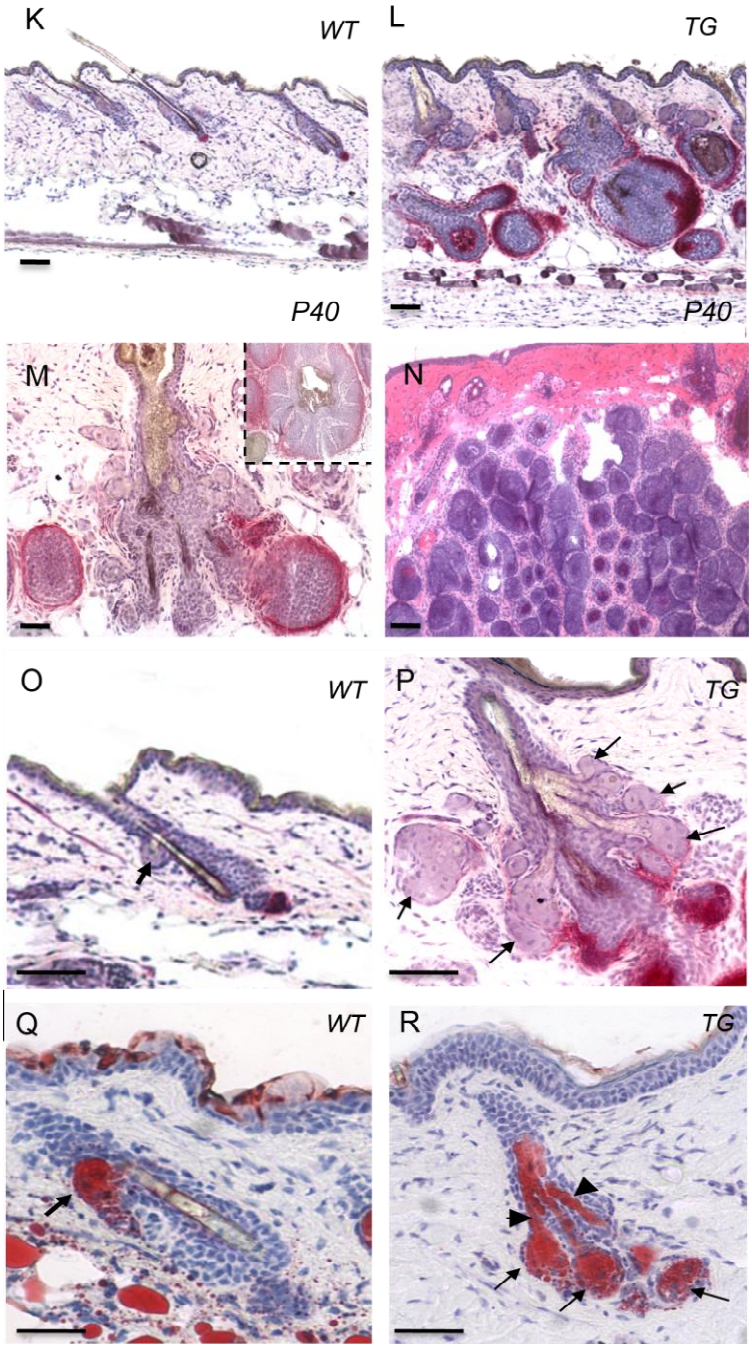

Fig 3.3. Development of the HF-derived tumours in K14-Noggin mice (*continued*)

In TG skin, hair follicles grow continuously without catagen and telogen phases (**B, D, F, H, L**), resulting in trichofolliculoma-like tumour formation (**M, N**); (**O-P**) Hyperplastic sebaceous glands (P, arrows) in TG versus WT skin (O, arrow); (**Q-R**) Enlarged and lipid-enriched sebaceous glands in TG skin (arrows indicate lobules, arrowheads point ducts). Scale bar, 50 μm

Hair follicles in the TG mice continued to grow resulting in marked enlargement of the lower portion of the hair follicles, and an increase in thickness of the ORS (**Fig. 3H**, arrow). These microscopic changes strongly correlated with the progression of hair loss.

Moreover, during the first postnatal hair cycle (P23-P40), hair follicles in the TG mice showed outgrowth of additional epithelial buds ("tumour placodes") from the ORS leading subsequently to formation of large tumour-like structures (**Fig. 3.3J and 3.3L**).

Interestingly, an increased alkaline phosphatase (AP) activity was detected in the HF connective tissue and dermal cells adjacent to the newly formed epithelial buds (**Fig. 3.3J** arrows), while in WT skin AP was seen exclusively in dermal papilla fibroblasts (**Fig. 3.3I** arrow), suggesting a marked remodeling of the mesenchymal hair follicle compartment upon the epithelium-derived noggin over-expression.

Nearly 100% of 6 month-old mice showed macroscopically visible skin tumours distributed randomly on the dorsal and ventral skin surfaces. Histological analysis of the tumours revealed multiple, densely packed, and disoriented hair follicle-like structures emerging from a single focal source with a tendency to form an epithelioid cyst containing keratinized substance in the center (**Fig. 3.3M** and **3.3N**).

Tumours contained hyperplastic matrix cells and rudimentary hair shafts, were surrounded by mesenchymal cells with strong alkaline phosphatase activity and morphologically resembled human trichfolliculomas.

In addition, adult *K14-Noggin* mice showed a significant enlargement of the sebaceous glands with an increase in number of lobules and elongated

ducts compared to WT mice (**Fig. 3.3O and 3.3P,** arrows). Oil-red staining revealed increased production and accumulation of lipid components in the hyperplastic lobules and multiple widened and ramified ducts (**Fig. 3.3Q and 3.3R**, arrows and arrowheads).

3.1.4. Expression of the markers of cell proliferation and apoptosis in the skin of K14-Noggin mice

During anagen phase, pulse DNA labeling with BrdU (50ug/g) revealed the presence of actively proliferating cells in the hair matrix only in the WT skin (**Fig. 3.4A**, arrows), while in TG skin, there was a marked increase in the number BrdU-labeled S phase cells in the hair follicle ORS (**Fig. 3.4B**, arrows).

These findings were confirmed by immunofluorescent detection of Ki67-antigen (**Fig. 3.4C and 3.4D**), another well characterized marker of cell proliferation (Scholzen and Gerdes, 2000).

However, in addition to the presence of proliferating cells in the hair matrix and the hair follicle ORS, Ki67-positive cells were detected at the peripheral portion of HF-like outgrowths (**Fig. 3.4F**) in the TG skin.

During catagen phase, WT hair follicles underwent an apoptosis-driven regression and showed numerous apoptotic cells in the lower portion of the hair follicles as was determined by TUNEL assay (**Fig. 3.4G**).

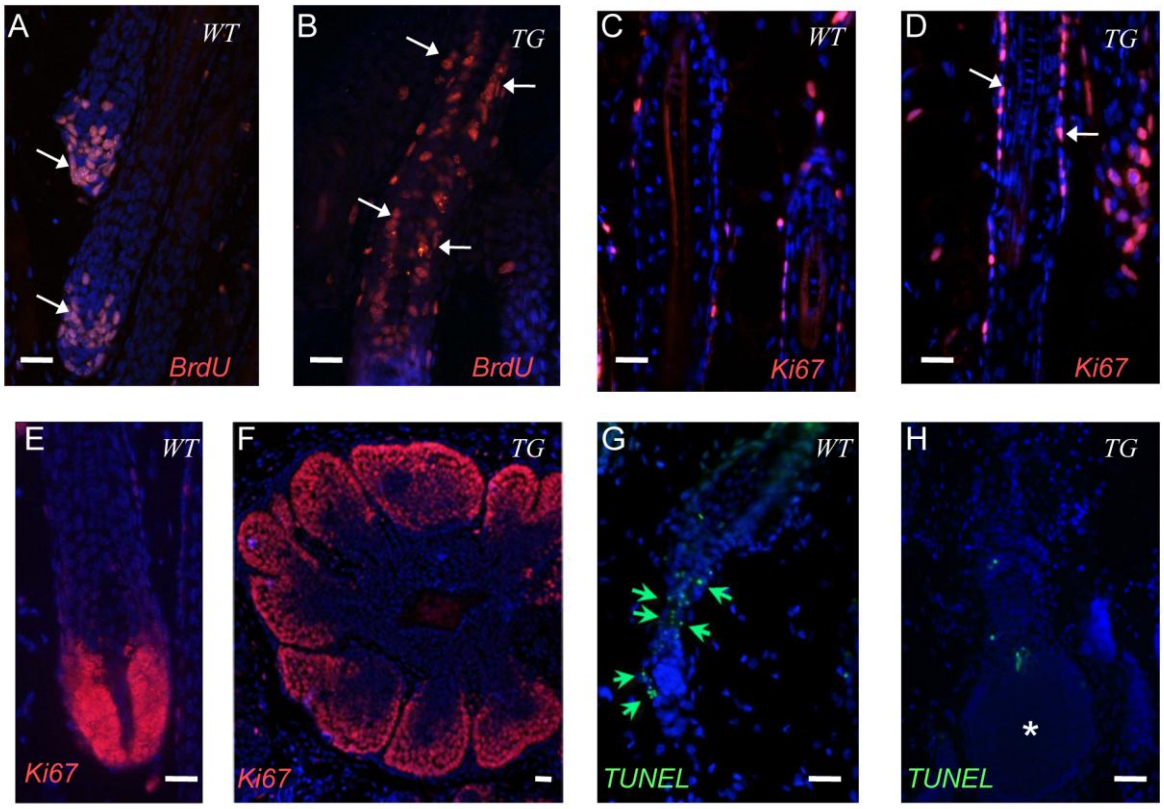

Fig 3.4. Increased proliferation and inhibition of apoptosis in hair follicles of K14-Noggin mice

(**A**, **B**) Increased number of BrdU-positive proliferating cells in the hair follicle ORS (arrows) of TG mice (B), compared to WT HFs (A, arrows indicate matrix cells); (**C, D**) Ki67 expression in the hair follicle ORS of WT (C) and TG (D) skin; (E,F) Ki67 expression in the matrix cells of WT HFs(E) and HF-derived tumours (F); (**G, H**) TUNEL staining in WT (G) and TG (H) skin (arrows indicate apoptotic cells). Scale bars, 25 μm

In contrast, the first anagen-catagen transition was arrested in the *K14-Noggin* HFs, which showed lack of TUNEL-positive cells (**Fig. 3.4H**, asterisk). Moreover, no apoptosis was detected in the HF-derived tumours (data not shown).

Therefore, the transgenic overexpression of Noggin resulted in a significant activation of cell proliferation and inhibition of apoptosis in the HF epithelium.

3.1.5. Markers of skin stem cells and early progenitors are increased in the skin of K14-Noggin mice

Several lines of evidence suggest that BMP signalling is involved in maintenance and/or activity of stem cells in different tissues, including the skin (He *et al.*, 2004; Kobielak *et al.*, 2007; Varga, 2005; Zhang *et al.*, 2006; Zhang and Li, 2005). To investigate a role for the transgenic overexpression of BMP antagonist Noggin in the control of stem cells activity, expression of selected markers of skin stem cells and their early progenies was analyzed. In particular, detection of the keratin 15, Lhx2, and Sox9 was carried out in the TG skin in comparison to WT mice. In addition, pulse-chase experiment with the IdU and CldU for detection proliferating and label retaining cells (LRC) was performed.

Expression of the *Krt15* transcript was analyzed by RNA *in situ* hybridization technique. In WT skin, *Krt15* expression was detected in a group of cells in the HF bulge, known as a HF stem cells niche (**Fig. 3.5A**). Interestingly, in the TG skin *Krt15*-positive cells were also detected in cells of the HF outer root sheath below the bulge (**Fig. 3.5B** and **3.5C**, arrowheads).

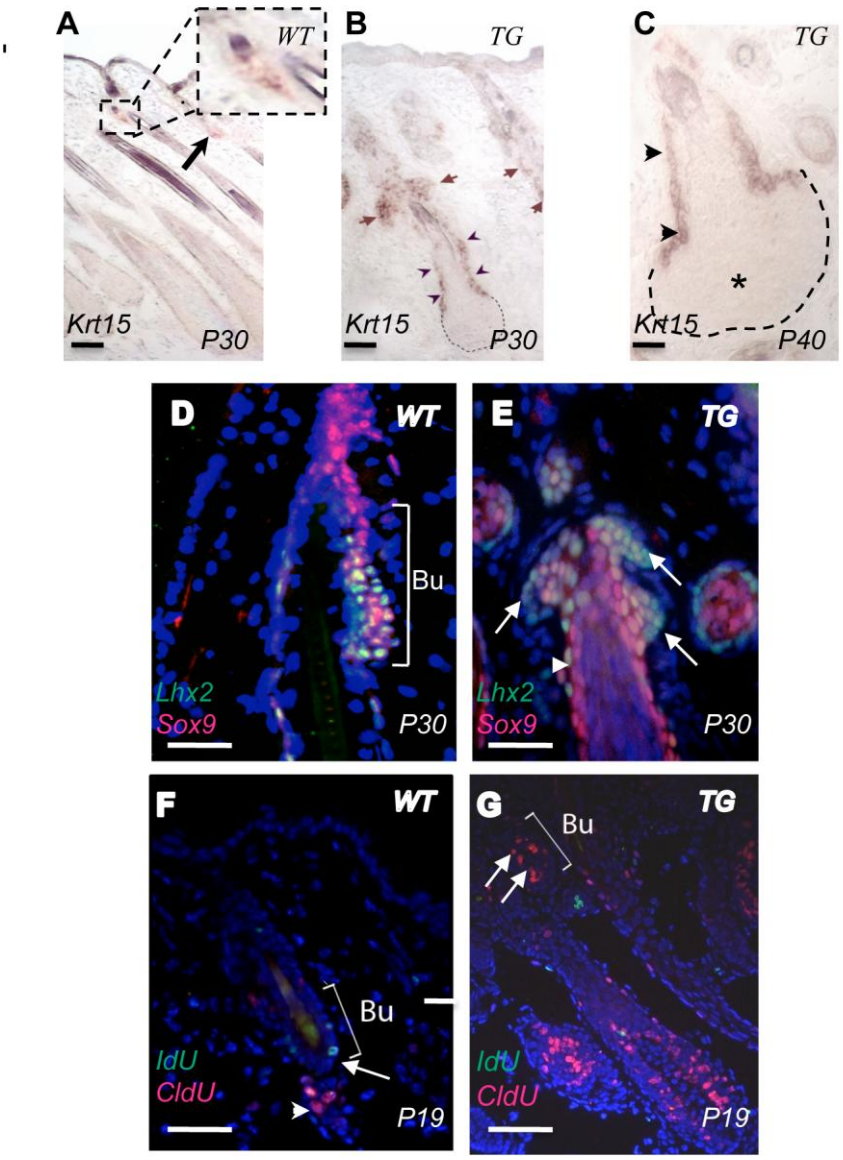

Fig. 3.5. Expression of stem cell markers in K14-Noggin skin

(**A, B, C**) in WT skin, K15 mRNA expression restricted to the bulge (BU) cells (A, insert, arrow), while in the TG skin (B, C) its expression is broader in the ORS (arrowheads) and tumour placodes (arrows); however K15 does not express in advanced tumours (C, asterisk); (**D**, **E**) Ectopic expression of Lhx2 and Sox9 in the ORS (arrowhead) and tumour placodes (arrows) in the TG (E) skin compared to WT (D); (**F**, **G**) Loss of LRC (green) and increased number of proliferating cells (red) in the TG HFs (G) compared to WT mice (F). Scale bars, 50 μm

Moreover, cells of the tumour placodes showed high expression of the *Krt15* (**Fig. 3.5B**, arrows), whereas in the more developed tumours its expression was strongly downregulated (**Fig. 3.5C**, asterisks).

Immunodetection of the DNA binding proteins Lhx2 and Sox9 also revealed nuclear expression of these transcription factors in the ORS cells beyond the bulge in the TG HFs, compared to WT mice (**Fig. 3.5D**).

Newly developing tumour placodes were positive for Lhx2 and Sox9 staining (**Fig. 3.5E**), while their expression in more advanced stages of the tumour development was markedly decreased (**Fig. 3.5 D**). However, these two antibodies predominantly detect different population of cells yet partially overlap in some cells. Sox9-positive cells were mainly detected in ORS and at the base of tumour placodes, while Lhx2-positive cells were located at the leading edge of growing tumour placodes (**Fig. 3.5D**).

However, in the chase experiment with IdU for detection LRC, TG mice showed almost no traces of the label in HFs even after 2 weeks of the chase (**Fig. 3.5F**, green staining), while 2 hours pulse labeling with CldU revealed active label-incorporation by the bulge cells at that time point (**Fig. 3.5F**, red staining, arrows), thus suggesting active proliferation of cells in the bulge area of the TG hair follicles. In contrast, some bulge cells in WT skin retained the IdU label after two weeks of the chase (**Fig. 3.5E**, arrows), and only few CldU-positive cells were detected in the secondary hair germ at P19 (**Fig. 3.5E**, arrowhead). Therefore, the Noggin-induced BMP inhibition leads to the loss of stem cells quiescence in the HF; instead, the cells located in the bulge area actively proliferate followed by their expansion beyond the stem cell niche.

Expression of the stem cell markers in tumour placodes suggests that the activated stem cells and/or their immediate progeny may contribute to the HF-derived tumour development.

3.1.6. Increase of epidermal thickness and cell proliferation in the interfollicular epidermis of K14-Noggin mice

To better characterize the epidermal phenotype of the K14-Noggin mice, a detailed morphological analysis of the interfollicular epidermis (IFE) was carried out. In contrast to WT mice, adult TG mice showed a significant increase in the thickness of the IFE (**Fig. 3.6A** and **3.6B**). A morphometric analysis revealed a two-fold increase in epidermal thickness in the TG skin compared to WT mice (32 ± 4 μm versus 15 ± 2 μm, $p<0.01$) (**Fig. 3.6C**). Moreover, changes in epidermal thickness are accompanied by increase in a number of layers of viable suprabasal keratinocytes. In WT skin, the IFE was composed of two-three nuclei containing cell layers (**Fig. 3.6A**); while in the TG epidermis, the number of such layers was increased up to six (**Fig. 3.6B**). Structurally, these layers in the TG skin were not distinguishable from the WT counterparts: basal keratinocytes had a cuboid shape versus more flatted shape of the suprabasal cells. Thus, the changes reflect hyperplastic processes in the IFE. However, some areas of mild and/or moderate dysplastic alterations were observed in the epidermal cells, mostly in the immediate proximity to the follicular epithelium (**Fig. 3.6B**, arrow). The dysplastic cells were characterized by changes their shape: they were seen as elongated, vertically oriented, and densely packed in both basal and suprabasal layers of the interfollicular epithelium (**Fig. 3.6B**, arrow).

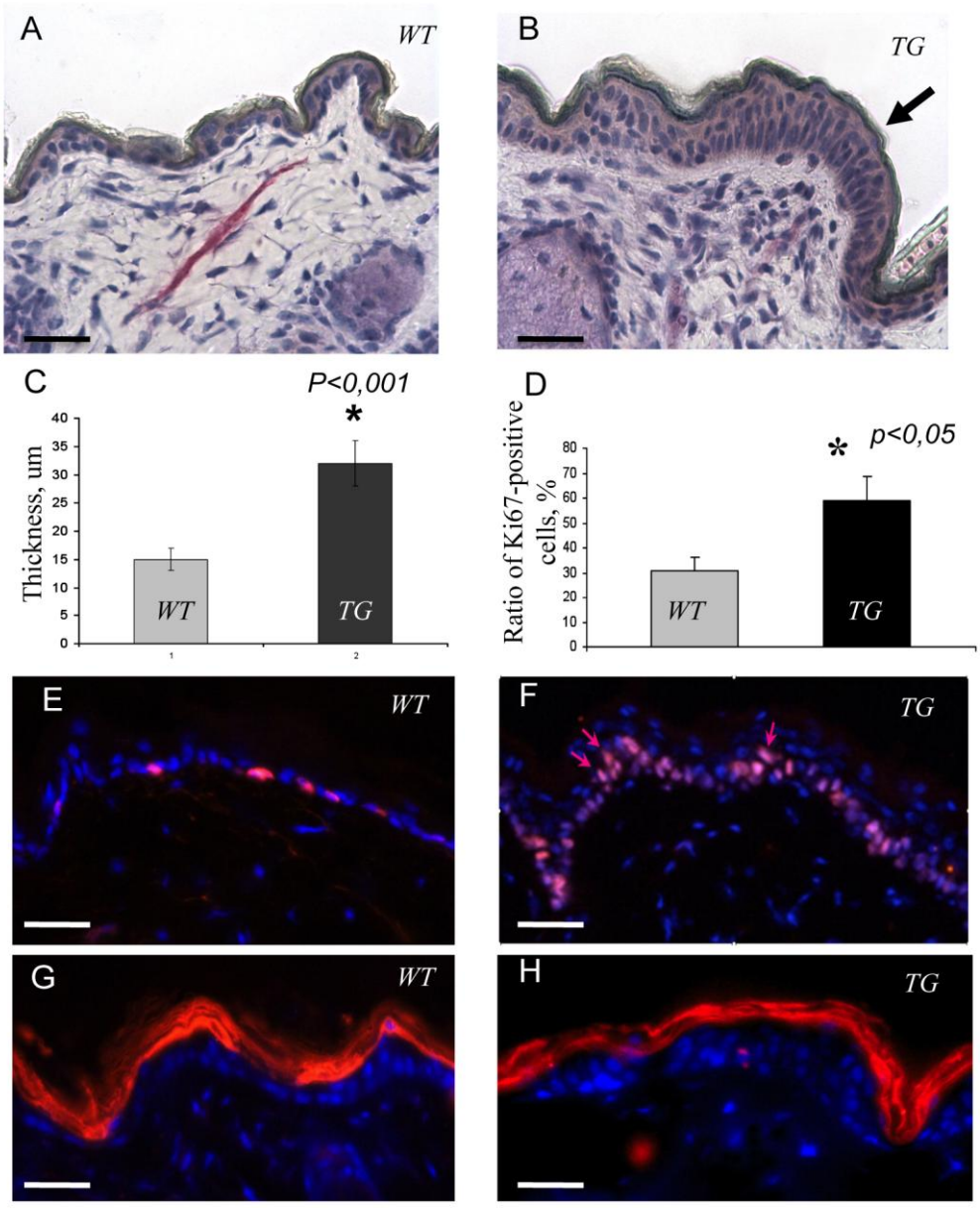

Fig. 3.6. Hyperplastic changes in the *K14-Noggin* epidermis

(A, B) H&AP staining shows increased thickness of the epidermis in TG skin (B) compared to WT (A) (arrow indicates an area of dysplasia); **(C)** Epidermal thickness in TG and WT skin; (**D)** Number of Ki67-positive cells in the basal layer of WT and TG epidermis; **(E, F)** Ki67 expression in the epidermis. Note increased Ki67-positive cells in TG skin (F), compared to WT mice (E) (arrows indicate Ki67-positive suprabasal cells); **(G, H)** Loricrin expression in WT (G) and TG (H) epidermis. Scale bars, 50 μm

To find out, whether the epidermal hyperplasia in the K14-Noggin skin was a result of active cell proliferation and/or altered differentiation, expression of the markers of proliferation and differentiation was analyzed.

Immunofluorescent detection of the Ki67-antigen (Ki67), which accumulates in actively proliferating cells, revealed a marked increase in the number of Ki67-positive cells in K14-Noggin epidermis versus WT mice (**Fig. 3.6D** and **3.6F**). Quantitative analysis showed at least a two-fold increase in a number of Ki67-positive cells in the basal layer of TG skin compared to WT mice (**Fig. 3.6D**).

Moreover, Ki67 expression was also seen in some suprabasal keratinocytes in the TG epidermis (**Fig. 3.6F**, arrows), while proliferating cells were not detected in the suprabasal layers of the WT counterparts (**Fig. 3.6E**). In TG epidermis, expression of loricrin, a marker of terminally differentiated keratinocytes, was detected in the outermost layers of the epidermis (**Fig. 3.6G**). Similar pattern of the loricrin expression was observed in WT skin (**Fig. 3.6H**).

Therefore, the process of keratinocytes differentiation was not affected by overexpression of *Noggin* in the adult TG skin. Instead, Noggin-induced BMP suppression resulted in the stimulation of cell proliferation in the IFE.

3.1.7. K14-Noggin mice are more susceptible to chemically-induced carcinogenesis

K14-Noggin mice showed a marked increase in cell proliferation in the interfollicular epidermis. However, no tumours arising from the interfollicular epidermis were detected within one and more years of observation.

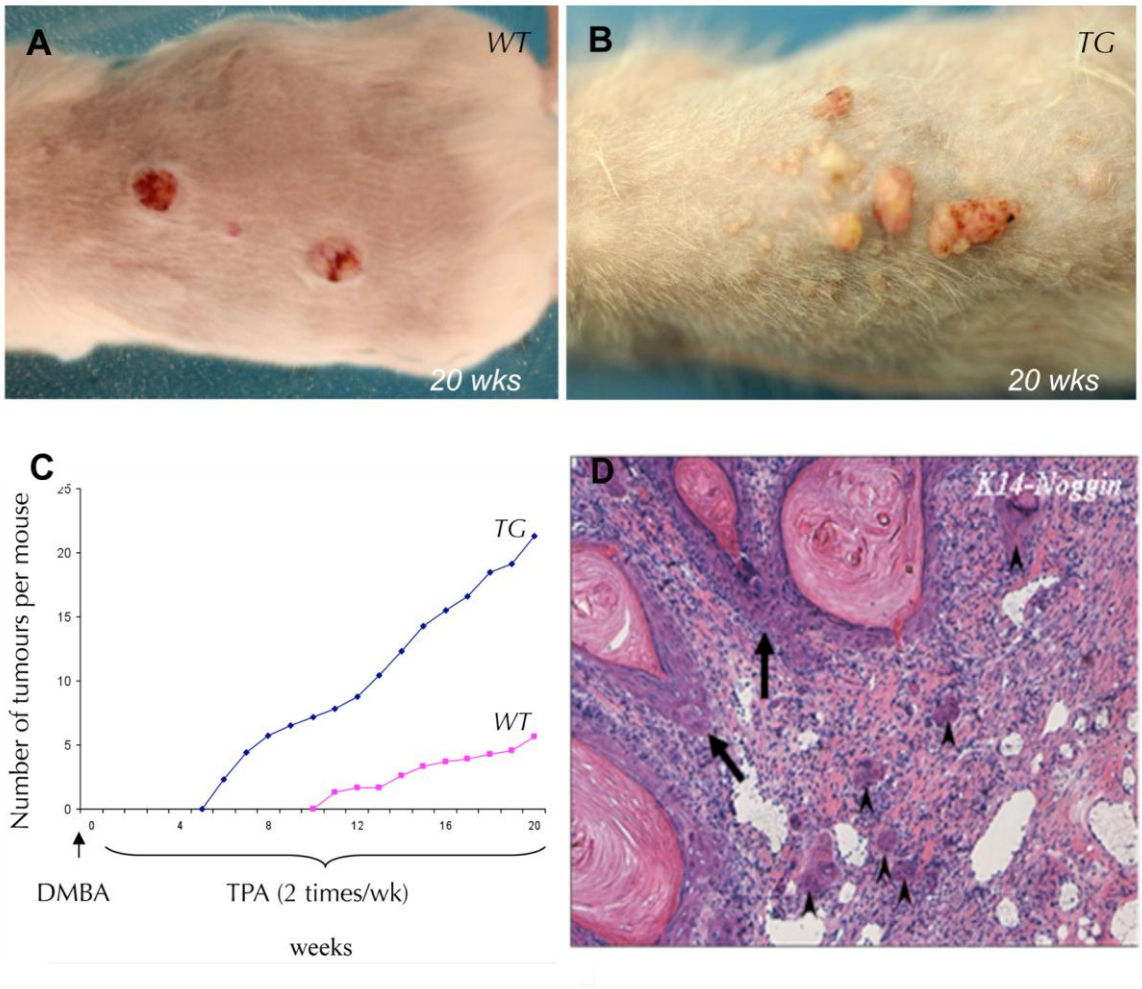

Fig. 3.7. Chemically induced tumour development in *K14-Noggin* skin

(**A, B**) Papilloma-like tumour development in WT (A) and TG (B) skin; (**C**) The tumours emerged much earlier and a greater amount in the TG skin compared to WT mice; (**D**) Squamous cell carcinoma (arrows) in K14-Noggin mice after application of the DMBA/TPA protocol [note numerous foci of invasion into the dermis (arrowheads)].

To test whether Noggin overexpression in the epidermis affects development of the chemically-induced skin tumour, the skin of WT and TG mice was treated with chemical carcinogen DMBA.

Single dose of DMBA application followed by weekly TPA treatment during 20 weeks resulted in a papilloma-like tumour development in both WT and TG skin (**Fig. 3.7A** and **3.7B**). However, in TG skin the first tumours emerged as early as 6 weeks after DMBA treatment (**Fig. 3.7C**). In contrast, WT mice developed papilloma-like tumours much later by 11 weeks of the treatment (**Fig. 3.7C**).

Moreover, weekly monitoring of mice revealed a significant difference in the frequency of the tumours between the TG and WT mice: over 5-fold increase in a total number of skin tumours in TG compared to WT mice by the end of the experiment (**Fig. 3.7C**).

Histological examination of skin tumours in WT mice did not show any signs of malignancy. In contrast, atypical cells were detected in the tumours of TG mice with squamous cell carcinoma-like metastases in the dermis (**Fig. 3.7D**).

Thus, inhibition of the BMP signalling in the skin greatly enhances the susceptibility to chemical carcinogenesis and promotes malignant transformation of benign tumours into SCCs.

3.2. Global gene expression profiling of the trichofolliculoma-like tumours in K14-Noggin mice

3.2.1. Microarray analysis of gene expression in the HF-derived tumours.

To find out the possible molecular mechanisms leading to tumour development upon inhibition of the BMP signalling in the skin, whole genome microarray analysis was employed.

Epithelial cells of the hair matrix of anagen hair follicles and cells of follicular tumours were isolated by laser capture microdissection (LCM) (**Fig. 3.8**) followed by RNA extraction. The RNA samples were further processed for microarray profiling (Agilent platform) and qRT-PCR.

Analysis of the microarray data showed two-fold or higher changes in expression of 390 genes encoding the adhesion/extracellular matrix molecules, cell cycle/apoptosis and cytoskeleton/cell motility markers, molecules involved in cell differentiation, metabolism, signalling and transcription (**Fig. 3.9, Appendix A, B**).

Expression of several genes (*Msx1*, *Msx2*, *Id1*, *Id2*, *Id4*) that are known as direct targets of the BMP signalling was down-regulated in the follicular tumours **(Appendix B**), suggesting that the microarray profiling adequately represents transcriptional changes upon Noggin-induced BMP inhibition.

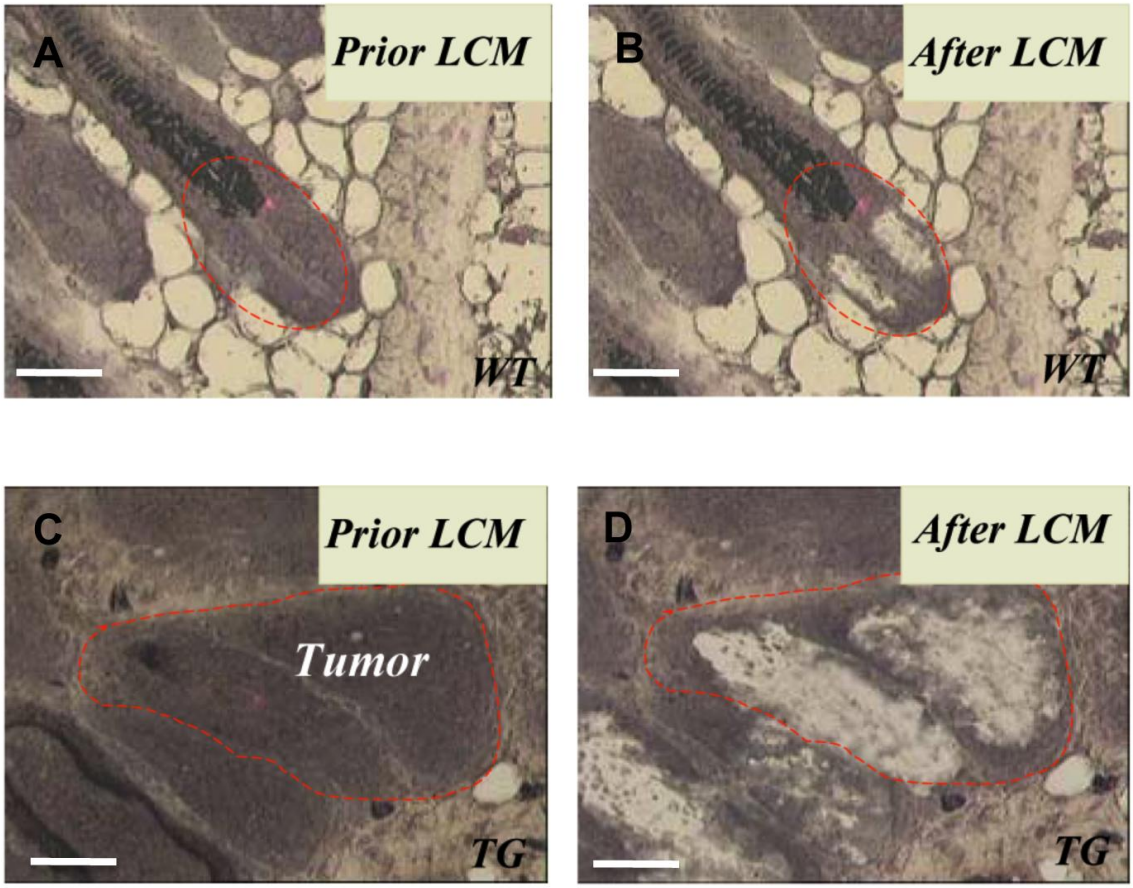

Fig. 3.8. Laser-capture microdissection (LCM) of normal hair matrix and HF-derived tumours

(**A**, **B)** HF matrix cells before (A) and after (B) LCM; (**C**,**D**) HF-derived tumours before (C) and after (D) LCM. Scale bars, 50 um

The analysis of the microarray data revealed a misbalanced expression of a large group of Homeobox (Hox) genes, which are transcription factors playing a crucial role during development and malignant transformation in many organs including epithelial tissues (Nunes, 2003).

There was an over four-fold increase in expression of *Hoxa9* and *Hoxd11*, while expression of *Hoxa7*, *Hoxb7*, *Hoxc13*, and *Hoxd4* was markedly decreased in the HF-derived tumours (**Appendix B**). This suggests that some Hox genes may serve as targets for BMP signalling. The possible mechanism of the BMP-dependant regulation of specific Hox genes expression is of particular interest to understand the complex function of the BMP signalling and how they relate to growth, development, and pathogenesis.

In contrast to WT mice, HF-derived tumours also showed a marked increase in expression of genes encoding selected components of signalling pathways implicated in cutaneous tumourigenesis: Wnt (*Wnt5a, Wnt6, Wnt9b, Wnt10b, Fzd2, Fzd7*), Hedgehog (*Shh, Dhh, Smo*), Pdgf (*PdgfA, PdgfB*), Ras, etc.

Substantial changes were observed in the expression of the genes involved in the regulation of cell proliferation. Expression of genes that are involved in inhibition of cell proliferation (*Igfbp4*, *Bmp8a*, *Id2*, *Id4*) was decreased in the tumour cells (**Appendix B**). Furthermore, expression levels of cyclin D2, cyclin M3 and cyclin F were three-fold higher in the tumours compared to the hair matrix of WT mice. Expression of several antiapoptotic markers (*Bcl6*, *Faim2*, *Bag3*) was also increased, while an apoptotic gene encoding effecter caspase-7 was down-regulated in the tumours compared to WT HFs (**Appendix B**).

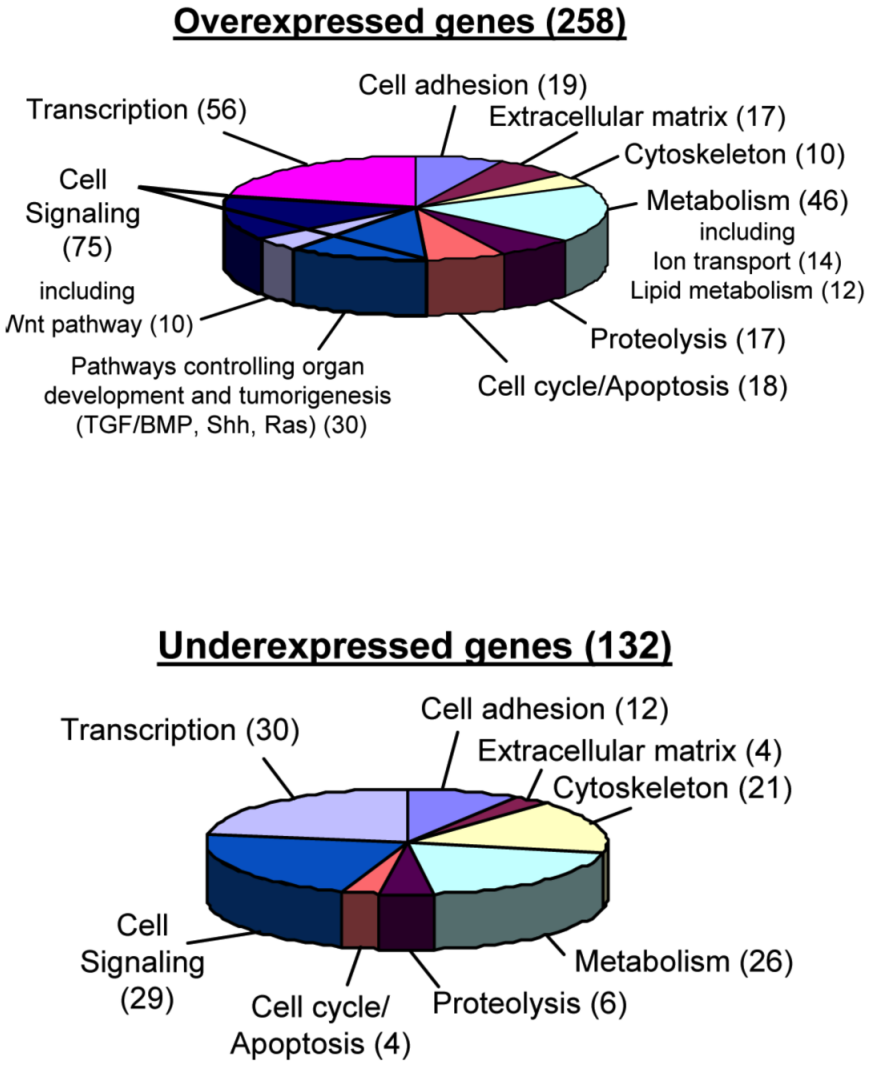

Fig.3.9. Functional annotation of the over- and under-represented genes in the tumours of K14-Noggin mice

Analysis of the microarray data showed two-fold or higher changes in expression of 390 genes encoding the adhesion/extracellular matrix molecules, cell cycle/apoptosis and cytoskeleton/cell motility markers, molecules involved in cell differentiation, metabolism, signalling and transcription

In addition, expression of the selected HF-specific keratins, keratin-associated proteins and trichohyalin decreased, suggesting alterations in the cell differentiation in the skin with Noggin overexpression (**Appendix B**).

Interestingly, expression of *Lhx2* and *Sox9* encoding transcription factors, markers of the HF stem cells/progenitors, was three-fold up-regulated in the tumours (**Appendix A**), confirming the result of immunostaining for these proteins (**Fig. 3.5D and 3.5E**).

Furthermore, Noggin overexpression resulted in a up-regulation of genes encoding several pro-oncogenes (*ErbB2, c-Jun,* and *Ski*), while expression of *Hic2* and *Wfdc1*, which were considered as putative tumour suppressors and downregulated in several tumours, was decreased (**Appendix A**). In addition, a marked increase in expression of *Trp73*, a member of p53 family of tumour suppressors, was revealed by microarray analysis (**Appendix A**).

There are two major p73 isoforms, which possess opposite effects (Deyoung and Ellisen, 2007). However, the microarray data did not allow finding the difference between the isoforms, which expression was altered upon BMP inhibition.

Taken together, the results of microarray analysis suggest that Noggin-induced BMP inhibition leads to an alteration of transcription of numerous groups of genes. However, potential roles for a large number of genes that show differences in expression between hair matrix of WT mice and tumours in K14-Noggin mice remain to be further defined in terms of their contribution to the development of HF neoplasia in noggin transgenics. Some of the changes in gene expression can lead to predisposition and/or promote tumour

development, at least in part, through enhancing cell proliferation and/or impeding differentiation.

3.2.2. Validation of the microarray data by qRT-PCR.

To validate the data of microarray analysis, the RNA samples isolated by LCM were further processed for real-time PCR. Several group of genes which expression was altered in the HF-derived tumours were selected for the analysis.

Real-time PCR analysis confirmed the increased expression of genes encoding selected regulators of cell cycle machinaryv: *Bcl6* (4-fold), *Ccnd2* (3-fold), *Ccnm3* (3-fold), *Ccnf* (2-fold) (**Fig. 3.10**). In contrast, expression of the apoptotic gene Casp7 was two-fold decreased in the TG tumours versus hair matrix of WT mice (**Fig. 3.10**).

qRT-PCR also revealed misbalanced expression of several keratins in the tumour cells. Interestingly, a number of keratins that are normally expressed in differentiated hair matrix keratinocytes (*Krt1-c29, Krt2-20, Krt1-1*) were strongly downregulated, while expression of *Krt1-16, Krt2-5*, and *Krt1-17*, markers of undifferentiated keratinocytes of the epidermis and HFs, was markedly increased (**Fig. 3.10**). Furthermore, the results obtained by real-time PCR were consistent with microarray analysis in terms of changes in expression of the selected components of distinct signal transduction pathway.

In the tumour cells, expression of several Wnt ligands (*Wnt5a, Wnt10b*) was increased, while *Lef1* and Wnt antagonist *Wif1* were downregulated (**Fig. 3.10**).

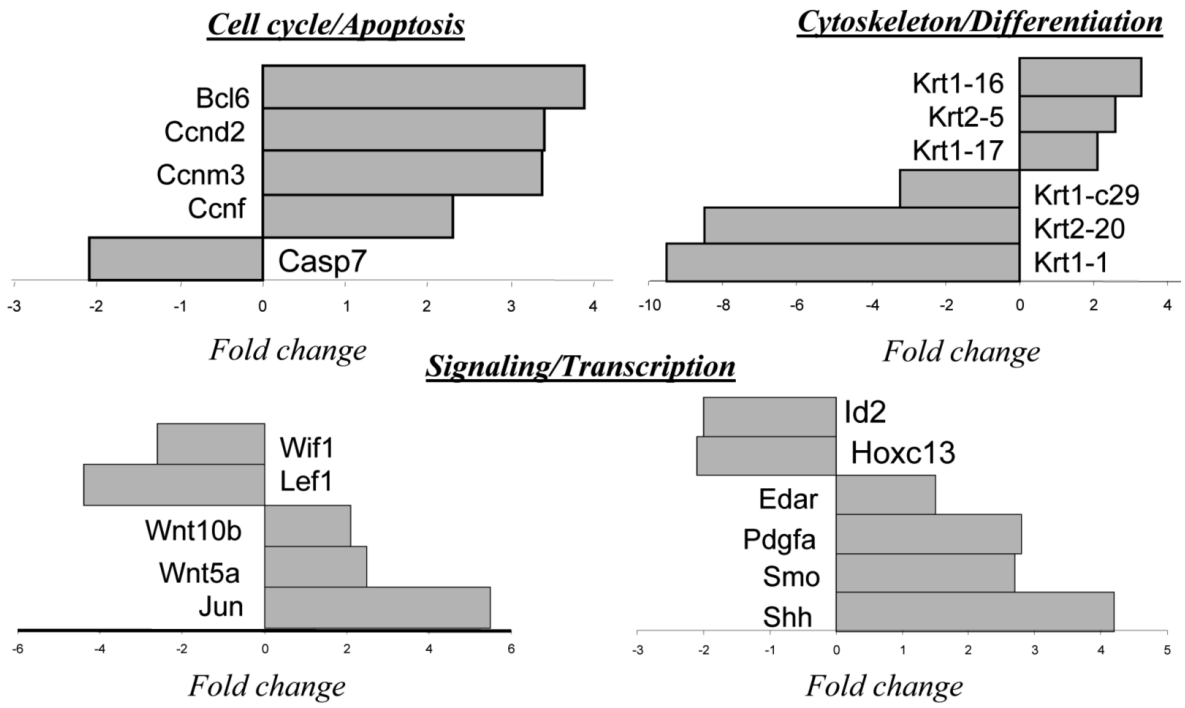

Fig. 3.10. qRT-PCR analysis of the selected genes in normal and neoplastic keratinocytes

qRT-PCR shows that development of the HF-derived is associated with activation of several pro-oncogenic pathways (Wnt, Hh, Pdgf), increase in expression of selected cell cycle activators and anti-apoptotic markers, while the programmeme of keratinocyte differentiation is changed toward a less differentiated state.

Some components of the Hh (*Shh, Smo*) and Eda signalling pathways (*Edar*) were also notably over-expressed in the tumour cells, as determined by real-time PCR (**Fig. 3.10**). Similar to microarray data, expression of *Pdgfa*, *Pdgfb*, and *Jun* was increased, while *Id2* and *Hoxc13* were decreased in the tumour cells compared to normal matrix keratinocytes (**Fig. 3.10**).

Thus, the results of real-time PCR analysis well correlate with the data obtained by microarray-based quantification. These data confirm that development of the HF-derived tumours upon Noggin-induced BMP inhibition is associated with activation of several pro-oncogenic pathways (Wnt, Hh, Pdgf), increase in expression of selected cell cycle-associated genes and anti-apoptotic markers, while the programmeme of keratinocyte differentiation showed alterations towards a less differentiated state. These results suggest that local concentration or activity of Noggin can significantly compromise tumour-suppressing activity of the BMP signalling in keratinocytes.

3. 3. Wnt and Shh signalling pathways in Noggin-induced tumourigenesis.

Microarray-based gene expression profiling revealed accelerated expression levels of several components of the Wnt and Shh signalling pathways (**Fig. 3.10, Appendix A**) in the K14-Noggin tumours. Both Wnt and Shh pathways are essential for HF development and postnatal remodeling (Fuchs, 2007), and, if abnormally activated, result in the development of a number of epithelial tumours, including SCC and BCC (Gat *et al.*, 1998; Grachtchouk *et al.*, 2000; Hutchin *et al.*, 2005; Niemann *et al.*, 2003a; Oro *et al.*, 1997; Yang *et al.*, 2008).

These data suggest that Wnt and Shh signalling pathways may contribute to the tumour development in K14-Noggin mice. This raised an important question about mechanisms underlying a crosstalk between the BMP, Wnt, and Shh pathways during tumour development in the skin epithelium.

3.3.1. Expression of key components of the Wnt signalling in Noggin-induced tumours.

To explore the role for the Wnt and Shh signalling pathways in a control of tumour development in the TG mice, the expression of key Wnt and Shh signalling components and selected targets was compared between WT HFs and TG tumours at distinct stages of their formation.

As it was described above (see Chapter 3.1.3), the tumour development begins with formation of epithelial placodes emerging from the hair follicle ORS. Immunostaining against Wnt10b, Lef1 and β-catenin revealed strong expression of these proteins in the tumour placodes, while their expression was markedly reduced in the fully developed tumours (**Fig.3.11A-C**), indicating that the active Wnt signalling was associated with the initiation of the tumours. Interestingly, the tumour placode cells, as it was shown above, were also enriched for the Keratin 15 mRNA and Lhx2 and Sox9 proteins, markers of the epithelial bulge stem cells (**Fig. 3.5 and 3.11C)**. In WT bulge stem cells, the Wnt signalling is active at the early stage of anagen, when the stem cells actively proliferate to give rise to newly developing HFs (Van Mater *et al.*, 2003). Thus, the mechanism of tumour initiation is likely similar to HF regeneration and involves the active Wnt signalling in controlling this process.

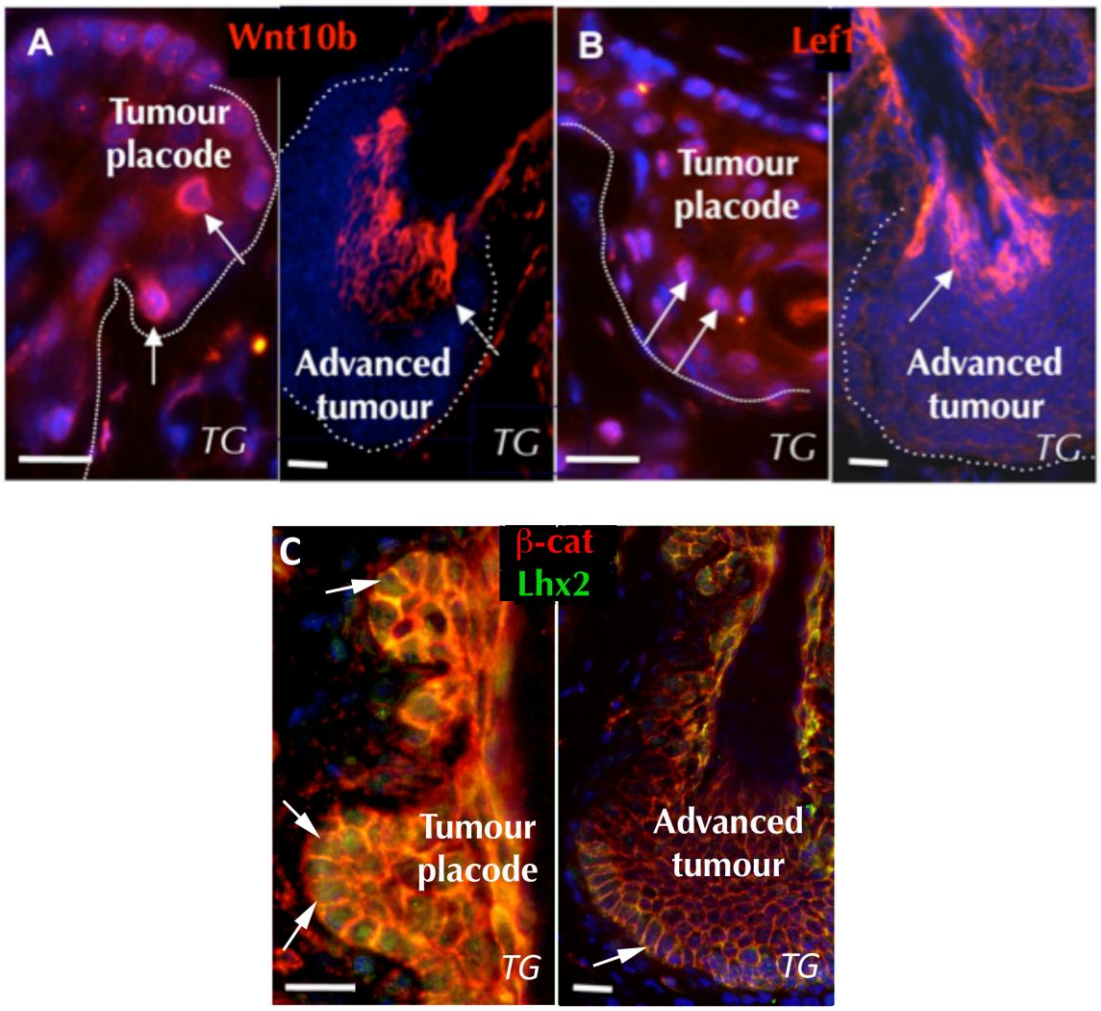

Fig. 3.11. Expression of selected components of Wnt signalling at distinct stages of the tumour development in K14-Noggin skin

(**A**) Wnt10b expresses in the tumour placode cells but not in advanced tumours (arrows indicate Wnt10b-positive cells); (**B**) Strong nuclear staining for Lef1 in tumour placodes compared to advanced tumours (arrows indicate lef1-positivecells); (**C**) Nuclear β-catenin and Lhx2 staining (arrows) in tumour placodes. Scale bars, 25 um

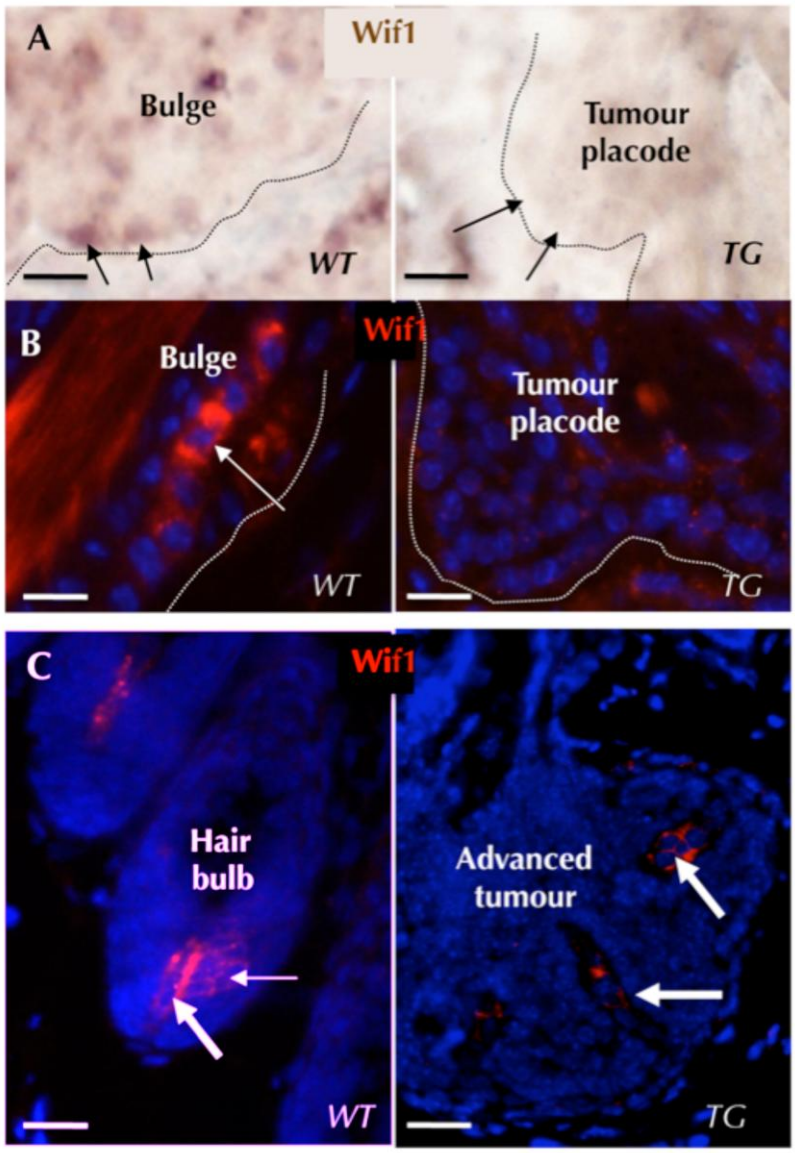

Fig. 3.12. Expression of Wif1 at distinct stages of the tumour development in K14-Noggin skin

(**A, B**) Wif1 mRNA (A) and protein (B) expression is down-regulated in the tumour placodes compared to anagen bulge region in WT skin (arrows indicate Wif1-positive cells); (**C**) In advanced tumours, Wif1 is expressed in the tumour stroma only, compared to the dermal papilla (big arrow) and a small cluster of matrix keratinocytes (small arrow) in WT anagen hair bulb. Scale bars, 25 um (A, B) and 50 um (C)

On another hand, the expression of the Wnt antagonist Wif1 was not observed in the cells of tumour placodes and fully developed tumours (**Fig 3.12A and 3.12B**). In contrast, Wif1 was actively expressed in the WT bulge and dermal papilla cells during the late anagen and telogen (**Fig 3.12A-C**), suggesting its involvement into the control of normal hair follicle stem cells and/or early progenitors activity. Down-regulation of the Wif1 in the TG skin suggests that Wif1 may serve as a BMP target mediating the crosstalk between the BMP and Wnt pathways in the skin.

3.3.2. Expression of key components of the Shh pathway in K14-Noggin tumours.

In contrast to the components of Wnt signalling pathway, transcripts for *Shh* were not detected in the tumour placodes, while *Ptch1*, *Ptch2*, *Gli1* and *Gli2* mRNAs showed expression levels similar to the fully developed tumours (**Fig. 3.13A** and **3.13B**). However, expression of the *Shh* markedly increased in the fully developed tumours compared to the tumour placodes (**Fig. 3.13B**) and to the hair matrix of normal anagen hair follicles (not shown). Consistently with these data, transcripts for Cyclin D1 and Cyclin D2, serving as the Wnt and Shh targets (Katoh, 2007), were seen in the tumour placodes and their expressions increased in fully developed tumours (**Fig. 3.13C** and **3.13D**)

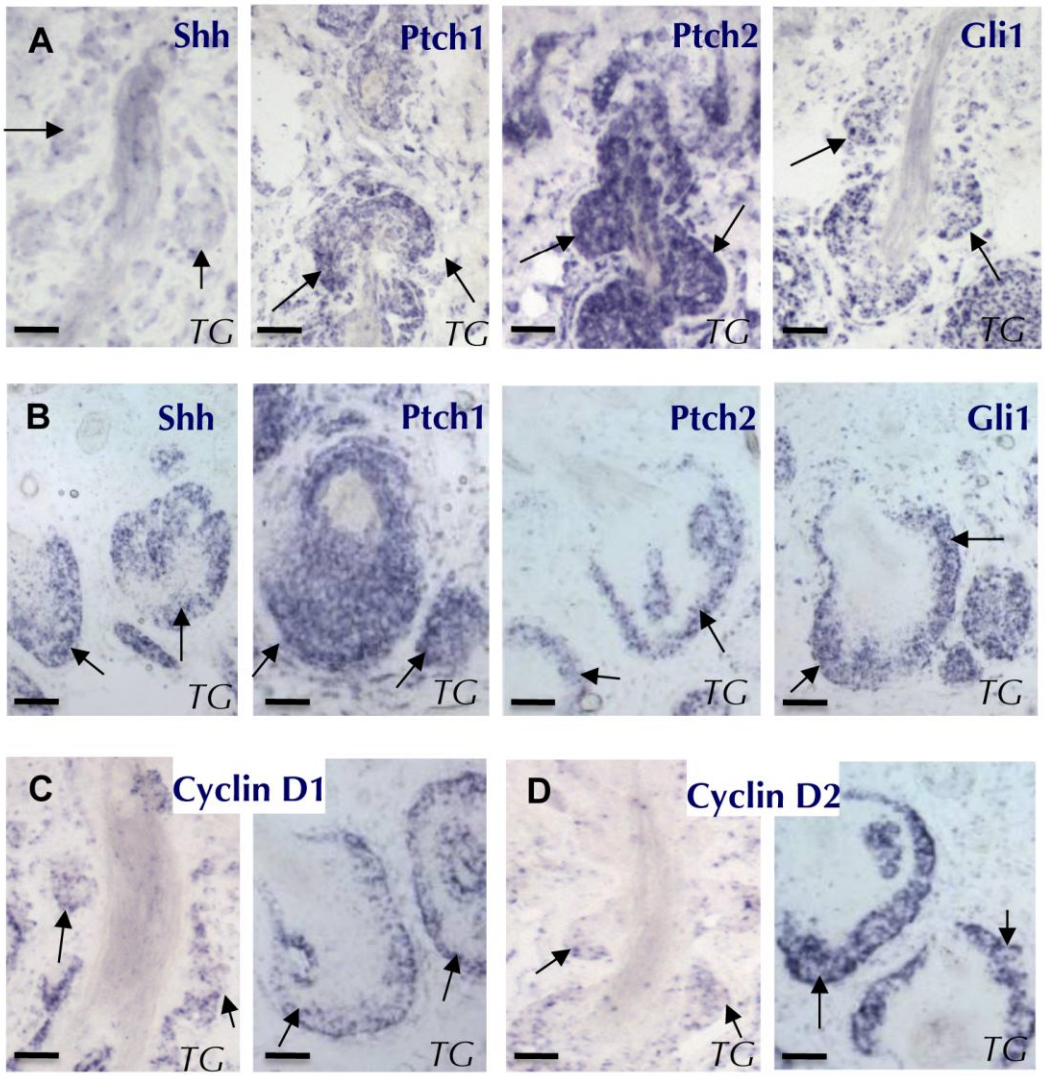

Fig. 3.13. Expression of selected genes of Shh pathway at distinct stages of the *K14-Noggin* tumourigenesis

(**A**, **B**) *Shh*, *Patch1/2* and Gli1*/2* transcript expression in tumour placodes (A) and advanced tumours (B); (**C**) Cyclin D1 mRNA expression in the tumour placodes (left) and advanced tumours (right). (**D**) Cyclin D2 mRNA expression in the tumour placodes (left) and advanced tumours (right). Arrows indicate sites of the expression. Scale bars, 50 um

3.3.3. Treatment of K14-Noggin mice with the Wnt and Shh antagonists

To better define the requirement for Wnt and Shh signalling for the development of hair follicle-derived tumours, in vivo pharmacological studies were performed using the corresponding Wnt and Shh inhibitors Aptosyn and Cyclopamine [reviewed in (Athar, 2006; Li *et al.*, 2002)].

To define whether inhibition of the Wnt and Shh signalling could affect the initiation and progression of the tumours, TG mice were treated either with Aptosyn or Cyclopamine daily s/c from postnatal day 10 (P10) and skin was harvested on P19, P21 and P28 (9, 11 and 18 days after beginning of the treatment, respectively).

In contrast to the vehicle-treated TG mice, mice treated with the Wnt antagonist Aptosyn showed significant ($p<0.05$) decrease in the number of HFs with new outgrowths arising from the outer root sheath (**Fig. 3.14A**-C). This accompanied by the decrease of nuclear beta-catenin staining in the HF keratinocytes, thus suggesting inhibition of the tumour initiation (**Fig. 3.14D**).

However, treatment of TG mice with Shh inhibitor Cyclopamine did not show any effects on tumour initiation and the formation of new outgrowths from the outer root sheath (data not shown).

Instead, Cyclopamine treatment resulted in significant ($p<0.001$) retardation of tumour progression in the TG mice compared to controls (**Fig. 3.15A-C**).

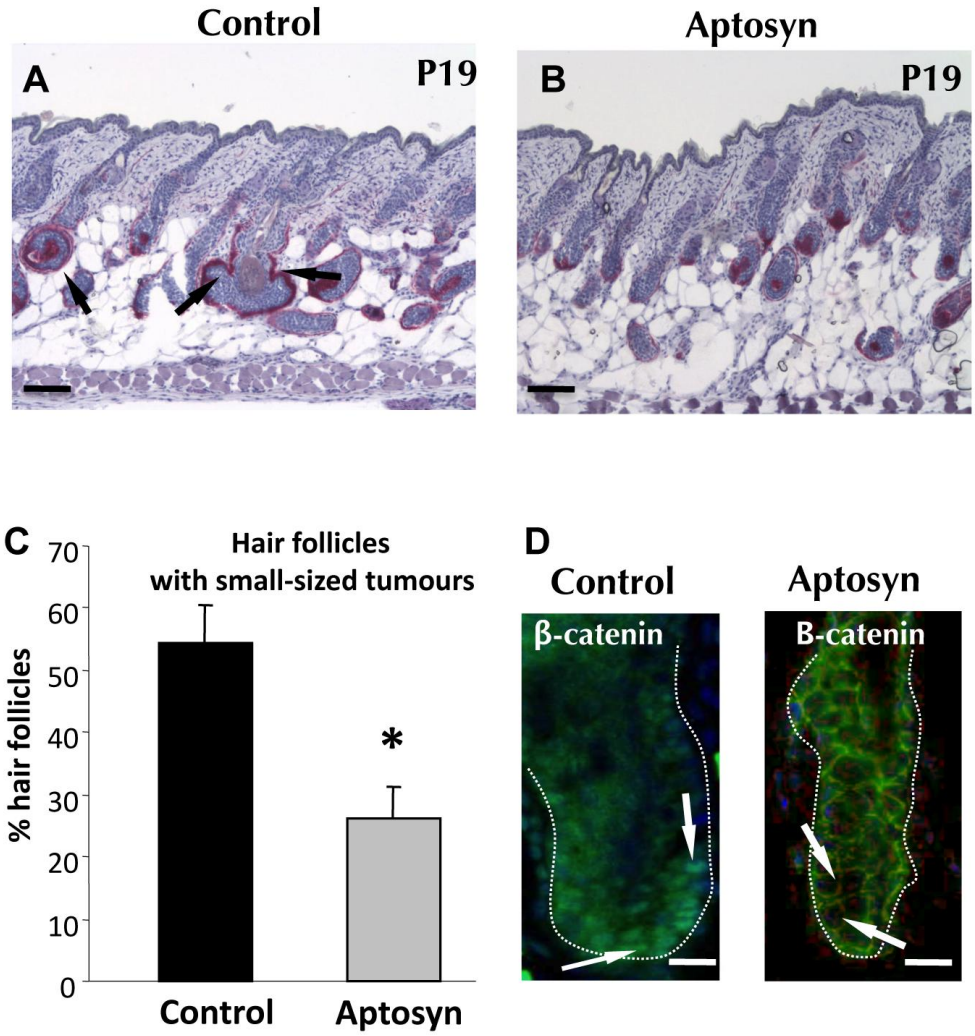

Fig. 3.14. Effect of the Wnt inhibitor Aptosyn on tumour development in K14-Noggin mice

(**A, B**) AP staining of skin section after control (A) and Aptosyn (B) treatment (arrows indicate tumour outgrowths); (**C**) Percentage of the HFs with tumours in the control and Aptosyn-treated groups ($p<0.05$); (**D**) β-catenin immunofluorescence in the HFs of the control and Aptosyn-treated skin (note decreased nuclear staining for β-catenin in Aptosyn treated HFs). Scale bars, 100 um (A, B) and 25 um (D)

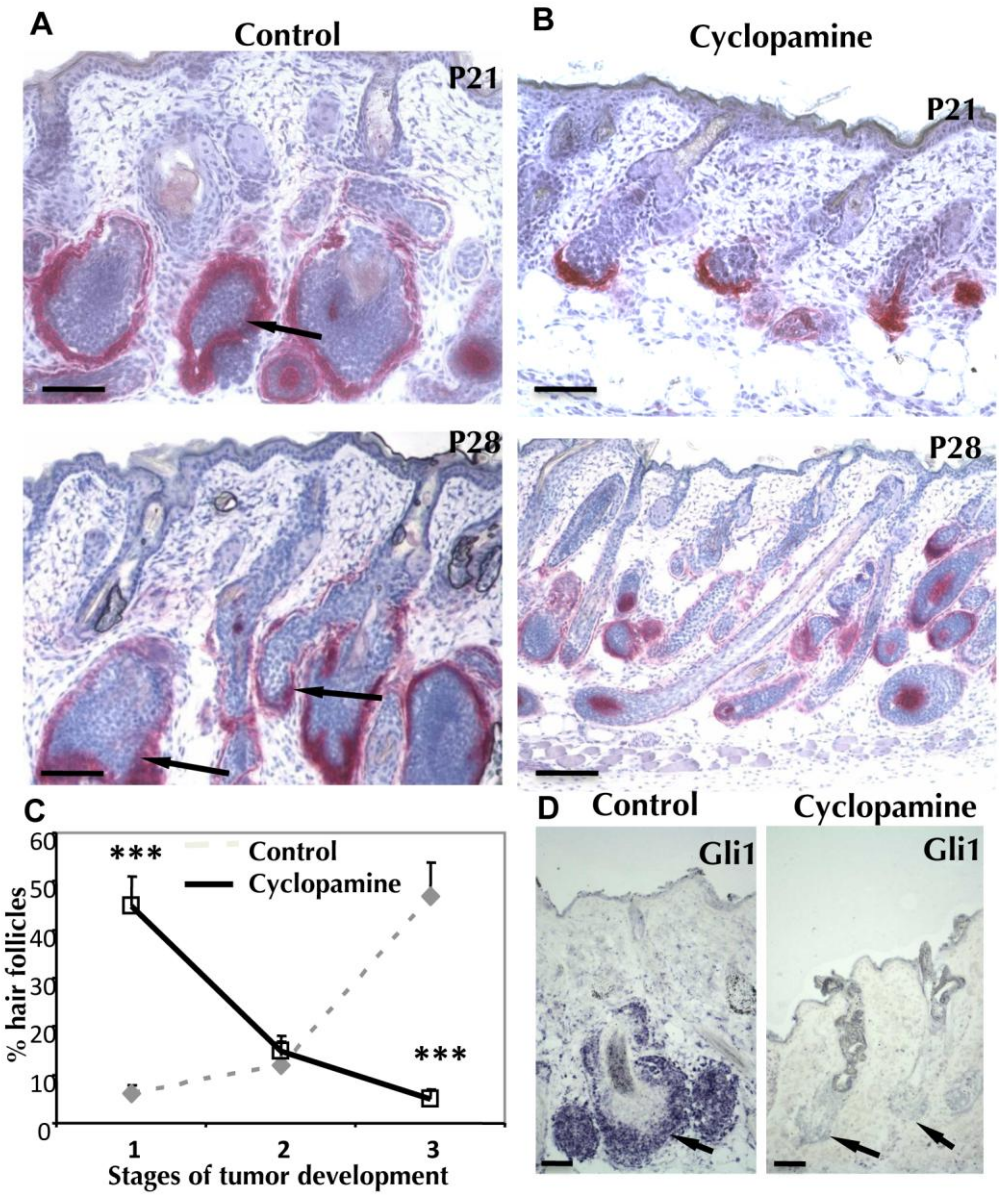

Fig. 3.15. Effect of Shh inhibitor Cyclopamine on the tumour development in K14-Noggin mice

(**A, B**) AP staining of sections at P21 and P28 after control (A) and Cyclopamin (B) treatments (arrows indicate tumour outgrowths); (**C**) Significant decrease in a number of HFs bearing large and medium-sized tumours in TG mice treated with Cyclopamine versus control; (**D**) Decreased Gli1 expression in the HF keratinocytes after Cyclopamine treatment (arrows). Scale bar, 50 um

Inhibition of tumour growth after Cyclopamine treatment was accompanied by decrease in expression of the Gli1 in the HF epithelium, as well as by down-regulation of the alkaline phosphatase activity in adjacent mesenchyme indicating that Cyclopamine may also affect epithelial-mesenchymal interactions in developing tumours (**Fig. 3.15C**). These data suggested that Wnt and Shh signalling are differentially involved in the control of tumour initiation and progression, respectively, and may serve as targets for the BMP regulation in the HF keratinocytes.

3.3.4. Effects of BMP4 on Wif1 and Shh expression *in vitro*

To further understand the mechanisms underlying the cross-talk between the BMP, Wnt and Shh signalling pathways in development of the HF-derived tumours, tumour cells were isolated from the TG mice and cultured in presence of different concentrations (200 ng/ml and 500 ng/ml) of recombinant BMP-4 protein. By real-time PCR, a marked increase of the *Wif1* and decrease of the *Shh* transcripts were detected in the tumour cells after 24-hour treatment with 500 ng/ml BMP-4 (**Fig. 3.16A**).

Similarly, Western blotting revealed a dose-dependent increase in the expression of the Wif1 protein, while Shh protein was markedly decreased in the BMP4 treated cells (**Fig. 3.16B**). Thus, BMP signalling pathway is indeed involved in the control of Wif1 and Shh expression in the tumour cells cultured *in vitro*.

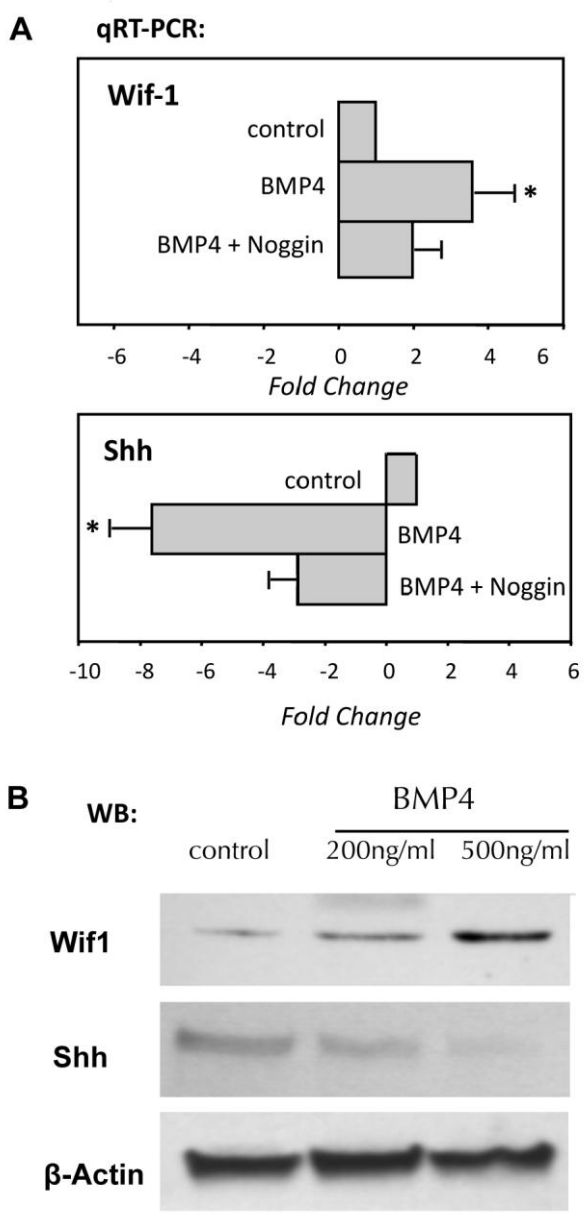

Fig. 3.16. Wif1 and Shh expression in tumour cells after BMP4 treatment *in vitro*

(**A**) Wif1 and Shh mRNA expression in the tumour cells isolated from K14-Noggin mice after BMP4 treatment ($p<0.05$); (**B**) Western blot of the Wif1 and Shh proteins after BMP4 treatment.

3.3.5. Regulation of Wifi and Shh promoter activity by BMP pathway

To assess whether BMP signalling is capable of influencing the activities of the Wif1 and Shh promoters, HaCaT keratinocytes were transfected with the corresponding reporter plasmids containing human *Wif1* or *Shh* promoters (Kitazawa *et al.*, 1998; Reguart *et al.*, 2004) or with pGVB2L-Luc and p3GC2-Luc as negative and positive control vectors respectively. The cells were also co-transfected with vectors containing one of the constitutively active BMP receptors (Alk3QD/Alk6QD) and/or pCMVSmad1/Smad5 vectors (Ishida *et al.*, 2000). Cell transfection with the Alk3QD and pCMVSmad1 vectors resulted in about 2.5-fold increase of Wif1-Luc activity versus control (**Fig. 3.17**). However, lack of any effects was seen on the Shh-Luc activity (**Fig. 3.17**).

These data suggest that BMP signalling pathway positively regulates activity of the *Wif1* promoter and may indeed suppresses tumour initiation *in vivo* via stimulating the Wif1 expression and antagonizing the Wnt signalling pathway. These data also suggest that BMP signalling does not regulate Shh expression directly.

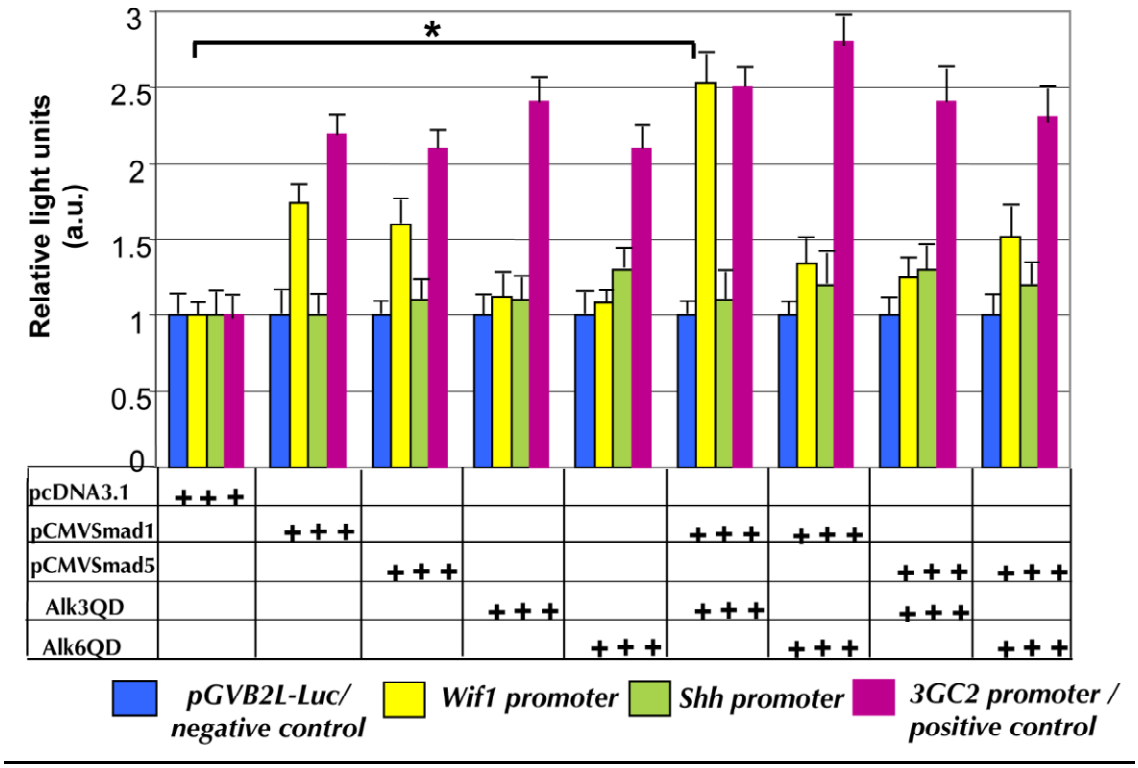

Fig. 3.17. Luciferase reporter assay with Wif1 and Shh promoter regions

Co-transfections with vectors containing constitutively active BMPR-IA (Alk3QD), BMPR-IB (Alk6QD), pCMVmSmad1 and/or pCMVSmad5 increase luciferase activity of the Wif1-Luc but Shh-Luc reporter plasmids containing human *Wif1 and Shh* promoter regions respectively; *p<0.05*

3.3.6. Chromatin immunoprecipitation (ChIP) of *Wif1* promoter region

In order to show that BMP-dependent Smad transcription factors bind to native chromatin within the *Wif1* promoter region to regulate its transcription, ChIP assay was performed.

Cross-linked chromatin was isolated from primary mouse keratinocytes after 3 hours of treatment with recombinant mouse BMP4/7 and, after sonication, immunoprecipitated with phospho-Smad1/5/8 antibodies. ChIPed DNA was analyzed by PCR with primers to different region within 2.5Kb of 5'UTR of *Wif1*.

PCR analysis showed an enrichment of a -326/+37 bp fragment of the *Wif1* 5'UTR with the pSmad1/5/8 antibodies, compared to non-immune IgG (**Fig. 3.18A**). Thus, these data demonstrate that BMP-dependent Smad transcription factors can be recruited to the *Wif1* promoter region *in vivo*.

Comparison of promoter sequences among different species using the whole genome mVISTA tool showed highly conserved regions between mouse and human within the -326/+37 fragment of the *Wif1* promoter (**Fig. 3.18B**).

Interestingly, several BMP-responsive elements (BREs) regulated by BMP-dependent Smad transcription factors were found within the analyzed promoter region of *Wif1*. The -326/+37 bp fragment of the *Wif1* promoter contains 7 conserved BREs that consist of GCCG and CGCC motifs (**Fig. 3.18B**). This further supports the idea that this region in the *Wif1* promoter is likely regulated by the BMP/Smad pathway.

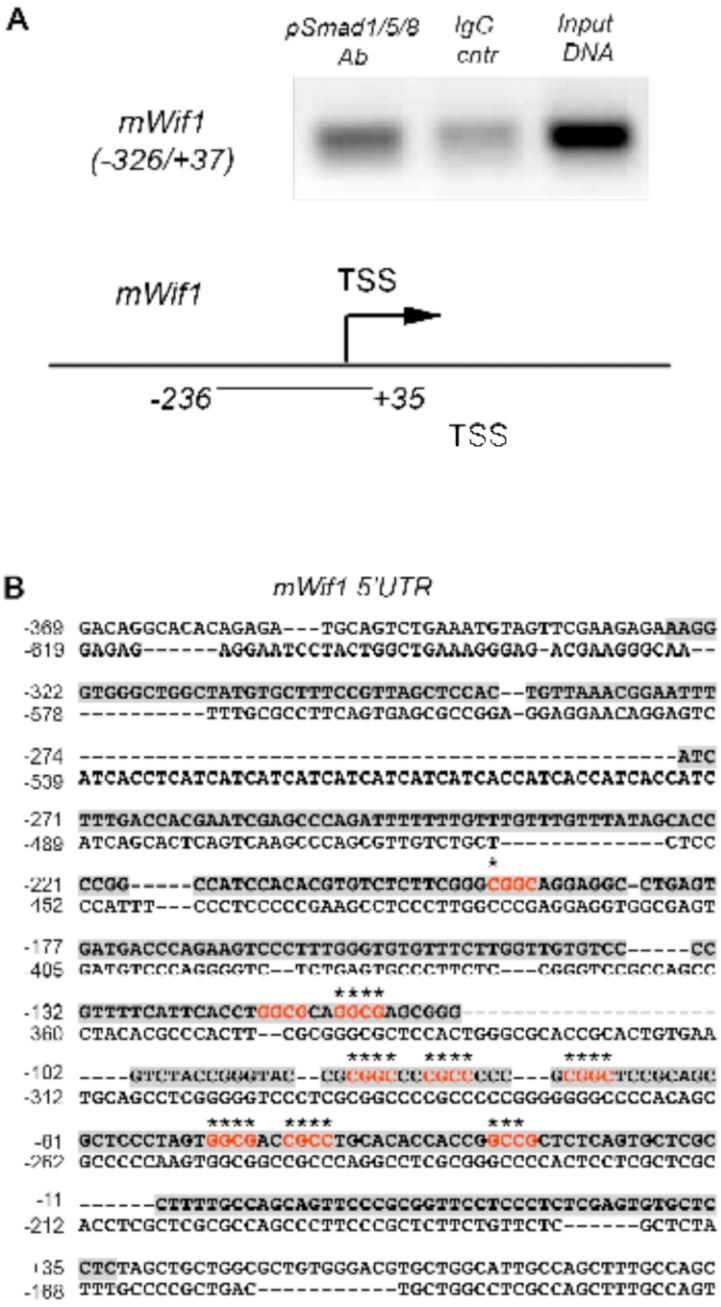

Fig. 3.18. Smad transcription factors bind to *Wif1* promoter region

(**A**) ChIP assay with pSmad1/5/8 antibodies shows an enrichment of -326/+37 bp region of the *Wif1* promoter; the scheme shows position of that binding region within the 5'UTR of *Wif1* (TSS, transcription start site, is shown as an arrow); (**B**) Alignment of mouse (top) and human (bottom) *Wif1* promoter sequences; PCR amplified region is shown as grey boxes. Smad1 binding sequences are shown in red. Asterisks indicate conserved nucleotides within the Smad1 binding sites between the mouse and human sequences.

IV. DISCUSSION

4.1 Overexpression of Noggin in the outer root sheath keratinocytes leads to tumour development

BMP signalling is involved in controlling a large number of biological functions including cell proliferation, differentiation, cell fate decision, and apoptosis in many different types of cells and tissues during embryonic development and postnatal life (Botchkarev, 2003).

In the skin, BMP signalling inhibits cell proliferation and promotes differentiation (D'Souza *et al.*, 2001; Drosdoff *et al.*, 1994; McDonnell *et al.*, 2001; Park and Morasso, 2002). Whilst BMP ligands over-expressed in the epidermis, transgenic mice become resistant to chemically-induced tumours (Blessing *et al.*, 1995; Wach *et al.*, 2001). Furthermore, the chemically-induced tumour development is associated with down-regulation of BMP-dependent Smad1 and Smad5 proteins (He *et al.*, 2001). Taken together, these data support a tumour-suppressive role for BMP signalling in the skin. However, molecular mechanisms of the tumour-protective function of BMPs are mainly remained unclear.

In this project, a transgenic mouse model was employed to further elucidate tumour-suppressive role of the BMP signalling in skin carcinogenesis. BMPs activity was specifically compromised in the epidermis by Keratin 14-driven over-expression of the BMP antagonist Noggin. Elevated expression of the Noggin at both mRNA and protein levels was associated with significant reduction of phospho-Smad1/5/8 expression in the TG skin suggesting that at least canonical BMP-Smad pathway was markedly downregulated in the K14-Noggin mice.

Almost all K14-Noggin mice spontaneously develop macroscopically visible skin tumours by 3-6 months of age. However, the first signs of tumour growth were histologically detected as early as within 3 weeks of postnatal life. At the same time the first signs of hair lost on back skin were detected. The morphological analysis revealed marked increase of hair follicle size, which followed by progressive growth of the HFs without entry to the regression (catagen) and resting (telogen) phases. The hyperplastic changes eventually lead to formation of tumours, which show morphologically striking similarity to human HF-derived trichofolliculoma. These data suggest an involvment of BMP signalling in the development of this type of neoplasia in human skin. However, it remains to be determined whether trichofolliculoma development in humans is associated with mutations in genes encoding the components of BMP signalling pathway, or, similar to *K14-Noggin* mice, its development is regulated by biochemical changes at the levels and/or activity of the BMP antagonists.

Early stages of the tumours development were accompanied by ectopic cell proliferation in the outer root sheath, at the sites of Noggin over-expression, as it was evident by appearance of numerous Ki67- and BrDU-positive cells. In contrast, no TUNEL-positive cells were detected in the ORS compared to WT littermates during catagen, suggesting the apoptotic process was strongly down-regulated upon Noggin over-expression.

The tumour-suppressive function of the BMP pathway in the skin is more likely to involve an ability to maintain a quiescent state of the bulge stem cells in HFs. In the pulse-chase experiment with injection of IdU and CldU for simultaneous detection of the label retaining cells (LRC) and proliferating cells, IdU label was not detected in the bulge region two weeks after chasing, while

an increased amount of proliferating CldU-positive cells was observed in comparison to WT littermates. These data suggest a loss of quiescent cells in the bulge of TG HFs; instead of that they actively proliferate. The fact that these proliferating bulge cells remain their stem cells identity is supported by the expression of such stem/multipotent progenitor cell markers as Lhx2 and Sox9. Moreover, Lhx2- and Sox9-positive cells were present not only in the bulge area but they also appear beyond the stem cell niche. Interestingly, Lhx2- and Sox9-positive cells are present in the new tumour buds growing from the HF outer root sheath pointing out onto the involvement of the HF stem cells and/or their progeny to the tumour initiation. Thus, the keratinocyte-specific inhibition of the BMP signalling in the K14-Noggin mice leads to loss of quiescent state and activation of the bulge stem/progenitor cells, followed by their expansion and tumour formation. These results support previous observations of the BMP involvement in the regulation of HF stem cell activity (Horsley *et al.*, 2008; Kobielak *et al.*, 2007)

Taken together, these data are consistent with previous observations demonstrating that BMP signalling operates as a potent tumour suppressor in the epidermis and HF (Andl *et al.*, 2004; Blessing *et al.*, 1996; Ming Kwan *et al.*, 2004; Zhang *et al.*, 2006). Here we show that anti-tumour effects of BMPs in skin strongly depend on the local concentrations of BMP inhibitor Noggin, which may significantly compromise tumour suppressor function of the BMPs.

Similar effects of Noggin are also described in the intestinal epithelium, in which Noggin overexpression results in a formation of ectopic crypts and development of a phenotype resembling juvenile polyposis (Haramis *et al.*, 2004). Interestingly that another BMP antagonist, Gremlin, is widely expressed

in many different forms of cancers including basal cell carcinoma, and is also capable of promoting proliferation of tumour cells inhibited by BMPs (Sneddon *et al.*, 2006). Collectively, these data suggest that BMP antagonists may significantly affect intrinsic anti-tumour potential in the skin and their expression and activity appears to be critical for the normal versus neoplastic fate decision in keratinocytes.

Interestingly, another transgenic mouse model over-expressing Noggin under control of the K14 promoter was recently developed (Plikus *et al.*, 2004). These mice were generated using chicken *noggin* cDNA, while TG mice used here over-express the mouse *noggin* cDNA. Both TG mice showed some similarities in skin phenotype, such as lack of zig-zag hairs and hyperplasia of nails (Plikus *et al.*, 2004). However, in contrast to the TG mice generated here, those mice did not show formation of any tumours. Perhaps, levels of transgene expression and differences in activity of the mouse and chicken Noggin may differentially affect the capacity of the BMP ligands to bind BMP receptors and influence keratinocyte proliferation/differentiation and cell fate decision.

4.2 Noggin overexpression induces epidermal hyperplasia and increases susceptibility to chemical carcinogenesis in the interfollicular epidermis

In K14-Noggin mice, K14 promoter drives Noggin transgene expression in the basal layer of the interfollicular epidermis (IFE). Such atopic Noggin expression resulted in profound hyperplastic changes in the IFE. A two-fold

increase in epidermal thickness was accompanied by a significant increase in a number of proliferating Ki67-positive cells in the basal layer and aberrant appearance of these cells in suprabasal layers of the IFE. This is consistent with in vitro findings, which showed that BMP signalling in vitro inhibits cell proliferation and promotes cell differentiation (D'Souza *et al.*, 2001; Drosdoff *et al.*, 1994; McDonnell *et al.*, 2001; Park and Morasso, 2002). However, in our in vivo model, inhibition of the BMP pathway by Noggin does not compromise the process of terminal differentiation. Immunodetection of the Loricrin, a component of the cornified envelope, did not reveal any differences in its expression in keratinocytes of the stratum corneum between TG and WT epidermis.

Despite the marked increase in the keratinocyte proliferation rate and epidermal hyperplasia, no obvious signs of neoplastic process were detected within the IFE in *K14-Noggin* mice during 1.5 years of observation, although some areas of mild and moderate dysplastic alterations were seen in the epidermis near HFs.

Increased proliferative potential is one of the common features of tumour cells (Hanahan and Weinberg, 2000), and could predispose to tumour development in IFE in *K14-Noggin* mice. To test this hypothesis, DMBA/TPA chemical carcinogenesis protocol was employed. A single dose of DMBA as a carcinogen followed by multiple applications of the tumour promoter TPA for 20 weeks led to the development of papilloma-like tumours and SCC (Glick *et al.*, 2007; Yuspa *et al.*, 1994). In this protocol, transgenic Noggin over-expression resulted in a significantly faster appearance and a greater number of the papilloma-like tumours compared to WT skin. Moreover, the TG mice showed

more rapid malignant transformation of the benign tumours into SCC with appearance of local metastases in the dermis. In contrast, no signs of SCC development were detected in WT skin during 25 weeks of the experiment.

Taken together, an inhibition of the BMP pathway by Noggin markedly enhances proliferative potential of the IFE keratinocytes without altering their differentiation programmeme. Although, this is not enough to induce a spontaneous neoplastic process, but greatly increases susceptibility of the epidermis to chemically-induced tumourigenesis. However, a question still remains unanswered what mechanism prevents tumour formation in IFE versus follicular epithelium upon Noggin over-expression? Most likely, follicular and epidermal keratinocytes show differential response to extracellular BMP ligands, which is primarily dependent on expression of the BMP receptors on the cell surface. At the same time, these two groups of keratinocytes may expose to different network of signalling molecules regulating homeostasis in distinct skin compartments. Finally, there could be other mechanisms contributing to the tumour protection in the HFs and IFE, such as differences in mesenchymal components underlying the IFE and HF keratinocytes (basal membrane of the epidermis and HF connective tissue sheath).

4.3 Inhibition of BMP signalling leads to activation of pro-oncogenic Wnt and Shh pathways in K14-Noggin skin

An intrinsic response of every cell exposed to an external signalling molecule is a changing of the transcriptional programmeme in the nucleus,

which includes transcriptional activation and/or repression of distinct sets of genes. These genes representing direct targets for a particular signalling pathway may in turn regulate transcription of other genes (indirect targets) or can interfere with components of other signalling pathways creating a network of regulatory molecules. The cellular response in this case will reflect summarizing effects of the complex interactions between the components of different signalling pathways rather than only a direct effect of the primary molecule triggered the signal transduction. Apparently, the way of acting of the BMP signalling fully supports the mechanism mentioned above. In several model systems, different interactions between selected components of the BMP pathway and other signalling and regulatory molecules have been recently described, i.e. interactions between the BMP and Wnt signalling (Di Pasquale and Brivanlou, 2009; Ishitani *et al.*, 1999; Kamiya *et al.*, 2008; Lintern *et al.*, 2009; Piccolo *et al.*, 1999), BMP and Shh (Laurikkala *et al.*, 2003; Zeller and Zuniga, 2007; Zuniga *et al.*, 2000), BMP and Edar (Pummila *et al.*, 2007), BMP and p53 (Chandar *et al.*, 2005; Nakamura *et al.*, 2003), or Ras pathways (Liu *et al.*, 2003a).

By employing global microarray approach, genome-wide gene expression profiles were compared between the HF-derived tumours and normal hair matrix keratinocytes. The microarray and real-time PCR analyses showed two-fold or higher changes in expression of 390 genes in the HF-derived tumour cells versus hair matrix keratinocytes. These genes encode the adhesion/extracellular matrix molecules, cell cycle/apoptosis and cytoskeleton/cell motility markers, molecules involved in the control of cell differentiation, metabolism, signalling and transcription. It is unlikely that all

these genes are direct targets of the BMP signalling. However, the changes in transcription activity of the genes are likely to contribute to tumour development in K14-Noggin mice.

Importantly, the HF-derived tumours showed marked increase in expression of the genes encoding the selected components of signalling pathways implicated in cutaneous tumourigenesis (Wnt, Hedgehog, Pdgf-A/B, Ras, etc.), while expression of genes involved in inhibition of the cell proliferation (Igfbp4, Bmp8a, Id2, Id4) was decreased. These data suggested the involvement of the pro-oncogenic signalling pathways listed above in the development of HF derived tumours in K14-Noggin mice.

4.3.1. Noggin-induced activation of the Wnt signalling is crucial for initiation of hair follicle-derived tumours

Expression of several Wnt ligands (*Wnt5a*, *Wnt6, Wnt9, Wnt10b*) and their receptors (*Fz2, Fz7*) was markedly up-regulated in the K14-Noggin tumour cells compared to normal HF keratinocytes. By the date, there is no evidence showing direct regulation of the Wnt ligands expression by the BMP signalling pathway. However, activation of Wnt signalling may have a great impact on development of the tumours in K14-Noggin mice. It was recently shown that overexpression of the active form of β-catenin, a key component of the Wnt pathway, results in conversion of the HFs into benign tumours (pilomatricoma and trichofoliculoma) in mouse skin (Gat *et al.*, 1998; Lo Celso *et al.*, 2004).

It is possible to suggest a close functional relationship between the inhibition of BMP signalling and activation of Wnt/β-catenin pathway in the development of the trichofoliculomas in K14-Noggin mice. Indeed, nuclear

staining for β-catenin was detected in the outer root sheath and tumour placodes in the TG skin. Expression of the transcriptional co-factor of β-catenin, Lef-1, was also detected in the tumour placodes. Finally, inhibition of the Wnt pathway by pharmacological manipulation significantly decreases a number of tumour placodes arising from the outer root sheath accompanied by the decrease of nuclear β-catenin staining in the HF keratinocytes, thus suggesting an inhibition of the tumour initiation.

Interestingly, there is an important antagonistic interplay between Wnt and BMP pathways during HF induction in embryonic skin and anagen initiation in adult skin. Several reports show that activation of the Wnt signalling is a crucial step in HF induction and anagen initiation (Andl *et al.*, 2002; DasGupta *et al.*, 2002; Huelsken *et al.*, 2001; Merrill *et al.*, 2001; Plikus *et al.*, 2008; van Genderen *et al.*, 1994; Van Mater *et al.*, 2003). In contrast, inhibition of the BMP pathway activity was shown as an essential factor for hair follicle induction and new hair growth stimulation (Botchkarev *et al.*, 2002; Botchkarev *et al.*, 1999a; Botchkarev, 2001; Guha *et al.*, 2004; Plikus *et al.*, 2008). However, little is known about the mechanism governing the dynamic opposite interplay between these two pathways. It was previously reported that up-regulation of the BMP antagonist Noggin expression occurs in the bulge and secondary hair germ during the early anagen phase when HF stem cells are activated and migrate downward to generate new HFs (Botchkarev, 2001; Zhang *et al.*, 2006). Noggin operates through antagonistic interactions with BMPs, which result in up-regulation of the transcription factor Lef-1, a crucial component of the Wnt/β-catenin pathway (Botchkarev *et al.*, 1999a). Moreover, BMP inhibition via *BMPR1A* deficiency enhances β-catenin stabilization in the bulge cells through

a pathway involving PTEN inhibition and PI3K/AKT activation (Kobielak *et al.*, 2007; Zhang *et al.*, 2006).

However, our data revealed another mechanism for the antagonistic cross-talk between the BMP and Wnt pathways in the HFs. Data presented here suggest that an extracellular Wnt antagonist Wif1, which operates as a potent tumour suppressor in other organs (lungs, prostate, breast, etc) (Rubin *et al.*, 2006), may serve as a target for BMP signalling. Wif1 is expressed in the bulge cells of telogen and late anagen HFs in WT skin, while its expression is markedly down-regulated in the developing tumour placodes in K14-Noggin skin. We show here that Wif1 indeed represents as a direct target of the BMP signalling: i) Wif1 expression in tumour cells is positively regulated by BMP4; ii) BMP signalling pathway regulates the Wif1 promoter activity in HaCaT keratinocytes; iii) Smad1/5 bind the Wif1 promoter in ChIP assay. These data suggest that tumour suppressive activity of the BMP signalling in the keratinocytes is mediated, at least in part, via positive regulation of the Wif1 expression

4.3.2. Upregulation of Shh pathway promotes tumour progression upon Noggin-induced BMP suppression

Microarray and RT-qPCR data show increased mRNA expression of several components of the Hedgehog signalling pathway in K14-Noggin tumours, such as *Shh*, *Dhh*, *Smo* (**Fig. 3.13**). Increased expression of the Shh is also detected on a protein level. Moreover, several selected target genes of Shh pathway (*Ptch1/2, Gli1/2, Ccnd1/2*) are highly expressed in the tumours, thus suggesting an activation of the Shh signalling pathway during Noggin-

induced tumourigenesis.

Similarly to Wnt activity, Shh signalling stimulates proliferation and self-renewal of the stem cell/multipotent progenitor cells in both embryonic and adult tissues of different origins, e.g. hemopoietic, neuronal, epidermal and gastrointestinal (Ramalho-Santos *et al.*, 2000) (Bhardwaj *et al.*, 2001) (Palma *et al.*, 2005) (Zhou *et al.*, 2006).

In skin, activation of both Shh and Dhh in basal keratinocytes of the epidermis and in HFs results in hyperplasia of the epidermal progenitor cells and development of lesions reminiscent of human basal cell carcinoma (Adolphe *et al.*, 2004; Grachtchouk *et al.*, 2000). In contrast, loss of the Shh in skin causes defects in cell proliferation in hair placodes and arrests of HF growth at the early stage of the development (Chiang *et al.*, 1999; St-Jacques *et al.*, 1998). In epidermal keratinocytes, the pro-proliferative effect of the Shh signalling are mediated through inhibition of p21(CIP1/WAF1) (Fan and Khavari, 1999). In addition, Shh stimulates expression of cyclinD1 and cyclinD2 maintaining proliferation of non-differentiated progenitor cells, while more differentiated cells are not responsive to the Shh proliferative signalling (Kenney and Rowitch, 2000).

Thus, active Shh signalling may be involved in the tumourigenesis in the K14-Noggin skin promoting the proliferation of the HF stem cells or their non-differentiated progenies. Interestingly, inhibition of the Shh signalling by Smo inhibitor Cyclopamin does not effect on the tumour initiation, but rather blocks the growth and progression of the tumour after tumour placode induction (**Fig. 3.15**).

Up-regulation of the Shh signalling in K14-Noggin mice suggests that BMP

signalling can antagonize Shh activity in HFs. Indeed, BMP4 treatment of *K14-Noggin* tumour cells decreases *Shh* mRNA expression in vitro (**Fig. 3.16**), a result that is consistent with data previously obtained (Botchkarev, 2001; Sneddon *et al.*, 2006).

It is unclear, however, which mechanisms may be involved in mediating the effects of BMPs on the Shh expression in keratinocytes. A reporter assay with *Shh* promoter region suggests that *Shh* expression is not under direct regulation by BMPs (**Fig. 3.17**).

Eda/Edar signalling may serve as one of the candidates in mediating the cross-talk between BMP and Shh pathways in trichogenic tumours: *Edar* expression is increased in the HF-derived tumours of K14-Noggin mice (**Fig. 3.10**); Edar has been shown to be a direct target for down-regulation by BMP signals (Mou *et al.*, 2006), and, in turn, is capable of positively regulating Shh expression (Pummila *et al.*, 2007).

Interestingly, recent observations demonstrate the Shh pathway as a downstream effecter of Wnt signalling during development of basal cell carcinoma (Yang *et al.*, 2008). Thus, stimulation of the Shh signalling may be a consequence of Noggin-induced activation of the Wnt pathway in K14-Noggin skin. It also remains to be determined whether other regulatory molecules, whose expression is changed in the tumours, are also capable of modulating a cross-talk between the BMP and Shh signalling pathways during the neoplastic process in HFs.

4.3.3. Model illustrating the involvement of the BMP signalling in the suppression of tumour formation in the skin

Taken together, these data support a concept that signalling pathways involved in the control of skin morphogenesis are highly relevant to the development of skin carcinogenesis (Bornstein *et al.*, 2007; Owens and Watt, 2003; Yang *et al.*, 2008). Accumulated evidences have changed the paradigm of cancer development, which can no longer be viewed purely in terms of a network of oncogenes and tumour suppressor genes (Hahn and Weinberg, 2002). The complex interplay of different signalling pathways that regulate the balance between proliferation and differentiation in normal tissues is frequently subverted in cancer (Taipale and Beachy, 2001).

The data presented here provide compelling evidence that the BMP antagonist Noggin can play an important role in skin tumourigenesis and may significantly compromise tumour suppressor function of BMPs in postnatal skin, at least in part via stimulating the Wnt and Shh signalling pathways (**Fig. 4.1**). Thus, levels and/or activity of BMP antagonists may determine the predisposition of skin epithelium to carcinogenesis, while pharmacological modulation of BMP activity may provide a new set of tools for skin cancer prevention and management.

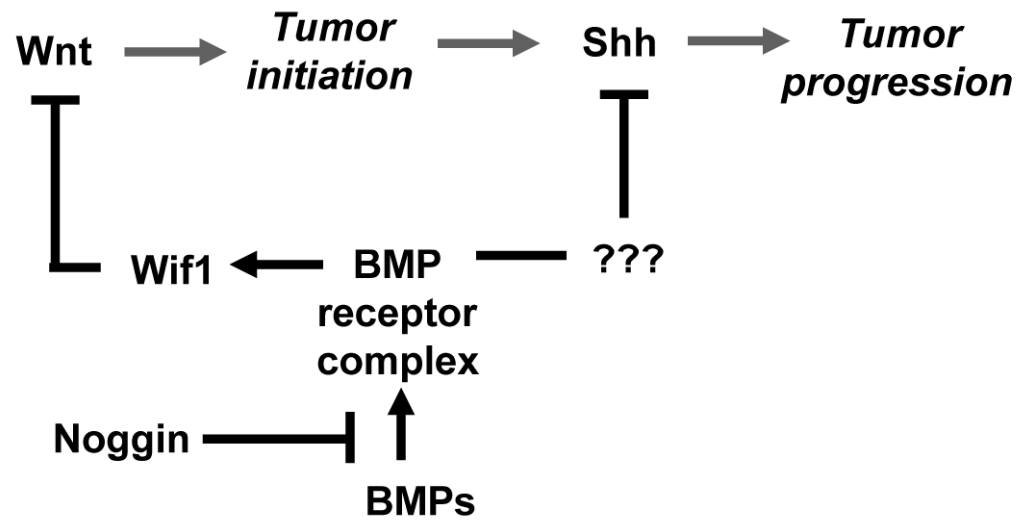

Fig. 4.1. Anti-tumour activity of the BMP signalling in keratinocytes

The scheme illustrates mechanisms of the effects of the BMP signalling on distinct stages (initiation and progression) of tumour formation

CONCLUSION

Based on the data presented above, the following conclusions are drawn:

1. Inhibition of the BMP activity in the murine epidermis and hair follicle by K14-driven Noggin overexpression results in spontaneous development of trichofolliculoma-like tumours, as well as in hyperplasia of the epidermis and sebaceous glands. Noggin induced BMP inhibition is associated with increased cell proliferation and decrease of apoptosis in the skin, as well as with activation and expansion of the epithelial stem cells and/or their early progeny. In the epidermis, decrease of the BMP activity significantly increases the susceptibility to chemical carcinogenesis.

2. Noggin overexpression leads to marked alterations in gene expression programmemes in keratinocytes and accompanied by the increase in the expression of genes encoding selected components of several pro-oncogenic signalling pathways (e.g., Wnt, Shh, Pdgf, etc.). Pharmacological treatment of the K14-Noggin mice with synthetic inhibitors of the Wnt and Shh signalling pathways suggests that these pathways are likely to contribute to the initiation and progression of the trichofolliculoma-like tumours.

3. Anti-tumourigenic effects of the BMP signalling in keratinocytes is mediated, at least in part, via cross-talk with the Wnt and Shh pathways:

a) Development of the hair follicle-derived tumours in K14-Noggin is associated with marked downregulation of the Wnt antagonist and tumour suppressor Wif1, while BMP4 directly stimulates expression of the Wif1 in cultured tumour cells. Smad1/5 bind the Wif1 promoter in the ChIP assay and

positively regulates the Wif1 promoter activity in the transient transfection assay. Thus, Wif1 can mediate the inhibitory effects of the BMPs on Wnt signalling pathway, as well as be part of the BMP-mediated anti-tumourigenic programmeme in the skin epithelium.

b) BMP signalling pathway negatively regulates Shh expression in keratinocytes. However, this effect is likely to be indirect and may mediated by other factors including Edar signalling.

4. Taken together, tumour suppressor function of the BMPs in the skin epithelium can be significantly compromised by a local concentration and/or activity of the BMP antagonist Noggin. Tumour suppressor activity of the BMPs in keratinocytes is mediated, at least in part, via regulation of the expression of factors antagonizing Wnt and Shh oncogenic pathways.

FUTURE WORK

Following studies could be helpful to further elucidate the molecular basis of tumour suppressor function of the BMP signalling and the complex interaction with other signalling pathways implicated in skin carcinogenesis:

- Generation of a transgenic mouse line overexpressing Wif1 under the control of skin specific promoters, such as K14: testing the susceptibility of these mice to chemical carcinogenesis and crossbreeding with K14-Noggin mice to rescue their tumour phenotype
- Isolation of cancer stem cells from chemically induced skin tumours by FACS sorting using cell surface markers (i.e. CD34) and treatment of them with BMP ligands to test their tumour initiation abilities in skin reconstitution assay
- Genome wide gene expression profiling of the cancer stem cells before and after BMP treatment and comparison with normal skin stem cells could bring some new insight into the tumour suppressor function of BMPs

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PUBLICATIONS

1. Sharov AA*, **Mardaryev AN***, Sharova TY, Grachtchouk M, Atoyan R, Byers HR, Seykora JT, Overbeek P, Dlugosz A, Botchkarev VA. (2009). Bone morphogenetic protein antagonist noggin promotes skin tumourigenesis via stimulation of the Wnt and Shh signalling pathways. Am J Pathol 175(3): 1303-14 [* equal contribution]

2. Fessing MY, Atoyan R, Shander B, **Mardaryev AN**, Botchkarev VV Jr, Poterlowicz K, Peng Y, Efimova T, Botchkarev VA. (2009) BMP Signalling Induces Cell-Type-Specific Changes in Gene Expression Programmes of Human Keratinocytes and Fibroblasts. J Invest Dermatol [Epub ahead of print]

3. Sharov AA, Sharova TY, **Mardaryev AN**, Tommasi di Vignano A, Atoyan R, Weiner L, Yang S, Brissette JL, Dotto GP, Botchkarev VA. (2006). Bone morphogenetic protein signalling regulates the size of hair follicles and modulates the expression of cell cycle-associated genes. Proc Natl Acad Sci U S A 103(48): 18166-71

Appendix A

Genes Overexpressed in the Tumour Cells of Hair Follicles of K14-Noggin Mice versus Matrix cells of Hair Follicles of Wild-Type Mice (258 genes)

Adhesion (19)	Gene Symbol	Accession Number	Fold Change
Neural cell adhesion molecule 1	Ncam1	NM_010875	↑**8.7**
Poliovirus receptor-related 3	Pvrl3	NM_021495	↑**7.7**
Coxsackievirus and adenovirus receptor	Cxadr	NM_009988	↑**4.8**
Lectin, galactose binding, soluble 7	Lgals7	NM_008496	↑**4.7**
Laminin, gamma 2	Lamc2	NM_008485	**f**↑**4.7**
Protocadherin beta 17	Pcdhb17	NM_053142	↑**3.7**
Protocadherin beta 10	Pcdhb10	NM_053135	↑**2.8**
odd Oz/ten-m homolog 2	Odz2	NM_011856	↑**2.8**
Integrin alpha X	Itgax	NM_021334	↑**2.7**
Protocadherin beta 21	Pcdhb21	NM_053146	↑**2.6**
Procollagen, type XVIII, alpha 1	Col18a1	NM_009929	↑**2.3**
Laminin alpha 5 chain	Lama5	U37501	↑**2.3**
Claudin 1	Cldn1	NM_016674	↑**2.2**
Protocadherin beta 22	Pcdhb22	NM_053147	↑**2.2**
Cell adhesion molecule 1	Cadm1	AF434663	↑**2.2**
Integrin beta 4	Itgb4	BC059192	↑**2.1**
Transmembrane protein 16B	Tmem16b	NM_153589	↑**2.0**
Protocadherin beta 13	Pcdhb13	NM_053138	↑**2.0**
Contactin associated protein 4	Cntnap4	NM_130457	↑**2.0**
Extracellular matrix (17)			
Semaphorin 4c precursor	Sema4c	XM_898566	↑**11.5**
EMI domain containing 2	Emid2	NM_024474	↑**7.5**
Muskelin 1	Mkln1	NM_013791	↑**6.6**
Procollagen, type XVII, alpha	Col17a1	BC003208	↑**5.9**
Phosphacan short isoform (RPTP-beta gene)	Ptprz1	AJ428208	↑**5.4**
Procollagen, type VII, alpha 1	Col7a1	NM_007738	↑**5.3**
Leucine rich repeat protein 1, neuronal	Lrrn1	NM_008516	↑**5.2**
EMI domain containing 1	Emid1	NM_080595	↑**4.5**
Hyaluronan synthase 3	Has3	NM_008217	↑**3.6**
Microfibrillar-associated protein 2	Mfap2	NM_008546	↑**2.8**

Heparan sulfate (glucosamine) 3-O-sulfotransferase 3B	Hs3st3b	NM_018805	↑**2.8**
Collagen type VI, alpha 3	Col6a3	NM_009935	↑**2.7**
Chondroitin sulfate proteoglycan 4	Cspg4	NM_139001	↑**2.3**
alpha-1 type IV collagen	Col4a-1	J04694	↑**2.3**
Procollagen, type XVIII, alpha 1	Col18a1	NM_009929	↑**2.2**
Fibronectin leucine rich transmembrane protein 3	Flrt3	NM_178382	↑**2.2**
Cytotoxic and regulatory T cell molecule	Crtam	NM_019465	↑**2.1**
Cell cycle (11)			
Deleted in bladder cancer 1	Dbccr1	NM_019967	↑**7.2**
Leucine rich repeat containing 4	Lrrc4	NM_138682	↑**6.9**
KH domain containing, RNA binding, signal transduction associated 3	Khdrbs3	NM_010158	↑**6.2**
Sex comb on midleg-like 2 cyclin-dependent kinase	Scml2	NM_133194	↑**3.9**
RAD50 homolog (S. cerevisiae)	Rad50	NM_009012	↑**3.6**
Cyclin D2	Ccnd2	NM_009829	↑**3.4***
Cyclin M3	Cnnm3	BC052714	↑**3.4***
Chromodomain helicase DNA binding protein 3	Chd3	BC020471	↑**2.6**
Musashi homolog 1(Drosophila)	Msi1h	NM_008629	↑**2.6**
Cyclin F	Ccnf	NM_007634	↑**2.3***
B-cell translocation gene 1, anti-proliferative	Btg1	BC018309	↑**2.3**
Apoptosis (7)			
B-cell CLL/lymphoma 11A (zinc finger protein)	Bcl11a	NM_016707	↑**4.7**
B-cell leukemia/lymphoma 6	Bcl6	NM_009744	↑**3.9***
Bcl-2 binding component 3	Bbc3	NM_133234	↑**3.4**
DNA-damage-inducible transcript 4	Ddit4	NM_029083	↑**3.2**
Transformation related protein 53 inducible nuclear protein 1	Trp53inp1	NM_021897	↑**2.3**
Fas apoptotic inhibitory molecule 2	Faim2	NM_028224	↑**2.2**
Bcl2-associated athanogene 3	Bag3	NM_013863	↑**2.0**
Cytoskeleton/Keratinocyte Differentiation (10)			
Keratin associated protein 6-1	Krtap6-1	NM_010672	↑**6.4**
Ankyrin repeat domain 2	Ankrd2	NM_021351	↑**5.5**
Erythrocyte protein band 4.9	Epb4.9	NM_013514	↑**3.9**
Keratin complex 1, acidic, gene 16	Krt1-16	NM_008470	↑**3.3***
Tektin 1	Tekt1	NM_011569	↑**3.1**
Microtubule-associated protein tau	Mapt	NM_010838	↑**2.8**
Keratin complex 2, basic, gene 5	Krt2-5	BC006780	↑**2.6***
Leiomodin 1 (smooth muscle)	Lmod1	NM_053106	↑**2.2**
Keratin complex 1, acidic, gene 17	Krt1-17	NM_010663	↑**2.1***
Ena-vasodilator stimulated phosphoprotein	Evl	NM_007965	↑**2.0**

Metabolism (46)			
Protein-tyrosine sulfotransferase 2	Tpst2	NM_009419	↑**8.2**
Glycine N-methyltransferase	Gnmt	NM_010321	↑**6.3**
AMP deaminase isoform M	Ampd1	XM_131103	↑**6.0**
Poly(ADP-ribose) polymerase 3	Adprt3	AY046317	↑**4.9**
Transmembrane channel-like gene family 7	Tmc7	NM_172476	↑**4.8**
Dehydrogenase/reductase (SDR family) member 6	Dhrs6	NM_027208	↑**4.4**
6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 2	Pfkfb2	BC018418	↑**3.9**
O-acyltransferase (membrane bound) domain containing 2	Oact2	NM_026037	↑**3.1**
Guanosine monophosphate reductase	Gmpr	NM_025508	↑**3.0**
Carbonic anhydrase 6	Car6	NM_009802	↑**3.0**
ADP-ribosylation factor 3	Arf3	NM_007478	↑**3.0**
Golgi associated, ARF binding protein 3	Gga3	NM_173048	↑**2.9**
Spermine oxidase	Smox	BC004831	↑**2.8**
Sialyltransferase 9	Siat9	NM_011375	↑**2.8**
Glutamate-ammonia ligase (glutamine synthase)	Glul	NM_008131	↑**2.3**
Ectonucleoside triphosphate diphosphohydrolase 2v	Entpd2	NM_009849	↑**2.2**
SEC24 related gene family, member D (S. cerevisiae)	Sec24d	BC026624	↑**2.2**
Syntaxin binding protein 4	Stxbp4	NM_011505	↑**2.2**
Xylosyltransferase II (XT-II gene)	Xylt2	AJ291751	↑**2.0**
Arylalkylamine N-acetyltransferase	Aanat	AF004109	↑**2.0**
Ion transport (14)t			
Calcium channel, voltage-dependent, T type, alpha 1G	Cacna1g	NM_009783	↑**16.4**
Ferritin light chain 2	Ftl2	NM_008049	↑**7.5**
ATPase, Na+/K+ transporting, beta 2	Atp1b2	NM_053146	↑**5.8**
ATPase, H+ transporting, V1 subunit C, isoform 2	Atp6v1c2	NM_133699	↑**5.0**
Potassium large conductance calcium-activated channel, subfamily M, alpha member 1	Kcnma1	NM_010610	↑**4.2**
FXYD domain-containing ion transport regulator 4	Fxyd4	NM_033648	↑**3.6**
Solute carrier organic anion transporter family, member 3a1	Slco3a1	NM_023908	↑**3.3**
Solute carrier family 22, member 4	Slc22a4	NM_019687	↑**3.2**
ATPase, Na+/K+ transporting, beta 1 polypeptide	Atp1b1	NM_009721	↑**2.8**
Progressive ankylosis	Ank	NM_020332	↑**2.7**
Chloride channel 2	Clcn2	NM_009900	↑**2.4**
ATPase, Cu++ transporting, beta polypeptide	Atp7b	NM_007511	↑**2.2**
Calcium channel, voltage-dependent, alpha 2/delta 2	Cacna2d2	NM_020263	↑**2.0**
ATPase, Na+/K+ transporting, beta 3 polypeptide	Atp1b3	NM_007502	↑**2.0**

Lipid metabolism (12)			
Arachidonate epidermis-type 12(S)-lipoxygenase	Alox12e	NM_145684	↑**17.5**
Apolipoprotein M	Apom	NM_018816	↑**6.5**
Peroxisomal biogenesis factor 11c	Pex11c	NM_026951	↑**6.5**
Phospholipase A2, group IIE	Pla2g2e	NM_012044	↑**5.2**
Arachidonate 5-lipoxygenase activating protein	Alox5ap	BC026209	↑**4.7**
Phosphatidylinositol-4-phosphate 5-kinase, type 1 alpha	Pip5k1a	NM_008846	↑**3.1**
ATP-binding cassette, sub-family G (WHITE), member 1	Abcg1	NM_009593	↑**2.9**
Iinositol 1,4,5-trisphosphate 3-kinase C	Itpkc	BC013227	↑**2.7**
Arachidonate lipoxygenase 3	Aloxe3	NM_011786	↑**2.4**
Phospholipase C, gamma 2	Plcg2	BC019654	↑**2.3**
Apolipoprotein E	Apoe	NM_009696	↑**2.1**
Sulfotransferase family 4A, member 1	Sult4a1	NM_013873	↑**2.0**
Proteolysis/Cytolysis (17)			
Alpha 1 microglobulin/bikunin	Ambp	NM_007443	↑**6.7**
Protease, serine, 12 neurotrypsin (motopsin)	Prss12	NM_008939	↑**5.3**
Protease, serine, 18	Prss18	NM_011177	↑**4.7**
Cathepsin H	Ctsh	NM_007801	↑**4.6**
Tetranectin (plasminogen binding protein)	Tna	NM_011606	↑**3.8**
Extracellular proteinase inhibitor	Expi	NM_007969	↑**3.7**
Kallikrein 7 (chymotryptic, stratum corneum)	Klk7	NM_011872	↑**3.1**
Protease, serine, 11 (Igf binding)	Prss1	NM_019564	↑**2.9**
Cathepsin S	Ctss	NM_021281	↑**2.8**
Cathepsin Q	Ctsq	NM_029636	↑**2.6**
Cathepsin L	Cts	NM_009984	↑**2.5**
P lysozyme structural	Lzp-s	NM_013590	↑**2.5**
Protective protein for beta-galactosidase	Ppgb	NM_008906	↑**2.4**
Tissue inhibitor of metalloproteinase 2	Timp2	NM_011594	↑**2.1**
Furin (paired basic amino acid cleaving enzyme) (), mRNA [6]	Furin	NM_01104	↑**2.1**
Mast cell protease 4	Mcpt4	NM_010779	↑**2.1**
Matrix metalloproteinase 7	Mmp7	NM_010810	↑**2.0**
Signalling (75)			
Signalling pathways controlling organ development/tumourigenesis (30)			
Transforming growth factor, beta 2	TGFβ2	NM_009367	↑**10.9**
Tumour necrosis factor receptor superfamily, member 10b	Tnfrsf10b	NM_020275	↑**10.2**
Transformation related protein 73	Trp73	NM_011642	↑**6.1**
EGF-like-domain, multiple 4	Egfl4	BC036727	↑**7.6**
PDZ and LIM domain 4	Pdlim4	NM_019417	↑**7.3**

Rab6b, member RAS oncogene family	Rab6B	NM_173781	↑**7.1**
Prokineticin 1	Pk1	AF487281	↑**7.0**
Platelet derived growth factor, alpha	Pdgfa	NM_008808	↑**5.5***
Retinoic acid receptor gamma 3	Rarg3	NP057424	↑**5.3**
Transmembrane protein with EGF-like and follistatin-like domains 2	Tmeff2	NM_019790	↑**5.1**
Ras homolg gene family, member G	Rhog	NM_019566	↑**5.0**
Cholecystokinin	Cck	NM_031161	↑**4.5**
Sonic hedgehog protein precursor (shh) homolog	Shh	NM_009170	↑**4.1***
RAB34, member of RAS oncogene family	Rab34	NM_033475	↑**4.1**
I-kappa-b-interacting ras-like protein 2	Nkiras2	NM_028024	↑**3.7**
Transcription factor hes-5 (hairy and enhancer of split 5)	Hes5	NM_010419	↑**3.6**
Calcitonin.	Calca	X97991	↑**3.5**
Desert hedgehog	Dhh	NM_007857	↑**2.7**
Ectodysplasin-A receptor	Edar	NM_010100	↑**2.6***
Tumour necrosis factor receptor superfamily, member 9	Tnfrsf9	NM_011612	↑**2.6**
Protein kinase C, theta	Prkcq	NM_008859	↑**2.6**
RAS-like, estrogen-regulated, growth-inhibitor	Rerg	BC026463	↑**2.5**
Tumour necrosis factor receptor superfamily, member 18	Tnfrsf18	NM_009400	↑**2.5**
Bone morphogenetic protein 7	Bmp7	NM_007557	↑**2.3**
Neurturin	Nrtn	NM_008738	↑**2.2**
Tumour necrosis factor (ligand) superfamily, member 15	Tnfsf15	NM_177371	↑**2.1**
Insulin-like 6	Insl6	NM_013754	↑**2.1**
Insulin-like growth factor binding protein 7	Igfbp7	NM_008048	↑**2.1**
Platelet derived growth factor, B polypeptide	Pdgfb	NM_011057	↑**2.0***
Smoothened homolog (Drosophila)	Smo	NM_176996	↑**2.0***
Wnt signalling (10)			
Kringle containing transmembrane protein	Kremen	NM_032396	↑**5.1**
Wingless-related MMTV integration site 5a	Wnt5a	NM_009525	↑**2.8***
Wingless-type MMTV integration site 9B	Wnt9b	NM_011719	↑**2.6**
Casein kinase 1, epsilon	Csnk1e	NM_013767	↑**2.4**
Low density lipoprotein receptor-related protein 4	Lrp4	NM_172668	↑**2.4**
Wingless related MMTV integration site 10b	Wnt10b	NM_011718	↑**2.3***
Wingless-related MMTV integration site 6	Wnt6	NM_009526	↑**2.2**
Frizzled homolog 7 (Drosophila)	Fzd7	NM_008057	↑**2.1**
Frizzled homolog 2 (Drosophila)	Fzd2	NM_020510	↑**2.1**
Wingless-related MMTV integration site 11	Wnt11	NM_009519	↑**2.0**
Other signalling pathway-related markers (35)			

Serine-threonine kinase receptor-associated protein	Strap	TC1066374	↑**6.2**
Transmembrane domain protein regulated in adipocytes	Tpra40	NM_011906	↑**4.8**
Guanine nucleotide binding protein, alpha z subunit	Gnaz	NM_010311	↑**4.7**
PTK2 protein tyrosine kinase 2 beta	Ptk2b	NM_172498	↑**4.6**
Erythropoietin receptor	Epor	NM_010149	↑**3.9**
Mps One Binder kinase activator-like 2C	Mobkl2c	AK084014	↑**3.8**
Mitochondrial tumour suppressor 1	Mtus1	BC030860	↑**3.7**
Oz/ten-m homolog 2 (Drosophila)	Odz2	NM_011856	↑**3.6**
Piwi like homolog 2 (Drosophila)	Piwil2	NM_021308	↑**3.2**
FMS-like tyrosine kinase 1	Flt1	NM_010228	↑**3.2**
Galanin receptor 3	Galr3	NM_015738	↑**3.0**
Reticulon 4 receptor	Rtn4r	NM_022982	↑**2.9**
Deiodinase, iodothyronine, type I	Dio1	NM_007860	↑**2.9**
Protein phosphatase 1A, magnesium dependent, alpha isoform	Ppm1a	NM_008910	↑**2.8**
Taste receptor, type 2, member 105	Tas2r105	NM_020501	↑**2.6**
GRP1(General receptor for phosphoinositides 1 ass. scaffold protein)	Grasp	NM_019518	↑**2.6**
Mitogen activated protein kinase kinase kinase 2	Map3k2	NM_011946	↑**2.6**
v-erb-b2 erythroblastic leukemia viral oncogene homolog 2	ErbB2	NM_001003817	↑**2.6**
Growth arrest specific 6	Gas6	NM_019521	↑**2.5**
Toll-like receptor 9	Tlr9	NM_03117	↑**2.4**
G protein-coupled receptor 49	Gpr49	NM_010195	↑**2.4**
Growth factor receptor bound protein 7	Grb7	NM_010346	↑**2.3**
Transmembrane channel-like gene family 4	Tmc4	BC040466	↑**2.3**
Heat shock protein, 25 kDA	Hspb1	NM_013560	↑**2.2**
Pellino 2	Peli2	NM_033602	↑**2.2**
Neogenin	Neo1	NM_008684	↑**2.2**
Icos ligand	Icosl	NM_015790	↑**2.1**
Protein tyrosine phosphatase PTPT9	Ptprs	D28530	↑**2.1**
MIS type II receptor	Misri	AF503863	↑**2.1**
G protein-coupled receptor RE2	Gpr161	AY255596	↑**2.1**
Melanocortin 4 receptor	Mc4r	NM_016977	↑**2.1**
Stromal interaction molecule 1	Stim1	NM_009287	↑**2.0**
Paternally expressed 12	Peg12	NM_013788	↑**2.0**
Feline sarcoma oncogene	Fes	BC038130	↑**2.0**
Macrophage stimulating 1 receptor (c-met-related tyrosine kinase)	Mst1r	NM_009074	↑**2.0**
Transcription (56)			
LIM domain only 1	Lmo	NM_057173	↑**16.7**
Homeo box A9	Hoxa9	NM_010456	↑**5.6**

Interleukin enhancer binding factor 2	Ilf2	NM_026374	↑**5.6**
BTB and CNC homology 2	Bach2	BC051242	↑**5.0**
Activating transcription factor 6	Atf6	NM_001081304	↑**4.9**
Paired like homeodomain factor 1	Prop1	NM_008936	↑**4.9**
B-cell CLL/lymphoma 11A (zinc finger protein)	Bcl11a	NM_016707	↑**4.7**
E74-like factor 4	Elf4	NM_019680	↑**4.3**
Iroquois related homeobox 4 (Drosophila)	Irx4	NM_018885	↑**4.0**
Homeo box D11	Hoxd11	NM_008273	↑**4.0**
similar to zinc finger and BTB domain containing 45	ZBTB45	NM_001024699	↑**4,0**
Tripartite motif-containing 45	Trim45	NM_194343	↑**4,0**
LIM homeobox protein 2	Lhx2	NM_010710	↑**3.9**
SRY-box containing gene 13	Sox13	NM_011439	↑**3.8**
Early growth response 2	Egr2	X06746	↑**3.7**
Mesoderm posterior 1	Mesp1	NM_008588	↑**3.6**
SRY-box containing gene 7	Sox7	NM_011446	↑**3.6**
Iroquois related homeobox 2 (Drosophila)	Irx2	NM_010574	↑**3.5**
Signal transducer and activator of transcription 3, transcript variant 3	Stat3	ИС019168	↑**3.4**
Iroquois-class homeobox protein Irx6	Irx6	AF165986	↑**3.4**
Transcription factor AP-2, epsilon	Tcfap2e	NM_198960	↑**3.3**
SRY-box containing gene 9	Sox9	BC004064	↑**3.3**
SRY-box containing gene 5	Sox5	NM_011444	↑**3.2**
T-cell leukemia, homeobox 2	Tlx2	NM_009392	↑**3.2**
Zinc finger protein 30	Zfp30	NM_013705	↑**3.2**
Basic transcription element binding protein 1	Bteb1	NM_010638	↑**3.1**
H6 homeo box 1	Hmx1	NM_010445	↑**3.0**
Notch-regulated ankyrin repeat protein	Nrarp	NM_025980	↑**2.9**
Sine oculis-related homeobox 1 homolog (Drosophila)	Six1	NM_009189	↑**2.9**
General transcription factor IIE, polypeptide 1 (alpha subunit, 56kDa)	Gtf2e1	NM_028812	↑**2.9**
Paired box gene 6	Pax6	AF457142	↑**2.8**
Snail homolog 2 (Drosophila)	Snai2	NM_011415	↑**2.8**
Zinc finger protein 287	Zfp287	NM_133208	↑**2.7**
ISL1 transcription factor, LIM/homeodomain (islet 1)	Isl1	NM_021459	↑**2.7**
RE1-silencing transcription factor (REST) co-repressor 1	Rcor1	NM_054048	↑**2.7**
SRY-box containing gene 7	Sox7	NM_011446	↑**2.7**
Transforming growth factor beta 1 induced transcript 4	TGFβ1i4	NM_009366	↑**2.6**
TATA box binding protein	Tbp	NM_013684	↑**2.6**
Jun oncogene	Jun	NM_010591	↑**2.6***
Ladybird homeobox homolog 1 (Drosophila)	Lbx1	NM_010691	↑**2.5**
Transcription factor CP2-like 3	Tcfcp2l3	NM_026496	↑**2.5**

Sloan-Kettering viral oncogene homolog	Ski	NM_011385	↑**2.4**
Insulin related protein 2 (islet 2)	Isl2	NM_027397	↑**2.4**
Transcription factor 3	Tcf3	NM_009332	↑**2.4**
Forkhead box K1, transcript variant 2,	Foxk1	NM_010812	↑**2.3**
Transcription factor CP2-like 3	Grhl2	BC004783	↑**2.3**
Zinc finger protein 423	Zfp423	NM_033327	↑**2.3**
LPS-induced TN factor	Litaf	NM_019980	↑**2.2**
Scleraxis	Scx	S78079	↑**2.2**
Zinc finger protein 354A	Zfp354a	NM_009329	↑**2.0**
Circadian locomoter output cycles kaput	Clock	NM_007715	↑**2.0**
Nuclear receptor co-repressor 2	Ncor2	NM_011424	↑**2.0**
SRY-box containing gene 18	Sox18	NM_009236	↑**2.0**
Zinc finger protein 339, transcript variant A	Zfp339	NM_026924	↑**2.0**
Homeo box A4	Hoxa4	NM_008265	↑**2.0**
Lipin 1, transcript variant 2	Lpin 1	BC042462	↑**2.0**

Differences in expression validated by real-time PCR are shown by asterisks.

Appendix B

Genes Underexpressed in the Tumour cells of Hair Follicles of K14-Noggin Mice Versus Matrix cells of Hair Follicles of Wild-Type Mice (132 genes)

Adhesion (12)	Gene Symbol	Accession Number	Fold Change
Claudin 8	Cldn8	NM_018778	↓**4.3**
Transmembrane 4 superfamily member 13	Tm4sf13	NM_025359	↓**4.1**
Protocadherin 8	Pcdh8	NM_021543	↓**4.0**
Cadherin EGF LAG seven-pass G-type receptor 1	Celsr1	NM_009886	↓**3.6**
Synaptogyrin 1	Syngr1	NM_009303	↓**3.1**
Gap junction membrane channel protein beta 5	Gjb5	NM_010291	↓**2.9**
Protocadherin 10	Pcdh10	NM_011043	↓**2.9**
Embigin	Emb	NM_010330	↓**2.7**
Plasma membrane associated protein, S3-12	S3-12	NM_020568	↓**2.4**
Ttight junction protein 2	Tjp2	NM_011597	↓**2.1**
Nexilin	Nexn	NM_199465	↓**2.0**
Transmembrane 4 superfamily member 6	Tm4sf6	NM_019656	↓**2.0**
Extracellular matrix (4)			
Proteoglycan, secretory granule	Prg	NM_011157	↓**6.4**
Heparan sulfate (glucosamine) 3-O-sulfotransferase 1	Hs3st1	NM_010474	↓**6.2**
Glycoprotein m6b	Gpm6b	NM_023122	↓**6.2**
Fibronectin type III domain containing 5	Fndc5	NM_027402	↓**5.2**
Cell cycle/Apoptosis (4)			
SAM and SH3 domain containing 1	Sash1	NM_175155	↓**4.0**
Ring finger protein 34	Rnf34	NM_030564	↓**2.7**
Caspase 7	Casp7	NM_007611	↓**2.3***
Tumour differentially expressed 1	Tde1	NM_012032	↓**2.1**
Cytoskeleton/Keratinocyte Differentiation (21)			
Keratin associated protein 16-9	Krtap16-9	NM_130876	↓**44.3**
Keratin associated protein 8-1	Krtap8-1	D86423	↓**20.4**
Keratin complex 1, acidic, gene 1	Krt1-1	NM_010659	↓**18.2***

Crystallin, beta A4	Cryba4	NM_021351	↓**18.2**
Fatty acid binding protein 4, adipocyte	Fabp4	BC002148	↓**18.2**
Keratin associated protein 14	Krtap14	NM_013707	↓**17.8**
Keratin hair type II	Krt2-20	AY028606	↓**9.9***
Keratin complex 1, acidic, gene 2	Krt1-2	NM_010665	↓**8.3**
Keratin complex 1, acidic, gene 3	Krt1-3	XM_981536	↓**7.7**
Loricrin	Lor	NM_008508	↓**7.7**
Keratin associated protein 16-7	Krtap16-7	NM_130875	↓**5.0**
Keratin associated protein 16-10	Krtap16-10	NM_183296	↓**4.6**
Keratin associated protein 3-2	Krtap3-2	NM_025720	↓**4.1**
Prominin 2	Prom2	NM_178047	↓**3.0**
Keratin complex-1, acidic, gene C29	Krt1-c29	NM_010666	↓**2.5***
Keratin complex 2, basic, gene 1	Krt2-1	NM_008473	↓**2.4**
Keratin associated protein 3-1	Krtap3-1	XM_894811	↓**2.3**
Transglutaminase 1	Tgm1	NM_019984	↓**2.3**
Der1-like domain family, member 1	Derl1	NM_024207	↓**2.3**
Keratin-associated protein 16.6	Krtap16.6	AF345296	↓**2.2**
RIKEN cDNA A030014E15 gene	A030014E15Rik	XM_896395	↓**2.2**
Metabolism (26)			
Lipid metabolism (5)			
Sterol O-acyltransferase 1	Soat1	NM_009230	↓**4.4**
Aldehyde dehydrogenase family 1, subfamily A3	Aldh1a3	NM_053080	↓**4.1**
N-acylsphingosine amidohydrolase (alkaline ceramidase) 3	Asah3	NM_175731	↓**3.9**
Sphingomyelin phosphodiesterase 1, acid lysosomal	Smpd1	NM_011421	↓**3.4**
Lysophospholipase 2	Lypla2	NM_011942	↓**2.9**
Other metabolic pathways (21)			
Selenium binding protein 1	Selenbp1	NM_009150	↓**31.1**
Solute carrier family 6, member 13	Slc6a13	BC023117	↓**28.2**
Selenium binding protein 2	Selenbp2	NM_019414	↓**21.0**
Fructose bisphosphatase 1	Fbp1	NM_019395	↓**14.8**
Solute carrier family 7, member 8	Slc7a8	NM_016972	↓**6.8**
S100 calcium binding protein A3	S100a3	NM_011310	↓**6.3**
Microsomal glutathione S-transferase 1	Mgst1	NM_019946	↓**5.8**
Hormonally upregulated Neu-associated kinase	Hunk	NM_015755	↓**4.9**
S100 calcium binding protein A4	S100a4	AK021069	↓**4.8**
Cytidine 5'-triphosphate synthase	Ctps	NM_016748	↓**4.4**
Monoglyceride lipase	Mgll	NM_011844	↓**4.2**
SA rat hypertension-associated homolog	Sah	NM_016870	↓**3.3**
Prolyl endopeptidase	Prep	NM_011156	↓**3.3**

L-threonine dehydrogenase	Tdh	NM_021480	↓**3.1**
Fibroblast activation protein	Fap	NM_007986	↓**2.7**
B-box and SPRY domain containing	Bspry	NM_138653	↓**2.4**
Enolase 3, beta muscle	Eno3	NM_007933	↓**2.3**
Ornithine decarboxylase, structural	Odc	NM_013614	↓**2.1**
Glycine C-acetyltransferase	Gcat	NM_013847	↓**2.0**
Lleucine aminopeptidase 3	Lap3	NM_024434	↓**2.0**
Silver	Si	NM_021882	↓**2.0**
Proteolysis/Cytolysis (6)			
Stefin A1	Stfa1	NM_001001332	↓**38.7**
Serine (or cysteine) proteinase inhibitor, clade B, member 13	Serpinb13	NM_172852	↓**12.9**
Stefin 2-like	Stfa2l1	NM_173869	↓**10.3**
Stefin A3	Stfa3	NM_025288	↓**7.2**
Pitrilysin metalloprotease 1	Pitrm1	BC006917	↓**3.1**
Cathepsin E	Ctse	NM_007799	↓**2.1**
Signalling (29)			
Phosphatidylinositol 3-kinase catalytic subunit, beta isoform	Pik3cb	NM_029094	↓**26.9**
Major urinary protein 1	Mup1	NM_031188	↓**20.8**
Glial cell line derived neurotrophic factor receptor alpha 3	Gfra3	NM_010280	↓**12.8**
Rhophilin, Rho GTPase binding protein 2	Rhpn2	NM_027897	↓**11.4**
Major urinary protein 3	Mup3	NM_010845	↓**9.6**
Tumour necrosis factor receptor superfamily, member 13c	Tnfrsf13c	NM_028075	↓**7.0**
Bone morphogenetic protein 8a	Bmp8a	NM_007558	↓**6.7**
BMP and activin membrane-bound inhibitor, homolog	Bambi	NM_026505	↓**6.4**
Transient receptor potential cation channel, subfamily M, member 1	Trpm1	AF047714	↓**6.0**
Guanine nucleotide binding protein 13, gamma	Gng13	NM_022422	↓**5.1**
Secretin	Sct	NM_011328	↓**4.6**
Mitogen-activated protein kinase kinase kinase 6	Map3k6	NM_016693	↓**3.8**
TANK-binding kinase 1	Tbk1	NM_019786	↓**3.6**
Cadherin EGF LAG seven-pass G-type receptor 1	Celsr1	NM_009886	↓**3.1**
Four and a half LIM domains 2	Fhl2	NM_010212	↓**2.9**
Synaptotagmin-like 2	Sytl2	NM_031394	↓**2.7**
Plexin domain containing 2	Plxdc2	NM_026162	↓**2.6**
Wnt inhibitory factor 1	Wif1	NM_001915	↓**2.5***
Insulin-like growth factor binding protein 4	Igfbp4	NM_010517	↓**2.4**
Interferon gamma receptor	Ifngr	NM_010511	↓**2.4**
RAB32, member RAS oncogene family	Rab32	NM_026405	↓**2.3**

Estrogen-related receptor beta like 1	Esrrbl1	NM_028680	↓**2.3**
Presenilin 2	Psen2	NM_011183	↓**2.1**
Endothelin 3	Edn3	NM_007903	↓**2.1**
Myelocytomatosis oncogene	Myc	NM_010849	↓**2.1**
Docking protein 4	Dok4	NM_053246	↓**2.1**
Tumour necrosis factor receptor superfamily, member 25	Tnfrsf25	NM_033042	↓**2.1**
Protein C receptor, endothelial	Procr	NM_011171	↓**2.0**
Frizzled homolog 10 (Drosophila)	Fzd10	NM_175284	↓**2.0**
Transcription (30)			
Dachshund 1 (Drosophila)	Dach1	NM_001038610	↓**28.9**
Homeo box A7	Hoxa7	NM_010455	↓**7.3**
E74-like factor 5	Elf5	NM_010125	↓**7.0**
Four and a half LIM domains 1	Fhl1	NM_010211	↓**6.7**
Hhomeo box B8	Hoxb8	X13721	↓**4.5**
Trans-acting transcription factor 6	Sp6	NM_03118	↓**4.2**
Inhibitor of DNA binding 2	Id2	NM_010496	↓**3.4***
AT motif binding factor 1	Atbf1	NM_007496	↓**3.2**
Zinc finger protein 185	Zfp185	NM_009549	↓**3.1**
Four and a half LIM domains 2	Fhl2	NM_010212	↓**3.1**
Homeo box B7	Hoxb7	NM_010460	↓**3,0**
Hypermethylated in cancer 2	Hic2	NM_178922	↓**3,0**
Transcription factor AP-2 beta	Tcfap2b	NM_009334	↓**2.9**
Zinc finger protein 185	Zfp185	NM_009549	↓**2.9**
Homeo box C13	Hoxc13	AF193796	↓**2.9***
Naked cuticle 2 homolog (Drosophila)	Nkd2	NM_028186	↓**2.9**
Homeo box, msh-like 2	Msx2	NM_013601	↓**2.8**
Zic family member 1 (odd-paired homolog, Drosophila)	Zic1	NM_009573	↓**2.8**
WAP four-disulfide core domain 1	Wfdc1	NM_023395	↓**2.8**
Inhibitor of DNA binding 1	Id1	NM_010495	↓**2.4**
Homeo box, msh-like 1, mRNA	Msx1	NM_010835	↓**2.4**
Serologically defined colon cancer antigen 33	Sdccag33	BC017636	↓**2.4**
Homeo box A5	Hoxa5	NM_010453	↓**2.3**
Pleckstrin homology-like domain, family A, member 2	Phlda2	NM_009434	↓**2.2**
Hairy and enhancer of split 6 (Drosophila)	Hes6	NM_019479	↓**2.2**
Homeo box D4	Hoxd4	NM_010469	↓**2.2**
Inhibitor of DNA binding 4	Id4	NM_031166	↓**2.1**
Splicing factor, arginine/serine-rich 2 (SC-35)	Sfrs2	NM_011358	↓**2.1**
Lymphoid enhancer binding factor	Lef1	NM_010703	↓**2.1***
Iroquois related homeobox 3 (Drosophila)	Irx3	NM_008393	↓**2.1**

Differences in expression validated by real-time PCR are shown by asterisks.