ROLES OF WNT5A IN MODULATING ENAMEL EPITHELIUM TUMORIGENIC BEHAVIOR

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ABSTRACT

WALEERAT SUKARAWAN: Roles of WNT5A in Modulating Enamel Epithelium Tumorigenic Behavior
(Under the direction of Dr. J. Timothy Wright)

Odontogenic tumors originate from the remains of migrating enamel epithelium after the completion of normal tooth genesis. These enamel epithelium remnants exhibit the ability to recapitulate the events that occur during tooth formation. Several lines of evidence suggest that aberrance in the signaling pathways similar to the ones that are used during tooth development might be the cause of odontogenic tumorigenesis and maintenance. In this study we demonstrated that WNT5A (wingless-type MMTV integration site family, member 5A) expression was intense in both the epithelial component of ameloblastomas, the most common epithelial odontogenic tumor, and in the cervical loop area during normal human tooth formation. In addition, we discovered that enamel epithelium (LS-8) cell-derived clones expressing high (S) levels of WNT5A exhibited characteristics of tumorigenic cells, including growth factor independence, loss of anchorage dependence, loss of contact inhibition, tumor formation *in vivo* and increased cell migration when compared to controls (LS-8, EV). In order to elucidate the signaling pathways underlying these findings, pharmacological inhibitors specific for different downstream effectors in the known WNT5A signaling cascade were used in the *in vitro* cell growth transformation assay. We determined that specific JNK (c-Jun N-terminal kinase) (SP600125) and ROCK (Rho-associated, coiled-coil containing protein

kinase) (Y27632) inhibitors suppressed the tumorigenic growth properties of enamel epithelium overexpressing WNT5A. Furthermore, strong phosphorylated JNK expression was detected in ameloblastomas in a similar pattern to WNT5A expression. Finally, increased levels of phosphorylated JNK expression was observed in enamel epithelium clones that overexpressed WNT5A as well as cells treated with recombinant WNT5A. Our data indicate that the Rho/JNK pathway could be the downstream signal of WNT5A in modulating tumorigenic behaviors of enamel epithelium.

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AP-1	activating protein-1
APC	adenomatous polyposis coli
Aq	aqueous phase of Triton-X114 phase separation
AS	LS-8 clones derived expressing lower level of WNT5A
ATP	adenosine triphosphate
BMP	bone morphogenetic proteins
bp	base pairs
CaCN	calcineurin
CAMKII	calcium calmodulin-dependent protein kinase II
CDKs	cyclin dependent kinase
CK1	casein kinase 1
CL	cervical loops
CRD	cysteine-rich domain
DAB	3, 3'-diaminobenzidine
DAG	diacylglycerol
De	detergent phase of Triton-X114 phase separation
DKK	dickkopf
DM	dental mesenchyme
DMEM	Dulbecco’s modified Eagle's medium
DMSO	dimethyl sulfoxide

DNA	deoxyribonucleic acid
DP	dental papilla
DSH	dishevelled
DSPP	dentin sialophosphoprotein
DTT	dithiothreitol
EB	early bell stage of tooth development
ECM	extracellular matrices
EK	enamel knots
EMT	epithelial-mesenchymal transition
EV	empty vector
FAK	focal adhesion kinase
FBS	fetal bovine serum
FGFs	fibroblast growth factors
FITC	fluorescein isothiocyanate
FZD	frizzled
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GAPs	guanosine-5'-triphosphatase-activating proteins
GDIs	guanosine diphosphate dissociation inhibitors
GDP	guanosine diphosphate
GEFs	guanine nucleotide exchange factors
GSK-3ß	glycogen synthase kinase-3ß
GTP	guanosine-5'-triphosphate
h	hour

HRP	horse radish peroxidase
IE	inner enamel epithelium
IP3	inositol triphosphate
JNK	c-Jun N-terminal kinase
kD	kilodalton
LB	late bell stage of tooth development
LEF1	lymphoid enhancer binding factor 1
LRP	low density lipoprotein receptor-related protein
MAM	mature/secretory ameloblasts
MAPK	mitogen-activated protein kinase
MMPs	matix metalloprotease family
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt
NFAT	nuclear factor of activated T cells
NLK	nemo-like kinase
OD	odontoblasts
OE	outer enamel epithelium
PAM	pre-secretory ameloblasts
PBS	phosphate buffer saline
PCNA	proliferating cell nuclear antigen
PCP	planar cell polarity
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PI3K	phosphatidyl inositol 3 kinase

PKC	protein kinase C
pRb	growth-inhibitory protein, retinoblastoma
RIPA	radioimmunoprecipitation assay
RNA	ribonucleic acid
ROCK	Rho-associated, coiled-coil containing protein kinase
ROR2	receptor tyrosine kinase-like orphan receptor 2
RT	reverse transcription
RYK	receptor-like tyrosine kinase
S	LS-8 clones derived overexpressing higher level of WNT5A
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SH	LS-8 clones derived highly overexpressing WNT5A
SHH	sonic hedgehog
SL	LS-8 clones derived low overexpressing WNT5A
SM	LS-8 clones derived moderately overexpressing WNT5A
ß-TrCP	ß-transducin-repeat-containing protein
SV40	Simian virus 40
TCF	T cell factor
TGFβ1	transforming growth factor, beta 1
TNFs	tumor necrosis factors
UV	ultraviolet
VEGF	vascular endothelial growth factor
wg	drosophila wingless
WNT	wingless-type mouse mammary tumor virus integration site family

WT	wildtype LS-8 cells
α	alpha
β	beta
°C	degree Celsius
µ	micro
m	milli
n	nano
v/v	volume by volume

CHAPTER I

REVIEW OF LITERATURE

REVIEW OF LITERATURE

Regulation of enamel epithelium development (1-4)

Mammalian teeth develop from the oral ectoderm and the underlying neural crest derived mesenchyme that progress through a series of interactions during the early stages of embryogenesis. The connection between enamel epithelium and dental mesenchyme involves a variety of cell signaling required for mediating cellular processes during the progression of tooth development. Tooth development can be divided into four major stages: initiation and the formation of dental placode, tooth morphogenesis, differentiation of tooth forming cells, and secretion of dentin and enamel matrices. The universal signals during embryonic development such as, Wingless-type MMTV integration site family (WNT), Sonic Hedgehog (SHH), Fibroblast Growth Factors (FGFs), Tumor Necrosis Factors (TNFs), and Bone Morphogenetic Proteins (BMPs) are used repetitively at different, advanced stages of tooth synthesis (5).

Initiation of tooth development (5, 6)

The enamel epithelium plays important roles during the very early stages of tooth formation. It serves as an early signaling center to induce the odontogenic potential in the dental mesenchyme. The first stage of tooth development begins with formation of the dental placode, and thickening of the epithelial band along the oral ectoderm (1). This process is necessary for defining the tooth forming area as teeth further develop along this structure. A variety of signaling molecules involving multiple signaling pathways are active in this dental placode (2, 3). *Pitx2* is known to be the earliest dental marker

detected in future dental epithelial band (7). *Shh* and *lymphoid enhancer binding factor 1 (Lef-1)* are expressed early at the primary epithelial band at E11 in mouse embryonic development. Tissue recombination studies in mouse reveal that the first inductive signal comes from the enamel epithelium and after E12 in mouse embryo development, induction switches to the dental mesenchyme (8, 9).

Tooth morphogenesis (2, 10)

As tooth development proceeds, the early thickening enamel epithelium invaginates into the underlying neural crest derived mesenchyme and induces the mesenchymal condensation leading to the formation of the tooth bud (bud stage). At the late bud stage, groups of non-dividing cells at the tip of tooth bud form important structures called "the primary enamel knot" (11-13). Studies show that over 50 genes belonging to BMPs, FGFs, SHH and WNT signaling pathways are transcriptionally active in the primary enamel knot (http://bite-it.helsinki.fi). These reciprocal signals direct the morphogenesis of the tooth. The surrounding enamel epithelium undergoes massive proliferation and the flanking epithelium proliferates further into the condensed mesenchyme and forms the cervical loops (cap stage). The epithelial in growth is called the enamel organ that will eventually differentiate to become ameloblasts and produce the tooth enamel. Mesenchymal cells that are surrounded by the cervical loop form the dental papilla and become the odontoblast and dental pulp cells.

The final shape of the tooth crown is determined by the signals from the secondary enamel knot (2), a structure that is formed at the bell stage after the primary enamel knot is removed by apoptosis (14). Disruption of signals such as BMPs,

ectodysplasin A or FGFs during the bell stage leads to malformed cuspal patterns (15-18).

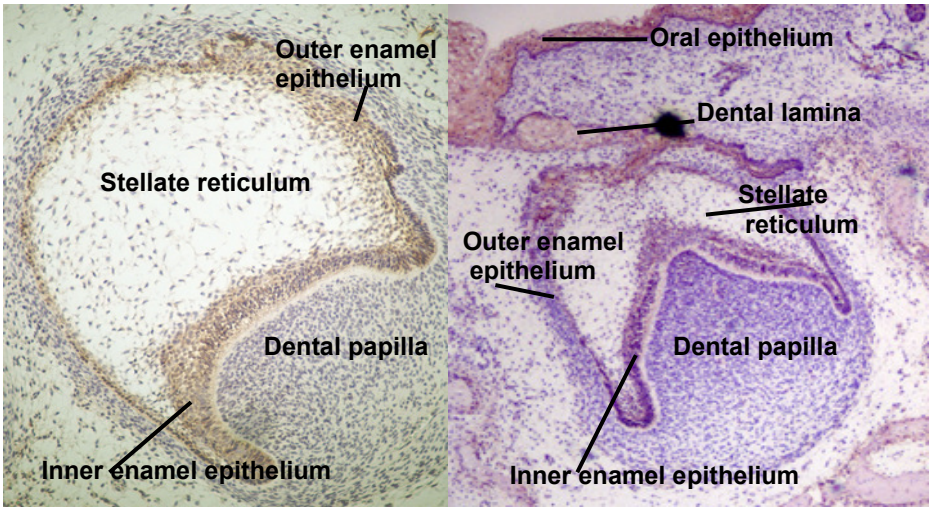


Figure 1.1 The cap and bell stages of tooth development. The enamel organ is expanding and undergoing massive histodifferentiation in the beginning of the early cap stage (left). The tooth has acquired its final shape and the structure is clearly distinguished in late bell stage (right).

Tooth-specialized cell differentiation and mineralization (10)

During tooth morphogenesis, the mass of tooth-specialized cells begin to differentiate into morphologically and functionally distinct components of tooth. Dentin and enamel, the mineralized structures of teeth, are formed by odontoblast and ameloblast differentiating from the mesenchyme and epithelium, respectively. The differentiation process begins at the bell stage of tooth development and continues in the cervical direction until the initiation of root development. The differentiation of odontoblasts and

ameloblasts is also regulated by the reciprocal epithelial-mesenchymal interactions similar to the morphogenetic regulation.

Odontoblasts

Preodontoblasts form a single layer of cylinder-shaped cells adjacent to the inner enamel epithelium. During differentiation, the preodontoblasts elongate and polarize to become odontoblasts that are tall columnar cells. A major function of odontoblasts is to secrete the dentin extracellular matrix that is rich in mainly type I collagen and contains non-collagenous proteins including proteoglycans, glycoproteins and dentin sialophosphoprotein (DSPP) (19). Odontoblast differentiation is tightly regulated by the inner dental epithelium (2, 20, 21). Studies show that TGFβ1 and -3 and BMP2, -4 and -6 can induce polarization of preodontoblasts and stimulate dentin matrix secretion (22, 23).

Ameloblasts

Enamel epithelium at the center of the enamel organ differentiates into the stellate reticulum cells that synthesize and secrete glycosaminoglycans. The cells at the periphery of the enamel organ differentiate into the outer enamel epithelium while the cells adjacent to the mesenchymal components differentiate into the inner enamel epithelium. Preameloblasts are derived from precursor cells of the inner enamel epithelium. During differentiation into secretory ameloblasts, they become highly columnar and polarized with oval-shaped nuclei elongated along the apical basal axis (1).

Ameloblasts go through a series of differentiation stages. During the secretory stage, functional ameloblasts produce and secrete an abundant of specific proteins into

the enamel matrix that will be largely replaced by the deposition of calcium and phosphate during the secretory and maturation stages of enamel formation (24). The principal components of the enamel matrix can be classified into two major categories; amelogenin, and nonamelogenin proteins. Amelogenin makes up approximately 90% of the enamel matrix and is one of the key factors in controlling crystal growth (25). Amelogenin is essential for the organization of the crystal pattern (26). Ameloblastin, enamelin, tuftelin, laminin 5 as well as proteolytic enzymes are also secreted by ameloblast (27). When the enamel matrix deposition is complete, the secretory ameloblasts shrink in size. Some secretory ameloblasts undergo apoptosis after maturation and the remaining cells, together with the outer enamel epithelium, form a protective layer on the enamel until the time of tooth eruption (28).

Ameloblast differentiation is tightly regulated by reciprocal epithelial-mesenchymal communications as well as by the presence of functional odontoblasts and/or predentin-dentin (29). It was recently shown that the main signal from odontoblasts inducing ameloblast differentiation was BMP4, and that activin release from the dental follicle antagonized the effect of BMP4 (30). SHH is also necessary for regulating cell proliferation within the dental epithelium and controlling cytodifferentiation of preameloblasts (31).

Odontogenic tumor (32, 33)

Odontogenic tumors are pathologies of the oral and maxillofacial region. They comprise a complex group of lesions from benign to malignant neoplastic tissue with metastatic capability. The term "odontogenic" refers to the derivation from a tooth-forming apparatus. Similar to normal odontogenesis, odontogenic tumors exhibit several characteristics of epithelium-mesenchyme interactions that occur during tooth formation. According to the latest World Health Organization classification in July 2005 (34), odontogenic tumors are classified according to their tissues of origin into epithelial, epithelial-ectomesenchymal, and ectomesenchymal neoplasms. Tumors of odontogenic epithelium contained only odontogenic epithelium. Epithelial-ectomesenchymal or mixed odontogenic tumors are composed of both odontogenic epithelium and ectomesenchyme. Lastly, odontogenic ectomesenchymal tumors are mostly composed of ectomesenchymal elements, but some odontogenic epithelium may be present without any role in pathogenesis.

Enamel epithelium remnants: origin of epithelial odontogenic tumor

After the formation of the crown, the inner and outer enamel epitheliums join together at the junctional zone called the cervical loop. Enamel epithelium continuously proliferates in an apical direction to the root area of the tooth and it is necessary for initiating the signals required for root formation. The fused extensions of these enamel epitheliums are call Hertwig´s epithelial root sheath. After tooth formation is completed, this Hertwig´s sheath later becomes fragmented but leaves quiescent small islands of epithelial cells called epithelial rests of Malassez in the periodontal ligament space.

Epithelial rests of Malassez are the only odontogenic epithelium cells that remain after teeth erupt and they still possess the potential to proliferate and differentiate (35-39). They are believed to be responsible for the development of several epithelial odontogenic cysts and tumors such as ameloblastoma (1, 40).

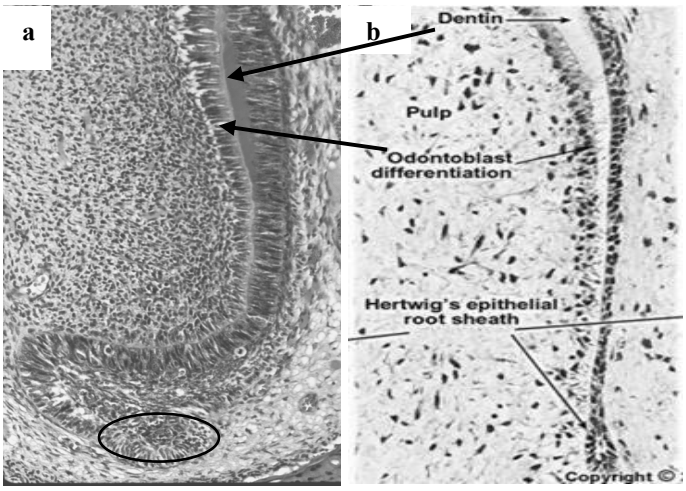


Figure 1.2 Enamel epithelium remnants: origin of odontogenic tumor. After tooth formation is completed, enamel epitheliums are left in the periodontal ligament area and potentially give rise to odontogenic tumor. (a) Tumor forming in the mouse incisor (circled) initiated from enamel epithelium remnants at the root area. (b) Hertwig´s epithelial root sheath leaves epithelial cells in the periodontal ligament area after tooth genesis. Modified from (1).

Ameloblastoma

Ameloblastoma, a usually benign but locally invasive tumor, is the most common odontogenic neoplasm derived from odontogenic epithelium. It has a high recurrence rate, persistent growth with the high ability to produce marked deformity of the face, but rarely becomes malignant. The term “ameloblastoma” is derived from the histology of the tumor that resembles the developing tooth germ. Ameloblastoma tends to recapitulate the incomplete events that occur during normal tooth formation (40, 41). It exhibits soft

tissue of the enamel organ and contains a mixture of tooth elements. For instance, one important feature of ameloblastoma is the island of odontogenic epithelium with the polarized cuboidal or columnar cells around the loosely angled shaped cells in a pattern similar to ameloblasts and stellate reticulum. In some cases, enamel matrix proteins are enumerated and can be found in the lesions.

Clinical features and tumor behavior

Ameloblastomas occur over a wide range of ages from children to the elderly. Due to the slow growing nature of this tumor, patients are usually diagnosed in the fourth and fifth decades of life. Gender predilection is still controversial. Ameloblastoma has a higher incidence in Asian populations (42-46). In particular, ameloblastoma is more commonly encountered in Africans, where it accounts for about 60% of odontogenic tumors (33). The frequency of ameloblastoma is rare in Caucasians representing about 11% of odontogenic tumors. Not surprisingly, it is more common in African-Americans (33). The most prevalent location of ameloblastoma is the mandible (80%), of which 70% occur in the molar region. In the maxilla, most lesions also arise in the molar area.

Ameloblastoma is a slow growing tumor and may be asymptomatic in the early stages of tumor development. In advanced stages, patients may present with swelling of the jaw bone and become aware of marked facial disfigurement. The enlargement of these tumors is usually confined in bone, but in advanced cases, bony perforation and soft tissue overgrowth can be found due to the extensive destruction of overlying bone. In the maxilla, minor bony expansion can be observed since the lesion can protrude into sinus spaces. Adjacent teeth usually become loosened and resorption of roots is common, but

rarely painful. Ameloblastoma has a high recurrence rate (50-90%), therefore surgical resection with a margin of normal bone is the preferred treatment.

Histopathology

Ameloblastomas can be classified according to their histological patterns as follicular, plexiform, granular cell, acanthomatous, desmoplastic and basal cell types (40). The follicular and plexiform types are the most common patterns and in some tumors the mix of both patterns exists. No consistent differences in terms of tumor behavior have been found among the different histological types (33, 41). In the follicular pattern (Figure 1.3, right), epithelial islands form around nests or follicles which resemble the enamel organ of developing teeth. Each follicle is occupied by stellate-reticulum like cells in the center surrounded by a single layer of reverse polarized ameloblast-like cells. In the plexiform type (Figure 1.3, left), tumor epithelium forms a network of interconnected strands. Each epithelium strand contains the same components as in the follicular pattern, thus each strand is connected by ameloblast-like cells that encircle the stellate reticulum-like cells. In some cases, stellate-reticulum like cells can be located outside of the epithelium strands.

Minor variations of other histological types include the acanthomatous type where the odontogenic epithelium exhibits squamous differentiation. The granular cell type is characterized by cells that have abundant granular eosinophilic cytoplasm in the tumor nests. The desmoplastic type demonstrates a high content of collagenized stroma that supports the tumor. The uncommon basal cell type contains nests of basaloid cells similar to basal cell carcinoma.

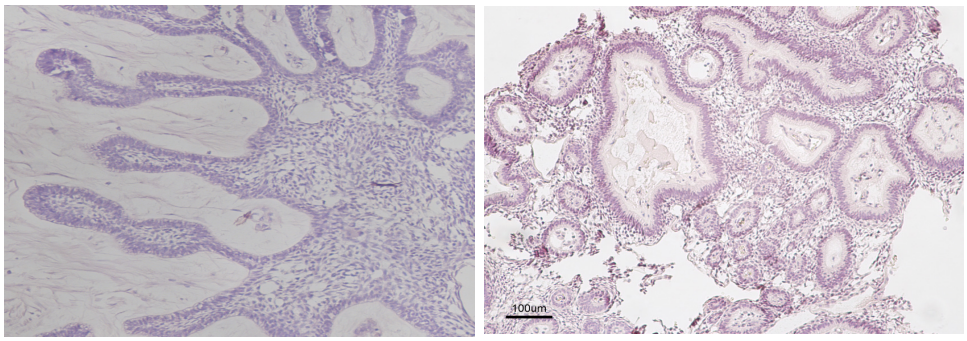


Figure 1.3 Histology of ameloblastoma. The plexiform and follicular patterns are the most common histological subtypes of ameloblastoma. Plexiform ameloblastoma shows the network of interconnected epithelium strands (Left). Follicular ameloblastoma shows the peripheral cuboidal/columnar cells and central stellate-reticulum like cells (Right).

Subtypes of ameloblastoma (41)

According to the new classification of head and neck tumors by World Health Organization in July 2005 (34), ameloblastoma is categorized in relation to age, distribution, localization, radioimaging features, and prognosis to four variants, solid/multicystic ameloblastoma, extraosseous/peripheral ameloblastoma, desmoplastic ameloblastoma and unicystic ameloblastoma.

In an analysis of the international literature, 3,677 cases of ameloblastoma were reviewed, of which 92% were solid or multicystic, 6% were unicystic, and 2% were peripheral (47).

Solid or multicystic ameloblastoma

The solid or multicystic variant of the ameloblastoma occurs in patients over a wide age range being more common in the third through seventh decade of life (48, 49). The most common radiographic feature is a multilocular radiolucency with cortical bone expansion. All histological patterns of ameloblastoma, follicular, plexiform, granular cell, acanthomatous, desmoplastic and basal cell types, can present as multicystic ameloblastoma.

Unicystic ameloblastoma

The unicystic type of ameloblastoma is most commonly seen in young patients, with about 50% of these tumors being diagnosed during the second decade of life (47). The most common radiographic presentation for the unicystic ameloblastoma is a well-defined unilocular radiolucency, usually associated with an unerupted tooth. Histologically, three variants of unicystic ameloblastoma are presented. Luminal unicystic ameloblastoma is a cyst lined by ameloblastic epithelium. The intraluminal unicystic ameloblastoma contains a localized nodular proliferation of ameloblastoma tissue into the cystic lumen. These nodules usually exhibit a typical plexiform pattern of ameloblastoma. A third variant is described as mural unicystic ameloblastoma where ameloblastic tissue infiltrates the fibrous wall of the cyst. This type is important to distinguish because the extent and depth of the ameloblastic infiltration of the tumor influences considerably the treatment procedure.

Peripheral ameloblastoma

This rare type of ameloblastoma usually occurs over a wide range of ages. The clinical presentation of peripheral or extraosseous ameloblastoma is a non-ulcerated sessile or pedunculated gingival lesion. The origin of this variant could be the rests of dental lamina or the basal cell of the surface epithelium (50).

Desmoplastic ameloblastoma (51-53)

Desmoplastic ameloblastoma is a rare variant that presents with several different characteristics from typical ameloblastoma. From the review of case reports (51, 53), the majority of these tumors are located at the anterior or premolar region of the jaws. Moreover, half of the lesions occur in the maxilla which is in contrast to the other forms of ameloblastomas that favor the mandibular molar area. Histological appearance of desmoplastic ameloblastoma is also different. This tumor exhibits small nests and/or strands of odontogenic epithelium that are separated by dense fibrous connective tissue (stromal desmoplasia) (52, 54, 55). Columnar or cuboidal cells with reverse polarity that resemble ameloblast cells can be seen in the lesion but are not the dominant feature. However, age and gender prevalence are similar to those found with other variants of ameloblastoma.

Treatment and prognosis

Treatment of ameloblastoma continues to be controversial due to the benign but locally aggressive nature of the tumor. Although aggressive surgical excision treatment is generally accepted as the most reliable and necessary approach, these tumors still

frequently recur. Recurrence rates of ameloblastoma are reportedly as high as 15% to 25% after radical excision treatment (56-59) and 75% to 90% after conservative excision treatment (57, 60, 61). Numerous treatments have been suggested for ameloblastoma depending on several factors such as, type of tumor, location, extent of disease, histological type, radiographic presentation as well as patient age. Treatments range from simple enucleation with bone curettage (62, 63) to radical resection (64). Unicystic ameloblastoma is well localized by the fibrous capsule of the cyst and can be curable by a conservative approach. The mural unicystic ameloblastoma where ameloblastic tissue infiltrates the fibrous wall of the cyst requires treatment with radical surgery. Multicystic ameloblastoma has more invasive characteristics and typically infiltrates the adjacent tissue and the cancellous space; a multicystic ameloblastoma therefore must be treated by wide resection of the jaw (65, 66). Malignant transformation of ameloblastoma is very rare, but may be classified as metastasizing ameloblastoma or the more aggressive ameloblastic carcinoma (67).

While ameloblastoma is likely curable after radical resection and when all soft and hard tissue margins are histological negative, recurrence can still occur in some cases. Several studies have tried to identify indicators to predict for recurrence of this tumor. Recurrent ameloblastoma is associated with high numbers of proliferating cell nuclear antigen (PCNA) positive cells compared with the primary tumors (68). Solid multicystic ameloblastoma also has more nuclear PCNA positive cells compared with the unicystic ameloblastoma that is less aggressive in behavior (68, 69). Other molecules involved in cell cycle, such as highly expressed *BCL2* and *BCLX,* are reported to support the aggressive behavior of these ameloblastomas (70, 71). Unfortunately, there are no

radiological, histological or molecular markers known to predict with reliability the recurrence tendency of ameloblastoma.

Molecular pathology

The molecular determinants driving initiation and maintenance of ameloblastomas and other odontogenic tumors remain unclear. The investigation and systematic study of the molecular mechanisms operating in these tumors is scarce due to the lack of appropriate cell culture and/or animal models. However, there is evidence from gene and protein expression studies that suggest alteration in signaling pathways that play a role during normal tooth development. It therefore seems likely that TNF, FGF, SHH and WNT pathways might contribute to the etiology of odontogenic tumor (72-75). There is no known genetic mutation related with pathogenesis of ameloblastoma. Studies have shown that TNFα can induce cell survival and proliferation in ameloblastoma cells, AM-1, through AKT and p44/42 mitogen activated protein kinase (MAPK) activation (76, 77). *BCL2* and *BCLX* are reported to support ameloblastoma cell survival by inhibition of apoptosis (70, 71). Aberrant expressions in both canonical and non-canonical WNT pathways such as WNT1, β-catenin and WNT5A, are found in ameloblastoma as well (78-81), however the molecular details of how alteration in WNT signaling or other pathways affect the tumorigenic behavior of enamel epithelium have not been studied.

Characteristic of tumorigenic cell (82)

Transformation of normal cells into tumorigenic cells is a multistep process that requires several alterations in cell behavior. Most tumorigenic cells share six essential changes in behavior that are self-sufficiency in growth signals, insensitivity to anti-growth signals, avoidance of programmed cell death, limitless cell replication, induced angiogenesis, invasion and metastasis (82). These processes can occur at different time points during tumorigenesis and they are mostly governed by mutations in anti-cancer defense mechanisms that lead to chromosome instability and accumulation of mutations (82).

Proliferation

Eukaryotic cell proliferation involves the progression through cell cycle. It consists of the following phases: G1 phase is the phase that cells enter after mitosis. At this stage, most cells leave the cell cycle and remain in the resting stage in a non-proliferative G0 phase. After receiving growth stimuli, cells can reenter the G1 phase and prepare for cell division. Next is the synthetic, S, phase where DNA is duplicated and preparation for cell division proceeds in the subsequent G2 phase. The final step in the cell cycle is mitotic, M, phase, where the parental cell and its duplicated chromosomes segregate into two daughter cells in G1 phase. Cell cycle is tightly regulated by a number of proteins that control the entry and exit of cells from different phases of the cell cycle. The most important "checkpoints" in the cell cycle occur at the late G1 phase and the late G2 phase where the accuracy of the genome is crucial. The major regulators of cell cycle are cyclin/cyclin dependent kinase (CDKs) complexes. Together with cyclins, CDKs play

an important role in phosphorylation and inactivation of the growth-inhibitory protein, retinoblastoma (pRb) (83). pRb induces regulation of the E2F transcription factor and is important for preventing cells in G1 phase from entering S phase, therefore deactivation of pRb results in increasing cell proliferation.

Many cancer cells have the ability to stimulate their own proliferation via the production of their own growth signals, activating mitogenic signals and deactivating the growth-inhibitor signals. Common mitogenic signals that are known to regulate cell cycle proteins for example; Ras/Raf/MAPK pathway, C-MYC and β-catenin are frequently upregulated in several types of cancer (84-86). Loss of growth inhibitory or tumor suppressor signals, such as p53 and p21, are often found and mostly involved in subsequent deactivation of pRb (87).

Limitless of cell replication

Normal cells possess a certain potential for the number of cell replications. After reaching the limit of doublings, they enter the state called senescence. This process is governed by the mechanism that shortens the ends of the chromosome, the telomeres. During each cell cycle, cells lose about 50-100 bp of telomeric DNA and this progressive shortening eventually prevents further replication of the chromosomes and results in the death of the cells (88).

Telomere maintenance is a key to limitless replication of tumorigenic cells. The chromosome end is protected by the enzyme "telomerase" which adds hexanucleotide repeats onto the ends of telomeric DNA (89). Upregulation of telomerase in tumors helps to protect the chromosome in the remaining part of the tumors by the mechanism called

alternative lengthening of the telomeres. Telomeres are thus protected at a length above a critical threshold, allowing unlimited replication potential of tumorigenic cells (90).

Apoptosis

Apoptosis is a mechanism of cell death that serves as an intrinsic protection system to eliminate unwanted or harmed cells from the body. Most tumorigenic cells acquire the ability to evade apoptosis to undergo malignant transformation (91). The process of apoptosis begins with the condensation and fragmentation of DNA, followed by cell shrinkage, membrane blebbing, and eventually phagocytosis of the remaining apoptotic bodies. In contrast to necrosis induced cell death, apoptosis occurs without inducing inflammation (92). Apoptosis can proceed via two different pathways. The first pathway involves the series of cleavage induced activations of several different proteases called caspases. A second pathway occurs in a caspase independent manner through the activation of proteases like cathepsins and calpains (93). Apoptosis can be induced by extrinsic and intrinsic stimuli, such as activation of the death receptors FAS and TNFs, oncogenic stress, hypoxia and ultraviolet (UV) irradiation (94). In response to cellular stress, p53, a tumor suppressor, is a key molecule in initiating apoptosis. Activated p53 is accumulated in the nucleus and triggers irreversible cell cycle arrest and apoptosis (95). p53 mutations are frequently observed in cancer cells. Another means for tumorigenic cells to escape apoptosis is via deactivation of pro-apoptotic proteins such as caspases, FLICE inhibitory protein, and the human BCL2 family, and/or upregulation of survival genes like the phosphatidyl inositol 3 kinase (PI3K) pathway (96).

Sustained angiogenesis

The ability to induce and maintain angiogenesis is necessary for the growth of tumors. New blood vessels provide oxygen and nutrients, and also take away metabolic waste products from tumors. Moreover, tumor proliferation is stimulated by abundant growth factors, such as FGFs, granulocyte colony-stimulating factors, and platelet-derived growth factor (PDGF), which are produced by vascular endothelial cells. One mechanism allowing tumors to acquire neovascularization is via the change in balance between angiogenesis inducers and inhibitors (97). Vascular endothelial growth factor (VEGF) and FGFs are common angiogenesis inducers and their expressions are upregulated in many cancers, whereas angiogenesis inhibitors such as thrombospondin-1 or β-interferon are downregulated (98). Notably, the amount of angiogenesis is related to metastasis and prognosis of cancers (99), therefore tumor angiogenesis offers a promising target for cancer therapy.

Metastasis

Although metastasis is a very rare event for odontogenic tumors, it can be observed in untreated or recurrent ameloblastoma (67). The metastatic process occurs through the following steps; loss of adhesion from primary tumor, breakdown of the extracellular matrices (ECM), cell migration into the blood or lymphatic vessels, transportation in the circulation, adhesion and migration out of vessels and establishing a tumor in a new environment.

Cell adhesion

Several classes of proteins involved in cell adhesion are altered in invasive or metastatic cells. Epithelial cells normally adhere to each other via diverse structures, such as tight junctions, adherens junction and desmosomes (100). They are also attached to ECM via the cell-matrix adhesion receptors called integrins. Integrins exist as heterodimers of α and β subunits that give rise to at least 24 different heterodimers, each of which can bind to specific ECM ligands (101). Activation of integrins plays an important role in cell adhesion and migration. Upon binding to ECM ligands, cytoplasmic tails of integrins sequester a range of proteins to form focal adhesion complexes at the ECM/integrin junction. Actin cytoskeleton-binding proteins, tensin and talin then bind to the cytoplasmic tail of integrins and thereby link the cytoskeleton to the focal adhesions (101).

Another important group of cell adhesion proteins is the cell-cell adhesion molecules and some of the most important proteins in that context are cadherins (E, N and H-cadherins) and selectins (E, L and P-selectins) families. These molecules are responsible for cell-cell adhesion and also for intravasation and extravasation of tumor cells. It is noteworthy that, loss of cell adhesion, especially repression of E-cadherin, indicates the events of epithelial-mesenchymal transition (EMT) and results in the initiation of cancer metastasis (102).

Extracellular matrix degradation

The ability to degrade ECM is critical for tumor cell invasion and metastasis. The ECM degradation process involves the regulation of extracellular proteases (103, 104).

Increased expression of protease genes and/or downregulation of protease inhibitors are associated with tumor cell invasion (105). Expression of ECM proteases can be regulated by several stimuli, for example epidermal growth factor, PDGF, TNF-α and interleukin 1 are reportedly inducing ECM protease production (106). The matixmetalloprotease family (MMPs) is believed to play an essential role in tumor invasion and metastasis since they are important for angiogenesis and cell migration.(107, 108)

Cell migration

Cell migration is a cyclical process that involves the dynamic of actin reorganization and ECM adhesion. Cell motility is a result of actin polymerization that leads to cell membrane protrusion at its front and retraction at its rear (109). Formation and disassembly of focal adhesion are keys of cell migration. Focal adhesion synthesis leads to the activation of several signaling molecules, for examples focal adhesion kinase (FAK), SRC, PI3K, and the RHO GTPases (109), consequently generate mechanical force for cell movement by inducing the interaction between myosin II and actin filaments (110).

Actin cytoskeleton reorganization

Actin is a cytoskeleton filament that plays a major role in controlling cell movement (109). Constant polymerization and remodeling of actin fibers is necessary for membrane protrusion at the cell's leading edge, which causes the cell to move forward, and allows the formation of the retractile stress fibers that are required for contraction of the cell at trailing edge (111). Actin polymerization occurs by the continuous addition of

actin monomers to the plus end until the elongation is ceased by the binding of actin capping proteins such as gelsolin to that end (109). On the minus end of actin filament, disassembly of actin monomers is controlled by cofilin (112). Two major structures of membrane protrusion can be found in motile cells, lamellipodia refers to a broad, flat and lattice-like membrane extension, whereas filopodia is thin, cylindrical and bundled in shape (113). Another major protein important in cell migration is myosin II. Adenosine triphosphate (ATP) hydrolysis by myosin II causes conformational change that allows myosin to move along and bind to actin filaments, therefore myosin II acts as a mechano-chemical enzyme that generates energy for cell movement from ATP hydrolysis (114).

The Rho family

The Rho family of small guanosine triphosphate (GTP)-binding proteins is known to be responsible for cell migration in many cell types. The Rho family is comprised of RHO (RHOA, B and C), RAC (RAC1, 2 and 3) and CDC42 that all play important roles in regulating cell movement at specific sites. At the leading edge of migrating cells, activation of RAC and CDC42 is required for lamellipodia and filopodia formation respectively (113), while activation of RHO is critical for cell contraction at the trailing edge (115). Like other proteins in the GTPase family, RHO GTPases exist in the dynamic active GTP-bound and inactive guanosine diphosphate (GDP)-bound forms. The cycle of RHO GTPase is regulated by: the guanine nucleotide exchange factors (GEFs) that facilitate the GDP-to-GTP exchange, the GTPase-activating proteins (GAPs) that enable hydrolyzation of GTP to GDP, and the GDP dissociation inhibitors (GDIs) that bind to the inactive form of RHO GTPases and inhibit the GDP-to-GTP exchange (115).

Focal adhesion kinase

The formation of new focal contact at the front end and cell adhesion disassembly at the rear end are fundamentals of cell migration. The rate of migration is determined by the strength and stability of focal adhesion (110, 116). Focal adhesion turnover is regulated by a tyrosine kinase called FAK. Focal contacts that are formed at the ECM-integrin junction activate the assembly of intracellular focal adhesion complexes and the recruitment of actin cytoskeleton together with several signaling molecules. This provides a link between focal adhesion complexes and the cytoskeleton (117). FAK is also important for activation of RAC and CDC42 (118, 119). An abundance of proteins participate in cell migration. For instance, β-actin, p130CAS and paxilin, are substrates for FAK phosphorylation. FAK itself can trigger cell proliferation by recruiting the adapter protein GRB2 that leads to activation of RAS (120).

The WNT family and WNT signaling pathways

The WNT family constitutes one of the major developmentally significant groups of signaling molecules that control a variety of processes such as cell fate specification, cell proliferation and differentiation, cell polarity, cell adhesion, cell migration, and cell death (121-124). All WNT proteins share the similar structure of 39-46 kD lipid-modified secretory glycoproteins containing 350-400 amino acids, with a highly conserved pattern of 23-24 cysteine residues and several asparagines-linked glycosylation sites (123). Conserved cysteine residues serve as the sites for palmitoylation, lipid modification which is critical for WNT protein function (125). The founding member of the WNT family is mouse *Wnt1* and Drosophila wingless (*wg*) (126). Orthologs of *Wnt1* and *wg* are

conserved among species and have been found in many organisms from C. elegans to human, with each organism possessing multiple related *Wnt* genes (127). At least nineteen WNT proteins have been identified in vertebrates to date (128).

WNT proteins are generally classified into two functional classes; the WNT1 class and the WNT5A class. The WNT1 class includes the inducers of a secondary body axis in *Xenopus* which are WNT1, WNT2, WNT3, WNT3A, WNT8A and WNT8B. Interaction by WNT proteins of this class to their receptors ultimately leads to the activation of the canonical WNT/β-catenin pathway (128). The WNT5A class includes WNT4, WNT5A, WNT5B, WNT6, WNT7A and WNT11 that do not induce secondary axis formation in *Xenopus*. The WNT5A class proteins interact with their receptors resulting in activation of several alternative downstream pathways, involving increasing intracellular calcium levels (128). However, recent evidence shows that WNT proteins can generate distinct physiological responses by triggering various intracellular pathways depending on the receptors and other contexts that present at the cell surface (129-131).

After WNT proteins are secreted, their activity can be modulated by a variety of regulators. In vertebrates, WNT antagonists, such as secreted frizzled-related protein and WNT inhibitory factor, directly bind to WNT protein and prevent the interaction of WNT proteins to their receptors (132, 133). Another potent WNT inhibitor is dickkopf (DKK). The mechanism of DKK inhibitory function is unclear but is likely due to the ability of DKK to bind to WNT co-receptors and induced co-receptor internalization (123).

Receptors and coreceptors in WNT pathway (127, 128)

Different WNT proteins initiate the various cellular responses due to the repertoire of receptors at the cell surface. The major receptors of the WNT ligands are Frizzleds (FZDs). Several members of the FZD family have been identified in many organisms with each organism possessing multiple *Fzd* genes. *Fzd* genes encode seven-transmembrane, G-protein-coupled proteins (134, 135) containing a cysteine-rich domain (CRD) at the amino-terminal that binds WNT protein with high affinity (136). A single WNT protein can bind to a combination of FZDs, including FZD homologs from different species (137, 138). Binding of WNT protein to FZD activates either the canonical or non-canonical WNT signaling pathway leading to specific biological functions. FZD2, FZD4 and FZD5 have been shown to be the receptor for WNT5A and can initiate diverse signaling pathways and cellular responses (131, 139-141).

In addition to FZDs, other co-receptors are necessary for the WNT signaling pathway. The binding of WNT ligands to Low density lipoprotein receptor-related protein5/6 (LRP5/6), a singlepass transmembrane receptor, is also required for activation of the canonical WNT signaling pathway (142, 143). Recently, other co-receptors for the WNT signaling pathway have been identified. RYK (receptor-like tyrosine kinase) belongs to the receptor tyrosine kinase family shown to regulate both canonical and non-canonical WNT signaling pathways via an unclear mechanism (144, 145). ROR2 (receptor tyrosine kinase like orphan receptor 2) is an orphan tyrosine kinase receptor that contains an extracellular CRD. Mouse ROR2 has been shown to be the alternative receptor for non-canonical WNT5A/JNK signaling involved in cell migration and cell

adhesion (146, 147). ROR2 is also necessary for mediating WNT5A antagonist effects on the canonical WNT signaling pathway (131).

The canonical WNT/β-catenin signaling pathway

The majority of WNT proteins including WNT1, WNT3A, and WNT8 activate downstream target gene expression by the transcriptional factor, β-catenin. In the absence of WNT protein, β-catenin in the cytoplasm is associated with the destruction complex of glycogen synthase kinase-3β (GSK-3β), axin and adenomatous polyposis coli (APC). β-catenin is subsequently phosphorylated at the conserved serine and threonine residues in the amino-terminal region by casein kinase 1 (CK1) and GSK-3β. These phosphoamino acids serve as the active sites for β-transducin-repeat-containing protein (β-TrCP) /Slimb, a component of the supercoiling factor ubiquitin ligase complex. β-TrCP/Slimb polyubiquitinates and targets β–catenin to degradation by proteasome. As a result, β-catenin in the cytoplasm cannot translocate into the nucleus and the transcription of Wnt target genes is silenced (148, 149).

In the presence of WNT protein, the binding of WNT ligand to FZD results in the formation of a WNT-FZD-LRP5/6 complex. This interaction is followed by recruitment and phosphorylation of dishevelled (DSH) that subsequently binds and inhibits GSK-3β activity (150, 151). In addition, DSH also recruits axin to the cell membrane where it binds to WNT-FZD-LRP5/6 complex and promotes axin degradation (152). Without the formation of the destruction complex, phosphorylation of β-catenin and its degradation are impaired. The accumulating β-catenin in cytoplasm then translocates to the nucleus. In the nucleus, the T cell factor (TCF) and LEF are associated with transcriptional

repressors, such as groucho, at the promoter region of WNT target gene. β-catenin activates TCF/LEF by displacing the transcriptional repressor and forming a complex with TCF/LEF (150, 153-155). This leads to the transcription of a variety of WNT target genes that play important roles for regulating cell fate, proliferation, differentiation, adhesion and survival such as *c-Myc* (156), *Cyclin D1* (157-159), *Cyclooxygenase 2*, *Vegf* and *Axin-2* (160, 161).

The non-canonical WNT signaling pathway

In the non-canonical WNT signaling pathway, the WNT5A class of proteins consists of WNT4, WNT5A, and WNT11 that function through a non β-catenin-dependent fashion, such as the planar cell polarity (PCP) pathway (157, 162-164) and the WNT calcium-dependent pathway (165, 166). However, the signaling mechanism in the non-canonical WNT pathway remains unclear.

WNT/calcium pathway

In the WNT/calcium pathway, binding of WNT5A to FZD receptors, especially FZD2, leads to several cellular mechanisms that involve increased intracellular calcium release. Several enzymes in the calcium signaling pathway including calcium calmodulin-dependent protein kinase II (CAMKII) or calcineurin (CaCN) and protein kinase C (PKC) are activated in WNT/calcium pathway (139, 166-168). Studies have shown that WNT5A activates protein kinase G and phosphodiesterases that in turn leads to a sharp decrease in cyclic guanosine monophosphate levels (169, 170). The mechanism of intracellular calcium release is believed to occur via stimulation of Gαo and Gαt2 in

the G-protein dependent signaling pathway (169, 171). As a result, phospholipase Cβ is activated and translocated to the cell membrane, where the second messengers inositol trisphosphate (IP3) and diacylglycerol (DAG) are generated (165, 172). IP3 then interacts with the sarco/endoplasmic reticulum calcium transporting ATPase pump at the endoplasmic reticulum membrane and induces the release of calcium, whereas DAG activates PKC. These processes have been shown to subsequently stimulate the nuclear factor of activated T cells (NFAT) transcriptional factors (173-175). WNT/calcium signaling has been observed in zebrafish embryos (165, 176, 177), mouse cells (169, 178), and in thyroid carcinoma cells (179). In addition, FZD3, -4, -5, and -6 are also known to mediate PKC activation (167, 168).

WNT/PCP pathway

WNT5A/PCP signaling is regulated by PDZ and DEP, the two conserved domains of DSH (180-182). Recruitment of DSH leads to activation of JNK (183, 184), a member of MAPKs family that is primarily responsive to environmental stress and cytokines (185). Engagement of DSH by WNT5A also involves the activation of RHOA/RAC small GTPase (182) and RHO-associated kinase, ROCK, (186, 187), which in turn leads to the regulation of cytoskeletal organization, as well as activation of JNK (180, 186). JNK phosphorylates C-JUN and activates transcriptional factor AP-1. However, the requirement of DSH and RHO GTPases on JNK activation is still controversial (183). The WNT/PCP pathway is essential in epithelial cell polarity of Drosophila and convergent extension movement during gastrulation of vertebrates (188).

Interaction between non-canonical and canonical WNT pathway

In addition to WNT5A activity through the non-canonical WNT signaling pathway, several studies have shown that non-canonical WNT5A signals can interact with the canonical WNT/β-catenin pathway in a variety of ways (139, 189). WNT5A activates nemo-like kinase (NLK) through CAMKII and in turn suppresses the transcriptional activity of TCF/LEF by inducing phosphorylation (139, 188, 190, 191). In addition, binding of WNT5A to ROR2 initiates RHO GTPase-independent activation of JNK that can inhibit the canonical WNT pathway (188). WNT5A also can promote β-catenin degradation in a GSK-3 β-independent fashion by increasing expression of *Siah2* (190).

Although it appears that particular WNT proteins preferentially stimulate the canonical or non-canonical pathways, the distinction is not absolute. WNT signaling specificity may be achieved, in part, by the combination of the cell surface receptor and intracellular context that differs from cell type to cell type (192, 193). Recently, WNT5A has been shown to activate or inhibit the canonical WNT signaling pathway depending on the expression of appropriate FZD receptors and the co-receptors. In the presence of FZD4 and LRP5, WNT5A can activate canonical WNT signaling pathway. On the other hand, WNT5A antagonizes canonical WNT signaling when ROR2 is available (131). The WNT5A/PCP cascade is also known to stabilize β-catenin (194). Moreover, C-JUN, a downstream target of the non-canonical pathway, is found to act synergistically with TCF/LEF to promote gene transcription in intestinal cancer (195). Evidently, WNT5A signals are complex and closely cross-linked. These findings support the model that signaling by WNT5A can be dictated by the availability of cell receptors and therefore

generate a variety of cellular responses. This ability of WNT5A to switch between two different forms of signaling is particularly interesting given that WNT5A has been considered as both a tumor suppressor and an oncogene in various cell types (196-198). Further study into the details of these mechanisms is needed to better understand the complexities of Wnt5a signaling and how WNT5A contributes to tumorigenesis.

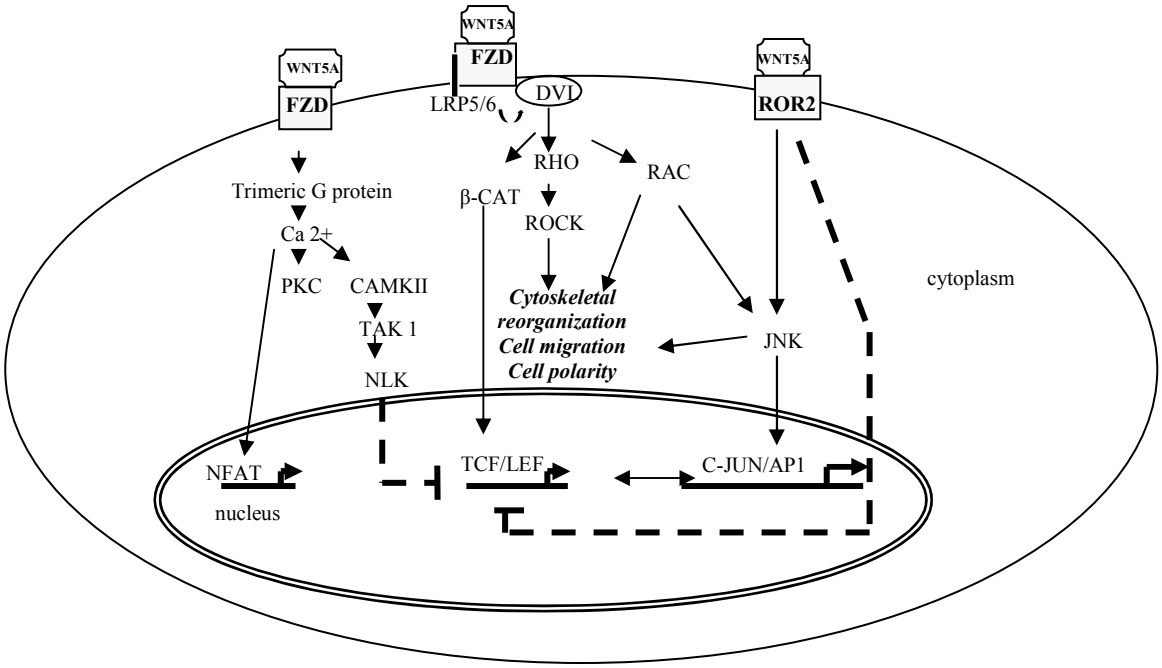


Figure 1.4 Schematic illustrations of the non-canonical WNT5A signaling pathways. WNT5A can initiate several downstream signaling pathways depending on cell surface receptors and intracellular contexts. These pathways include WNT5A/calcium, WNT5A/PCP and WNT5A antagonized Wnt/β-catenin pathways. Modified from (188).

WNT5A: structure and expression during embryogenic development

The first *Wnt5a* gene was identified in the mouse where its temporal expression correlates with tissue patterning and morphogenesis (199)**.** Orthologs of *Wnt5a* have also been found in other species such as drosophila, frog and zebrafish where their expressions are developmentally regulated. In humans, molecular cloning and sequencing

of *WNT5A* revealed cDNAs approximately 5.8 kb in length, encoding the mature protein that contains 380 amino acid residues. Human WNT5A shares over 93% homology to the reported sequences of WNT5A proteins from other species. WNT5A maintains a characteristic of the WNT protein family that posseses a hydrophobic signal sequence (CKCHGvSGSC), 24 conserved cysteine residues, 4 asparagine-linked oligosaccharide consensus sequences, and a tyrosine sulfation site (199). Due to the chemical structure, the majority of secreted WNT5A is bound to heparin sulfate proteoglycans present in the ECM (200). This chemical linkage greatly influences the WNT5A gradient needed, especially for cell polarity, cell movement and tissue patterning.

The primary regions of human *WNT5A* and mouse *Wnt5*a expression are the sites just prior to tissue differentiation and patterning. In mouse, *Wnt5a* is present in the distal compartments of several organs such as the central nervous system, limb, craniofacial processes, genitals and outer ear (147). The *Wnt5a* expression in differentiating cells is maintained over a period of time and finally diminishes at the terminal differentiation stage (201). WNT5A null mice exhibit perinatal lethality from defects in lung development and fail to develop multiple structures from the primary body axis, mandible, head and tongue. Interestingly, this defect in morphogenesis is due to a reduced rate of cell proliferation in tissue of the outgrowing structures (147) suggesting the indispensable nature of WNT5A during tissue development.

WNT5A signaling during tooth development

Several lines of evidence suggest an essential role of WNT signaling during tooth formation, especially on epithelial development (202, 203). Molecules in the WNT

signaling pathway show reiterative and overlapping expression during tooth development, mostly in the epithelial components (41). In mice, *Wnt4* and *Wnt6* are expressed in the facial, oral and dental epithelium. *Wnt10b* is localized to the presumptive dental epithelium. *Wnt3* shows restricted expression in the enamel knots (204). Furthermore, over- and ectopic expression of WNT3 in the epithelial compartment of postnatal lower incisor teeth causes progressive loss of ameloblasts and enamel in transgenic mice (205), supporting the role of WNT signal in regulating enamel epithelial development. Inhibition of WNT signaling either by blocking the WNT co-receptor or knockdown the LEF-1, nuclear mediator of WNT signal, results in the absence of all teeth in mice (206, 207). In LEF-1 null mice, tooth development is arrested at the bud stage (208) while overexpression of LEF-1 in epithelial cells in transgenic mice leads to an increased enamel epithelium and formation of tooth like structure (209).

Unlike other WNT proteins, studies have shown that *Wnt5a* expression is specifically found in dental mesenchyme during the early stages of mouse tooth development while its expression cannot be detected in the enamel epithelium (204, 210). The function of WNT5A during tooth development is largely unknown. However, the role of WNT5A in dental mesenchyme differentiation is speculative since WNT5A is known to counteract WNT canonical signaling and the WNT/β-catenin pathway is known to inhibit dental pulp stem cell differentiation (211).

Effects of WNT5A on cell behaviors

In the past, WNT5A has been defined as a non-transforming WNT protein due to the lack of transforming activity on mouse mammary epithelial cells (212, 213). WNT5A

has also been shown to reverse cell transformation (214, 215) and exhibit tumor suppressor activities in some studies (197, 216, 217). The role of WNT5A is still controversial, but in fact there is a multitude of evidence suggesting that WNT5A plays essential roles in cancer, especially in tumor progression (188). WNT5A can promote cell proliferation of progenitor cells (147, 218, 219), glioma cells (220), vascular endothelial cells (221) and several types of cancer cell (188, 222). Additionally, studies show that WNT5A also promotes cell growth and survival by inhibiting apoptosis in osteoblast (223) and vascular endothelial cells (221). Furthermore, forced expression of WNT5A in the deeper skin induces changes in the interfollicular epithelium mimicking true skin regeneration within the epithelial appendage, including formation of epithelia-lined cysts, sebaceous glands and hair follicles in the wound dermis (224). On the other hand, WNT5A suppresses cell proliferation in mammary epithelium, hematopoietic cell and lung (197, 213, 216, 217) or shows no effect on either apoptosis or cell proliferation in human mesenchymal stem cells and pituitary gland (225, 226).

The effect on cell migration is another well-known function of WNT5A. WNT5A increases cell migration and/or invasion in fibroblasts (200,227), vascular endothelial cells (221), breast epithelial cells (228), gastric cancer cells (229), pancreatic cancer cells (222) and melanoma cells (168,227). Increased cell migration is an anticipated role of WNT5A when considering the putative role of WNT5A in promoting cell polarization and gastrulation during embryonic development (230). WNT5A enhanced cell migration is known to be activated through RHOA/RAC small GTPase (182) and ROCK (186, 187), but a study in melanoma cell migration also suggests that WNT5A might signal via the PKC pathway and induce the loss of E-cadherin expression (231).WNT5A signals,

via ROR2, enhance migration in mouse embryonic fibroblasts by inducing filopodia formation and actin reorganization (227). WNT5A addition stimulates migration and invasion of NIH3T3 and L cells and is associated with an increased number of lamellipodia, activation of FAK and accelerated turnover of paxillin (229). In contrast, WNT5A has been found to suppress cell migration in breast epithelium (232), thyroid cell (179) and colon cancer cells (198).

The effect of WNT5A on cell adhesion is believed to be one of the mechanisms whereby WNT5A modulates cell function and behavior. WNT5A modifies cell adhesion and migration of mammary epithelium cells by affecting collagen binding with the discoidin receptor I (232) and regulating stromelysin-I and mesothelin expression (233) which contributes to the alteration of normal phenotypes and function of the mammary epithelium cell. The connection between increased cell attachment by WNT5A and cell migration varies considerably. For example, WNT5A can increase cell adhesion and cell migration in fibroblasts and melanoma cells (168,200), but WNT5A enhanced cell adhesion in breast epithelial cell and myocyte is believed to suppress their migration (232, 234).

Recently, the role of WNT5A in modulating the microenvironment has come into consideration. WNT5A increases production of MMP-1 in mouse mammary epithelial cells (233) and in endothelial cells (221). MMPs are well known for their essential role in ECM remodeling. WNT5A also promotes the formation of capillary networks by endothelial cells (221). Moreover, expression of WNT5A in melanoma is prominent at the edge of tumor invasion which strongly suggests a potential role of WNT5A in modifying tumor/stroma environment (188).

WNT5A expression and implication in cancers

The roles of WNT5A are implicated in a variety of cancers. Aberrant WNT5A expression has been found to correlate with aggressive and malignant behaviors of cells; however function of WNT5A in tumorigenesis is obscure and contradictory. Several lines of evidence suggest that WNT5A posses oncogenic properties. WNT5A is overexpressed in malignant melanoma (168), gastric cancer, lung cancer (235), pancreatic cancer (222), and prostate cancer (236, 237). High levels of WNT5A expression correlated with the advanced stage and poor prognosis of these cancers with a more aggressive phenotype and metastasis. In melanoma, the expression of WNT5A is increased relative to skin and overexpression of WNT5A in melanoma cells directly causes actin reorganization and increased cell invasion (168). WNT5A signaling is essential for macrophage-induced invasiveness in breast cancer and has been shown to regulate breast cancer cell migration as well as proteolytic activity of the macrophages (228). Furthermore, forced expression of WNT5A in the deeper mouse skin induces changes in the interfollicular epithelium, including formation of epithelia-lined cysts (188, 224). In addition, *WNT5A* was upregulated in astrocytoma and glioma (220), and *WNT5A* was found to be hypomethylated and overexpressed in 65% of prostate cancers (236). Interestingly, there is growing evidence suggesting that WNT5A is essential in tumor or malignant progression rather than in tumor initiation (188). In pancreatic cancer, *WNT5A* is upregulated in early pancreatic cancer genesis and the level is continuously increased during the progression from intraepithelial neoplasia to invasive adenocarcinoma (222). Moreover, *WNT5A* expression in squamous cell carcinoma and melanoma is associated with loss of E-cadherin and EMT which are the key events in metastasis (168, 238).

The opposite has also been reported. WNT5A has been considered as a tumor suppressor in some cancers, such as breast cancer, neuroblastoma, acute myeloid leukemia and primary dukes B colon cancer. In these tumors, the loss or reduction in *WNT5A* gene and protein expression correlated with aggressive phenotypes of cancer cells (196-198). *WNT5A* expression is downregulated in endometrial carcinoma cells (239) and pancreatic carcinoma (240) when compared with their normal cells of origin. WNT5A heterozygous mice developed chronic B cell lymphomas and it is noteworthy that *WNT5A* expression was lost in these malignant tissues (197). The tumor suppression mechanism of WNT5A is believed to be, in part, by interfering with the excess of canonical WNT/β-catenin signaling, particularly when it signals via CAMKII (188). In breast cancer, WNT5A expression is frequently reduced, which has been correlated with a higher incidence of early relapse and death (241). Notably, several other studies have examined the expression of *WNT5A* in breast cancer cell lines and tissues, but the results are contradictory (196, 242, 243).

It is obvious that WNT5A signaling is very complex and can be modified by the multitude of regulating factors. Not only the availability of receptors, downstream effectors and inhibitors, but also the nature of cell types and interactions between cells and the microenvironment all can play a role in regulation and determine the complexity and outcome of WNT5A signaling. Taken together, this knowledge strongly suggests that WNT5A could be a promising target for cancer therapy. Inhibition of WNT5A signaling in ameloblastoma could possibly serve an effective therapeutic approach to preserve the jaw from radical excision treatment and prevent the recurrence of this tumor.

CHAPTER II

Hypothesis

WNT5A modulates enamel epithelium tumorigenicity by promoting aggressive cell behavior via WNT5A/JNK pathway.

CHAPTER III

STUDY I

Characterization of WNT5A expression in normal and diseased enamel epithelium development

Specific aims

1. To characterize the temporal and spatial expression of WNT5A in normal and abnormal enamel epithelium.

INTRODUCTION

There is ample evidence strongly supporting the important role of the canonical WNT signaling pathway during normal tooth development (202, 203). Studies show that several molecules in the WNT-β catenin pathway, such as WNT3 and LEF-1, are essential for regulating mouse enamel epithelial development (205-207). For the non-canonical WNT5A signaling pathway, function of WNT5A during normal tooth formation has never been explored. Previous studies show that *Wnt5a* expression is specifically found in the dental mesenchyme during the early stages of normal mouse tooth development while its expression cannot be detected in the enamel epithelium (204, 210). Additionally, WNT5A could also play a role during enamel epithelium tumorigenesis as high WNT5A protein expression is detected in granular cells in ameloblastomas (81). Interestingly, we also observed high *WNT5A* gene expression in epithelial components of ameloblastoma using the laser-microdissection-microarray approach (unpublished data). High WNT5A expression in ameloblastomas could contribute to abnormal phenotype and aggressive cell behaviors of the tumorigenic enamel epithelium. In this chapter, expression of WNT5A during normal and diseased (ameloblastoma) enamel epithelium development are characterized. Temporal and spatial WNT5A expression in ameloblastomas as well as during normal tooth development will provide the better understanding of the possible role of WNT5A in odontogenic tumors.

EXPERIMENTAL PROCEDURES

Samples collection. Human and mouse tissue samples were obtained according to the Institutional Review Board and Institutional Animal Care and Use Committee approved protocols. First occurrence, multicystic/solid ameloblastoma tumors were collected from patients diagnosed with ameloblastoma through the Oral Pathology service at the University of North Carolina from 1998-present. Tumors were obtained along with the patients' clinicopathological and demographic data**.** Human primary tooth germs from 10^{th}-19 $6/7^{th}$ week old fetus were obtained from patients undergoing abortion through the University of North Carolina Department of Obstetrics & Gynecology. Any fetuses with known genetic anomalies or medical conditions were excluded from the study. Whole mouse heads were obtained from C57/BL6 at postnatal day 7. All tissue samples were fixed in formalin, embedded in paraffin and used for immunohistochemistry.

Immunohistochemistry. Goat anti-mouse WNT5A polyclonal antibody (1:200) and recombinant mouse WNT5A were obtained from R&D systems (Minneapolis, MN). Specificity and the sensitivity of anti-WNT5A antibody were tested on mouse small intestine, hair follicles and liver paraffin embedded sections. Blocking the anti-WNT5A antibody with recombinant mouse WNT5A (2 μg/ml) at 4 ^{o}C overnight was used as a negative control. After specificity and sensitivity of the antibody were verified, immunohistochemical staining was performed on 4 μm thick, paraffin-embedded sample sections using Elite Vectastain kit (Vector Laboratories, Burlingame, CA). Deparaffinized sections were preincubated with normal serum to block nonspecific binding and then incubated with anti-WNT5A antibody (1:200) at 4 ^{o}C overnight. A

biotinylated rabbit anti-goat antibody was used as the secondary antibody, and immunoreaction was visualized using 3, 3'-diaminobenzidine (DAB) substrate kit for peroxidase (Vector Laboratories, Burlingame, CA). Sections were counterstained with hematoxylin and evaluated for immunoreactivity.

Statistical analysis. Ameloblastoma samples were categorized into two groups, positive and negative, based on expression of WNT5A, and were then compared with regard to each clinicopathological variables including age, gender, tumor location and histological types. χ^2 test was used for statistical analysis and $P< 0.05$ was considered to be statistically significant.

RESULTS

Expression of WNT5A protein in ameloblastoma. First, we validated the specificity and sensitivity of anti-WNT5A antibody before using it in further experiments. Small intestine and skin from new born mice were used for positive controls and adult mouse liver was used as a negative control. Previous studies have shown these tissues serve as appropriate positive and negative controls (244-247). Strong WNT5A expression was detected in mouse small intestine and hair follicle (Figure 3.1 (a) and (c)) since WNT5A is essential for mouse small intestine elongation and hair follicle development (244, 246). When recombinant WNT5A (2 μg/ml) was used for antibody blocking, the immunoreactivity in both organs was diminished (Figure 3.1 (b) and (d)) indicating that anti-WNT5A antibody indeed detected the WNT5A protein. As previously reported weak or absent WNT5A expression was observed in adult mouse liver (Figure 3.1 (e)) (247).

The results of immunohistochemistry demonstrated the presence of WNT5A protein in ameloblastomas (Figure 3.2 (b) and (d)). The epithelial nests and/or strands exhibited strong positive staining (Figure 3.2 (e) and (f)). Slight WNT5A expression was also detected in the stromal component of ameloblastomas. WNT5A immunoreactivity in ameloblastoma was confirmed since the staining in both epithelium and stroma was absent when anti-WNT5A antibody was blocked with a recombinant WNT5A protein (Figure 3.2 (c)).

Among our 52 samples of first occurrence, solid/multicystic ameloblastoma, we found the major histological tumor subtypes was a mixture of plexiform and follicular patterns (44%), followed by follicular (29%) and plexiform (27%) patterns. Expression of

WNT5A was observed in 46 ameloblastomas (88%) regardless of the variation in histological subtype (Table 3.1). We detected comparable levels of WNT5A expression among histologically different ameloblastoma subtypes. Negative WNT5A expression was found in 6 cases (12%) and the major subtype of these ameloblastomas was the mixed plexiform and follicular pattern (67%). We were able to retrieve the clinicopathological features of tumors from 34 cases and the data along with statistical analysis are shown in Table 3.1. However, statistical analysis showed no significant correlation between WNT5A expression in ameloblastomas and any variables, such as age, gender, tumor location, and histological subtypes that we examined.

Expression of WNT5A protein during mouse and human enamel epithelium development. To better understand the possible role of WNT5A in odontogenic tumors, we performed immunohistochemical analysis of the WNT5A temporal and spatial expression during the development of normal mouse and human teeth. WNT5A expression during mouse tooth development demonstrated expression in the dental papilla and developing odontoblasts (Figure 3.3 (c) and (d)), but only slight or weak expression was present in pre-secretory ameloblasts during the early bell stage (Figure 3.3 (c) and (d)). Interestingly, during late bell stage, WNT5A expression was also detected in mature or secretory ameloblasts (Figure 3.3 (c)) while expression in odontoblasts and dental papilla still remained.

When we performed immunohistochemical analysis using human tooth germs (Figure 3.4), the WNT5A expression pattern was remarkably different compared with the mouse tooth buds. Human molars at cap stage (13-16 gestational weeks) showed WNT5A localized in both outer and inner enamel epithelium but was weakly detected in

dental mesenchyme (Figure 3.4 (a)). During early bell stage (16-18 gestational weeks), WNT5A expression was maintained in the inner enamel epithelium, outer enamel epithelium and pre-secretory ameloblasts with intense expression around the enamel knot and cervical loop areas (Figure 3.4 (c) and (d)). At late bell stage (18-20 gestational weeks) when there was some deposition of enamel matrix, WNT5A expression remained in the enamel knots and cervical loop areas but was absent from secretory ameloblasts as well as dental papilla and developing odontoblasts (Figure 3.4 (e) and (f)).

Table 3.1 Clinicopathological features of ameloblastoma in relation to WNT5A expression.

Variable	n	WNT5A positive	WNT5A negative	$\chi^2_{0.05}$
Age	34	30	4	0.78
0-20	9	8	1	
20-40	13	12	1	
>40	12	10	2	
Gender	34	30	4	0.92
Male	18	16	2	
Female	16	14	2	
Tumor location	34	30	4	0.73
Maxilla	11	10	1	
Mandible	23	20	3	
Histological types	52	46	6	0.53
Follicular	15	14	1	
Plexiform	14	13	1	
Mixed	23	19	4	

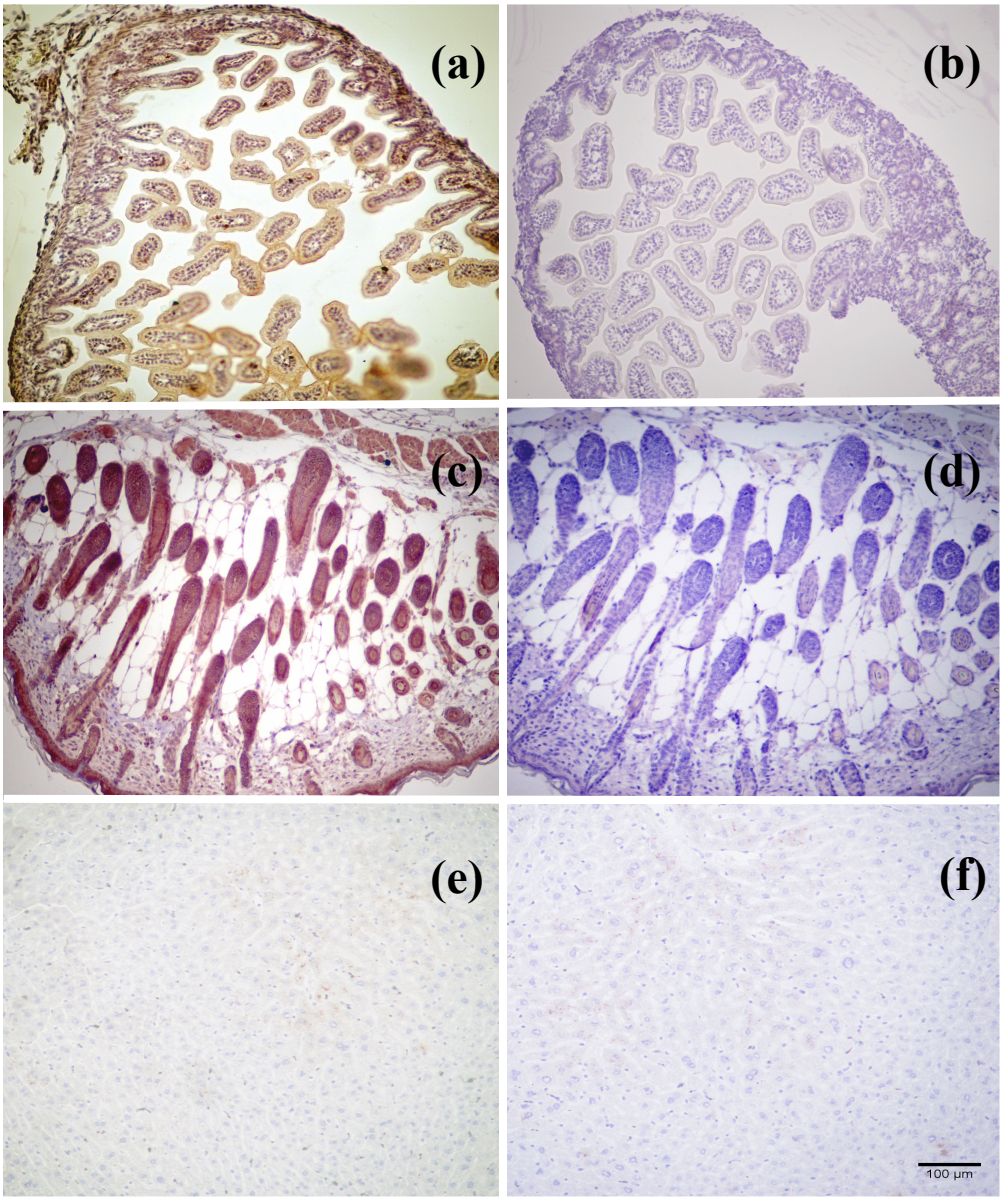


Figure 3.1 Validation of WNT5A antibody specificity. Strong WNT5A expression was detected in mouse small intestine (a) and hair follicles (c) and the immunoreactivity was diminished when the antibody was blocked with recombinant WNT5A (2 µg/ml) ((b) and (d)). Adult mouse liver was used as a negative control where no expression of WNT5A was detected when WNT5A antibody alone (e) and blocked WNT5A antibody (f) were used. (Antibody dilution 1:200), (Magnification x100), (Bar, 100 µm)

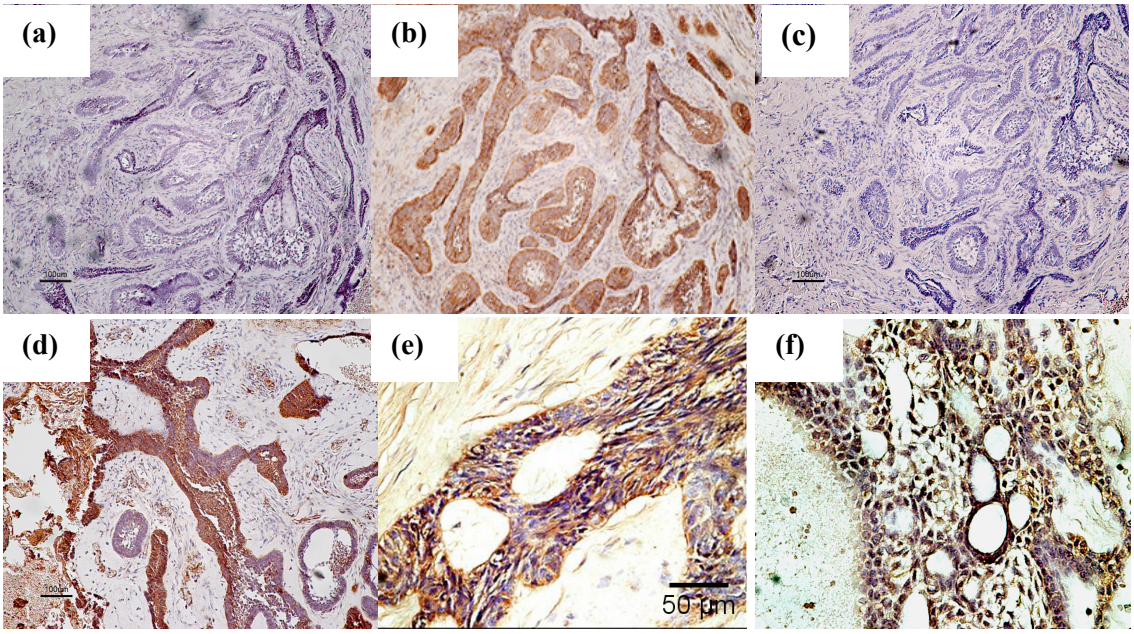


Figure 3.2 Expression of WNT5A in ameloblastoma. Ameloblastomas were immunostained with anti-WNT5A antibody (1:200) and images were capture under 100x (b and d) and 400x (e and f) magnification. Sections incubated with normal horse serum (a) and recombinant WNT5A blocked antibody (c) were used as negative controls. Bar in (a)-(d), 100 µm. Bar in (e)-(f), 50 µm. (*n*=52)

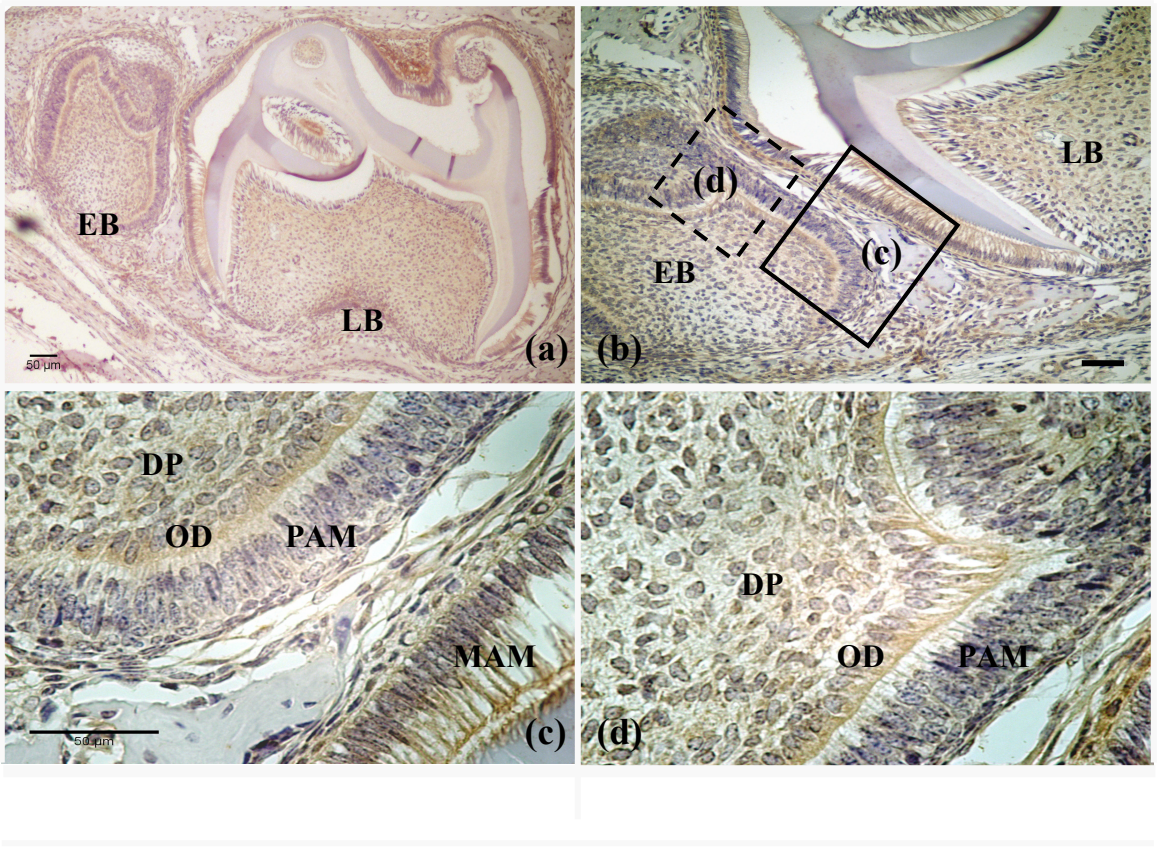


Figure 3.3 Expression of WNT5A during mouse tooth development. During the early bell stage (EB), intense expression of WNT5A was found in dental papilla (DP in (c) and (d)) and developing odontoblasts (OD in (c) and (d)) but slight or weak expression was seen in pre-secretory ameloblasts (PAM in (c) and (d)). At the late bell stage (LB), WNT5A expression was also detected in mature or secretory ameloblasts (MAM in (c)) while expression was sustained in odontoblasts and dental papilla ((LB) in (b)). (Antibody dilution 1:200), (Magnification, 40x (a), 100x (b), 400x (c) and (d)), (Bar, 50 µm), (*n*=3)

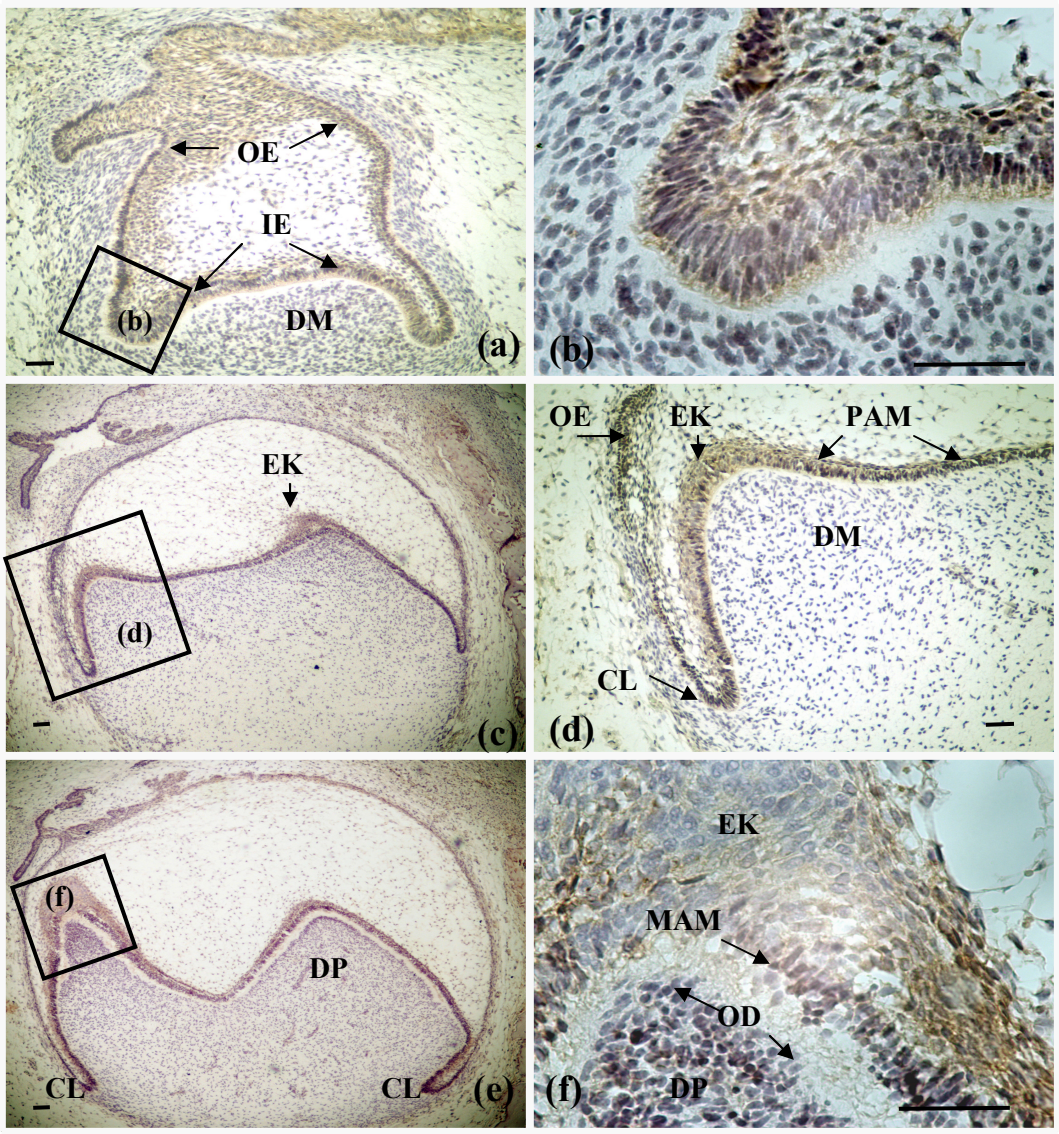


Figure 3.4 Expression of WNT5A during human tooth development. At the cap stage ((a) and (b)), WNT5A was localized in both outer (OE) and inner enamel epithelium (IE) but weakly detected in dental mesenchyme (DM). During the early bell stage ((c) and (d)), WNT5A remained in inner enamel epithelium and pre-secretory ameloblasts (PAM) with intense expression around enamel knots (EK) and cervical loops (CL). At the late bell stage ((e) and (f)), WNT5A expression remained in enamel knots (EK) and cervical loops area (CL) but was absent from mature or secretory ameloblasts (MAM) as well as dental papilla (DP) and developing odontoblasts (OD). (Magnification, 40x (c and e), 100x (a and d), 400x (b and f)), (Bar, 50 µm), (*n*=3)

DISCUSSION

Ameloblastomas are the most common epithelial odontogenic tumor and we show that they are highly consistent in their expression of WNT5A. While WNT5A expression is often associated with malignant tumors, ameloblastomas are benign but locally invasive and have a high recurrence rate (32, 40, 41). Detection of WNT5A expression in ameloblastoma has been reported by others (81) and our findings in this study also suggest the importance of WNT5A during both normal and diseased enamel epithelium development.

In the present study, it is notable that comparable levels of WNT5A expression were found in the plexiform, follicular and mixed plexiform/follicular histological subtypes, the two most common variants of ameloblastoma. WNT5A expression in granular pattern of ameloblastoma was detected in a previous study (81) but the expression of WNT5A in other histological subtypes has never been reported. Our study found no statistically significant correlation between WNT5A expression and other clinicopathological features including patient age, gender, tumor location and histological subtypes. A possible reason for this result could be the lack of variety of our samples since the recurrence and/or malignant ameloblastomas are excluded. Additionally, only partial demographic data were retrievable in our study. Aggressiveness was excluded from our variable since all samples are graded as benign tumors. One possible explanation could be the locally invasive nature of the tumor, the aggressiveness of ameloblastoma is not well characterized or graded and tumors are usually categorized as benign (32, 34, 41). Though there is a difference in occurrence rates observed between

subtypes, studies report no correlation among each histologically different pattern and aggressiveness of ameloblastoma (32, 40, 41).

The fact that WNT5A is shown to promote cancer aggressiveness and metastasis in many types of cancer (168, 188, 229, 231) could suggest the possible role of WNT5A in ameloblastoma progression. In melanoma, WNT5A was predominantly detected in the invading cells at the leading front of cancer invasion while the melanoma cells in other areas showed weaker WNT5A expression (168), suggesting the effect of differential WNT5A expression on tumor cell behavior. This could be extrapolated to the slow growing nature of ameloblastomas that might be regulated by the temporal WNT5A expression where tumors actively grow when WNT5A is expressed, and the tumor cells are in resting stage when WNT5A expression is downregulated.

The finding of WNT5A expression during human tooth formation in our study is strikingly different from what we and others have observed in mouse. To our knowledge, this is the first study of WNT5A expression during human tooth genesis. According to previous studies, *Wnt5a* is strictly expressed in dental mesenchyme but not in enamel epithelium during the bud, cap and early bell stages of mouse tooth development (204, 210). Interestingly, the expression of WNT5A differed between the human and mouse teeth and that varied at different developmental stages. Based on previous findings during mouse tooth formation, a major role of WNT5A is believed to involve dental mesenchyme development (204, 210). Although we cannot detect WNT5A expression in dental mesenchyme during human tooth development, the function of WNT5A in dental mesenchyme development is still debatable. Since WNT5A is an extracellular protein, a gradient of WNT5A expressed from the dental epithelium could influence adjacent

mesenchymal induction. In fact, expression of WNT5A in human enamel epithelial cells, especially at the enamel knot and cervical loop areas is logical when considering the putative roles of WNT5A in promoting cell polarization, tissue patterning and gastrulation during embryonic development (230, 248). Directional proliferation of enamel epithelium during tooth development is crucial for dental mesenchyme induction, tooth cusps patterning and root formation (2, 5). Likewise, cell polarization is an important step in ameloblast development (31); hence these processes might be governed or modified by WNT5A expression by enamel epithelial cells.

Enamel epithelial remnants from migrating enamel epithelium in the cervical loop areas are believed to give rise to odontogenic tumor (32, 40, 41). Interestingly, WNT5A expression in ameloblastoma is also intense in the epithelial compartment which is similar to WNT5A detection in cervical loop. In the cancer stem cell theory, we know that many cancer cells display a stem-cell like phenotype. The potential role of cervical loop cells strongly expressing WNT5A is consistent with enamel epithelium remnants from cervical loop being the cells giving rise to odontogenic tumors.

In summary, we demonstrated strong WNT5A expression in the epithelial compartment of ameloblastomas. Moreover, we also detected intense WNT5A expression in the normal odontogenic epithelial cells that are the origin of cell remnants (epithelial rests of Malassez) that gave rise to odontogenic tumorigenesis. These findings led us to speculate that WNT5A plays a role in enamel epithelium tumorigenesis. These observations led to development of our hypothesis that WNT5A modulates enamel epithelium tumorigenicity by promoting aggressive cell behavior which we investigate further and present in the Chapter IV and V.

CHAPTER IV

STUDY II

WNT5A modulates tumorigenic behaviors and increases cell migration in enamel epithelium.

Specific aims

1. To establish and characterize LS-8, enamel epithelium cell, derived stable clones expressing higher or lower levels of WNT5A.
2. To investigate the effect of WNT5A on transforming LS-8 derived stable clones behavior by cell transformation
3. To investigate the effect of WNT5A on LS-8 cell migration

INTRODUCTION

Tumorigenic cells exhibit several characteristics that distinguish them from the normal cell counterparts. Most tumor cells share essential changes in cell growth properties, including alteration in cell morphology, decreased dependence of growth factors, loss of contact inhibition of growth, ability to proliferate in suspension or semisolid culture medium, and ability to form tumors when transplanted into an immunocompromised host (82, 249). These growth alterations can be evaluated by the *in vitro* and *in vivo* growth transformation assays that are commonly used to determine the tumorigenic growth- regulating properties of oncogenes (249).

Multitudes of evidence suggest that WNT5A plays essential roles in cancer, especially in tumor progression (188). Despite the fact that WNT5A lacks transforming activity when introduced to mouse mammary epithelial cells (212, 213), studies show WNT5A displays growth promoting activities by increase cell proliferation in both progenitors (147, 218, 219) and cancer cells (188, 222), as well as inhibiting apoptosis in osteoblast (223) and vascular endothelial cell (221). High levels of WNT5A expression is often correlated with a more aggressive phenotype and metastasis of cancers (168, 222, 236, 237, 250). As previously shown in the chapter III, strong WNT5A expression was detected in the epithelial compartment of ameloblastomas leading us to speculate that WNT5A might promote aggressive growth behaviors of enamel epithelium.

In the present study, enamel epithelium cells (LS-8) were used to study the effect of WNT5A on enamel epithelium growth behavior. This seemed appropriate since the remnants of epithelium from the enamel organ that remains after tooth formation is believed to give rise to odontogenic tumors (32, 40, 41). LS-8 cells are derived from

enamel organ epithelium extracted form new born mouse first molars that are immortalized by simian viral (SV) 40 large T antigen (251). LS-8 cells exhibit characteristics of enamel epithelium including the expression and production of enamel matrix proteins, such as amelogenin and ameloblastin in a manner similar to primary enamel organ epitheliums (251-253).

In this chapter, effects of WNT5A in modulating tumorigenic growth behaviors of enamel epitheliums (LS-8) are demonstrated by *in vivo* and *in vitro* cell growth transformation assays. Alteration in enamel epithelium cell migration and cytoskeleton reorganization by WNT5A overexpression are evaluated by wound healing assay and immunofluorescence in the present study.

EXPERIMENTAL PROCEDURES

Cell culture. Mouse enamel epithelium cells established by immortalizing primary cultures of new born mouse enamel epithelium with SV40 large T antigen, LS-8 (generously provided by Dr. Malcolm Snead, University of Southern California), were cultured as previously described (251). Briefly, the cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS) (Gibco) and 100U/ml penicillin and 100 μg/ml streptomycin (Gibco). Cells were maintained in a humidified atmosphere of 95% air and 5% CO_2 at 37 ^{o}C and culture medium was replaced twice weekly.

Isolation of *Wnt5a* cDNA and constructs generation. Total RNA was isolated from mouse kidney, which is known to express *Wnt5a*, using TRIzol reagent (Invitrogen, Carlsbad, CA). Two μg of total RNA was used for reverse transcription (RT) and the cDNA was synthesized using the Superscript RT kit (Invitrogen) followed by polymerase chain reaction (PCR) with primers specific for full length mouse *Wnt5a*. Primer sequences were as follows; 5'-CACCATGAAGAAGCCCATTGG-3' and 5'-TTTGCACACGAACTGATCCAC-3'. The PCR products were verified by resolving in 1% agarose gel with ethidium bromide and photographed under UV light to confirm the expected size of the product at 1140 bp. The *Wnt5a*-PCR products were then ligated into pcDNA3.1 V5-His-TOPO mammalian expression vectors (Invitrogen), cloned by using TOPO TA cloning kit (Invitrogen). The molecular weight of the ligated insert was analyzed by restriction enzymes *HindIII* and *XhoI* (New England Biolabs) to demonstrate the correct size of full length *Wnt5a* were inserted in to the vectors. Plasmids were then

analyzed by sequencing at the UNC-CH DNA sequencing facility (University of North Carolina, Chapel Hill, NC) to determine the correct orientation of the insert.

Transfection and stable clone establishment. The sense, antisense constructs (pcDNA3.1/*Wnt5a* and pcDNA3.1/AS*Wnt5a*) or pcDNA 3.1/V5-HisA vector (empty vector), Invitrogen) were transfected into LS-8 cells using Fugene 6 transfection reagent (Roche Applied Science, Indianapolis, IN). After 48 h, cells were trypsinized and plated at a low density. Cells were maintained and selected in the presence of G418 (600 μg/ml) (Invitrogen) for up to 3-4 weeks to select the stably transfected clones. Single-cell derived stable clones were prepared by cloning rings and the expression of WNT5A from each clone was analyzed by Western blot.

Triton X-114 phase separation and Western blot analysis. To ensure the WNT5A secretion from stable clones, separation of WNT5A in conditioned medium was performed according to the protocol for WNT3 separation with modification (125). In brief, conditioned medium collected from stable clones was mixed 1:1 with ice cold 4.5% Triton X-114, 150 mM NaCl, 10 mM Tris-HCl, pH 7.5, incubated on ice for 5 min, then at 31 °C for 5 min, and centrifuged at 2,000*g* at 31 °C for 5 min. After two phases of Triton X-114 were separated, equal volumes of detergent and aqueous phases were then evaluated for the presence of WNT5A using Western blot.

Since the majority of secreted WNT5A was bound to the proteoglycans component of the ECM (200), WNT5A bound to the ECM and cell surface were also collected. The WNT5A detected from ECM and cell surface were normalized against β-actin and were used to compare the level of WNT5A expression. Cells were washed twice with phosphate buffer saline (PBS) and gently lysed in lysis buffer (20mM Tris-

HCl pH 7.5, 0.5 mM $MgCl_2$, 0.1% TritonX-100) containing the P1860 protease inhibitor cocktails (Sigma-Aldrich). The supernatant was collected for analysis.

Equal volumes of each sample for Western blot analysis were dissolved in sodium dodecyl sulfate (SDS) sample buffer (100mM Tris-HCl, pH 8.8, 0.01% bromophenol blue, 36% glycerol, and 4% SDS) in the presence of 10mM dithiothreitol (DTT), separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Invitrogen) and transferred to nitrocellulose membrane (Biorad). WNT5A protein was detected by anti-WNT5A antibody (R&D system) (1μg/ml) and the anti-goat conjugated with horse radish peroxidase (HRP) (Santa Cruz) was used as a secondary antibody. β-actin was detected by anti- β-actin antibody (cell signaling) and the secondary antibody, anti-rabbit conjugated with HRP (cell signaling). The immunoreactivity was visualized using the ECL Plus kit (GE Healthcare).

Quantitative real-time PCR. Total RNA was isolated from wildtype LS-8 (WT), empty vector clones (EV), LS-8 clones derived overexpressing higher (S) and lower (AS) levels of WNT5A using RNeasy kit (Qiagen). Two μg of total RNA was used for RT using SuperArray ReactionReady™ First Strand cDNA Synthesis Kit (SA Bioscience) containing random primers following the instructions provided in the user's manual. Real-time PCR was performed using RT^2 SYBR Green/ROX qPCR Master Mix (SA Bioscience) and sequence specific primers (SA Bioscience). Primers used were as follows; *Wnt5a* (#Mm.287544) and glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) (#Mm.343110). Real-time PCR was performed on an ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA) using the following cycling parameters: 10 min at 95°C; 40 cycles of 15 sec at 95°C, 1 min at 60°C. Dissociation curve analyses were

performed using the instrument's default setting immediately after each PCR run. The experiments were done in triplicate. The *Wnt5a* expression relative to *Gapdh* was determined and the fold changes were calculated by means of 2-ΔΔ*C*T method.

Cell proliferation assay. LS-8 , EV, S and AS clones were seeded in triplicate at a density of $1x10^5$ cell/well and maintained in DMEM containing 10% FBS for 7 days. Cells were trypsinized and counted at day 1, 3, 5 and 7. In addition, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) cell proliferation assay (CellTier 96®, Promega) was performed to confirm the results. LS-8 , EV, S and AS clones were seeded in triplicate at a density of $5x10^4$ cell/ml in a 96-well plate and maintained in DMEM containing 10% FBS for 7 days. At day 1, 3, 5 and 7 of culture, MTS solution was added into each well followed by incubation at 37 °C for 4 h. The amounts of formazan compound, produced by metabolically active cells were measured by absorbance at 490 nm. To confirm the reproducibility of the results, three independent experiments were performed.

Growth transformation assays. Serum dependence of cells for growth was determined as described previously (249, 254). Briefly, LS-8 , EV, S and AS clones were seeded in triplicate at a density of $1x10^5$ cell/well and maintained in DMEM containing 10% FBS and allowed to adhere to the culture dish. Then, culture medium was changed to DMEM containing 1% FBS and cells were cultured for 7 days. Cells were trypsinized and counted at day 1, 3, 5 and 7 to determine cell proliferation and viability. MTS assay was also performed as described above in the serum reduced condition to confirm the results.

To determine the ability of transfected cells to grow as multiple layers and form foci, focus formation assay was performed as previously described with modification (249, 254). LS-8, EV, S and AS clones were seeded in triplicate of 1x 10^5 cell/ 60-mm cultured dish and maintained in the cultured condition for 3 weeks. After that, cells were fixed with 3:1 (v/v) methanol: acetic acid stained with 0.4% crystal violet in 20% ethanol and photographed. Stained foci were counted to quantify the transforming activity.

To determine the anchorage-independent growth, soft agar assay was performed as previously described with modification (249, 254). LS-8, EV, S and AS clones were cultured as previously described. Monolayer of cells was trypsinized to obtain single-cell suspensions. A total of 5 x 10^3 cells were suspended in DMEM containing 0.3% agar and were then seeded onto a 0.5% agar base in six-well plates. DMEM containing 10% FBS was added to the top of the agar and was replaced twice weekly. Colony growth was assayed at 4 weeks after the initial seed. The number of colonies larger than 50 μm in diameter (containing >30 cells per colony) was counted in five random three-dimensional fields per well and photographed under 4x objective lens. To confirm the reproducibility of the results, three independent experiments for each assay were performed.

***In vivo* tumor formation.** To evaluate the tumorigenicity of the transfected cells, EV cells or cells highly overexpressing WNT5A (SH) were injected subcutaneously into the flanks of athymic nude mice (Jackson Labs) at seeding densities of 2×10^6 cells per site. Tumor development was monitored by personnel at animal studies core facility (Lineberger comprehensive cancer center, University of North Carolina, Chapel Hill, NC). Tumor measurements were taken by calipers three times per week over 12 weeks or until the tumor burden reached a volume of 1 cm^3. At this point, tumors were excised

immediately following euthanization, divided in half and fixed in 10% buffered formalin for histological analysis or RNA*later* solution (Ambion) for RT-PCR. For histological analysis, tumors were embedded in paraffin, and sections were cut and stained with hematoxylin and eosin. For RT-PCR analysis to confirm the tumor cell origins, total RNA was isolated from tumor tissue using TRIzol reagent (Invitrogen). Two μg of total RNA was used for reverse transcription and the cDNA was synthesized using the Superscript RT kit (Invitrogen) followed by PCR reaction. For WNT5A overexpressing tumors, PCR was performed using T7 promoter (5'-TAATACGACTCACTATAGGG-3') and *Wnt5a* (5'-TTTGCACACGAACTGATCCAC-3') specific primers to generate the 1150 bp products from pcDNA3.1/*Wnt5a* plasmid. For tumors formed by the EV group, PCR was done using T7 promoter (5'-TAATACGACTCACTATAGGG-3') and BGH (5'-CTAGAAGGCACAGTCGAGGCT-3') primers to verify the 250 bp products from pcDNA3.1 V5-HisA Empty vectors. The PCR products were visualized by resolving in 1% agarose gel with ethidium bromide and photographed under UV light.

Wound healing assay. Cells were grown on fibronectin (Sigma-Aldrich) coated 60-mm dish supplemented with completed medium (10% FBS) to created a monolayer of cells. After the cells reached 90% confluence, the medium was switched to 2.5% FBS and a wound was created by scratching a line across the monolayer of cells with a pipette tip (255). At 17 and 24 h after the scratch, cell migration was monitored. The number of cells migrated into the space at 24 h were counted in five random fields per group, photographed and fixed with 3:1 (v/v) methanol: acetic acid, and stained with 0.4% crystal violet in 20% ethanol. To confirm the reproducibility of the results, five independent experiments were performed with two replicates.

Actin filament staining. To visualize the change in actin filament organization, cells were grown on fibronectin coated glass coverslips, rinsed and fixed with 4% paraformaldehyde in PBS for 10 minutes at 4°C. Next, cells were permeabilized in 0.1% Triton X-100 in PBS for 10 minutes at 4°C. Actin filaments were stained with a 50 μg/ml of fluorescein isothiocyanate (FITC) phalloidin (Sigma-Aldrich) in PBS for 1 h at room temperature. The nuclei were counterstained with TOPRO-3 dye (invitrogen) and slides were mounted with Vectashield mounting medium (Vector Laboratories). Fluorescent images were examined with Olympus FluoView^{TM} FV500 confocal fluorescent microscope (Olympus Inc.).

Statistical analysis. All statistical analyses were performed using Sigma stat software. Data were expressed as mean$\pm$SD. Statistical differences were determined by one-way ANOVA followed by a Tukey-Kramer multiple comparison test at P=0.05.

RESULTS

Establishment and characterization of LS-8 derived stable clones. In order to determine the biological consequences of WNT5A expression in enamel epithelium cells, we over or underexpressed WNT5A in LS-8 cells. Full length mouse *Wnt5a* constructs were generated using *Wnt5a* template isolated from mouse kidney by specific PCR primers for mouse *Wnt5a* and were verified as bands at the expected size at 1140 bp (Figure 4.1). The *Wnt5a*-PCR products were then ligated into pcDNA3.1 V5-His-TOPO mammalian expression vector and analyzed by sequencing to determine the orientation of insert. A search in the database confirmed that the PCR products contained the coding region of the mouse *Wnt5a* cDNA and was 100% identical to the *Wnt5a* sequence in GenBank database.

We stably transfected LS-8 cell with sense (pcDNA3.1/*Wnt5a*) or antisense (pcDNA3.1/AS*Wnt5a*) constructs and stable clones were selected in the presence of G418 (600 μg/ml) in 4-5 weeks after transfection. The EV clones (pcDNA3.1/V5-His B) were subjected to the same selection procedure and were used as controls. To ensure the secretion of WNT5A protein, expression of WNT5A into the conditioned medium generated from each stable clone was verified by Western blot analysis. Due to WNT5A's highly hydrophobic nature, we adopted the Triton X-114 phase separation technique to concentrate and maintain solubility of WNT5A in conditioned medium (125, 200). Results shown in Figure 4.2 (A) illustrate that most secreted WNT5A partitioned to the detergent phase (De). Bands at the size of 42 kD (* in Figure 4.2 (A)) represented endogenous WNT5A expressed in LS-8 (WT), S and EV clones, while it disappeared from AS clone. Bands at approximately 47 kD (arrow in Figure 4.2 (A)) represented

WNT5A recombinant proteins which were only expressed in sense transfected clones and confirmed the production of WNT5A from transfected constructs.

When comparing the expression of WNT5A collected from cell surface and cell matrices, the level of WNT5A from S clones was classified into 3 groups; high, moderate and low overexpression (SH, SM and SL clones) as shown in Figure 4.2 (B). The endogenous WNT5A expression in EV clones was comparable to WT, while it was suppressed in AS stable clones (Figure 4.3). Expression of *Wnt5a* in each selected clone was then quantified by Real-Time PCR using specific primer with SYBR green detection system (Figure 4.4). Average expressions of *Wnt5a* in SH, SM and SL clones showed 7.97, 4.5 and 2.3 fold increases respectively when compared to WT and EV clones. *Wnt5a* expression was decreased in selected AS clones by 0.13 fold as confirmed by Real-time PCR. Three independent clones from each transfection were selected for the subsequent experiments.

Effects of WNT5A on *in vitro* cell growth transformation of enamel epithelium. We next evaluated the growth properties of selected stable clones by *in vitro* cell growth transformation assay. When seeded on culture dishes, EV, S and AS clones showed similar morphology to WT (Figure 4.5). No distinct change in cell morphology was observed in any of the groups. Increased expression of WNT5A in enamel epithelium did not affect cell proliferation when cells were maintained in medium containing 10% FBS (Figure 4.6 (A)). Interestingly, when cultured in serum reduced conditions that contained 1% FBS, only the WNT5A overexpressing clones survived in the serum starved condition. S clones had dramatically decreased rates of proliferation but were still able to survive up to 7 days in culture while WT, EV and AS clones did not

survive under reduced serum conditions (Figure 4.6 (B)). This result suggested that WNT5A could promote less dependence of growth factor property on enamel epithelium.

WNT5A overexpressing clones also exhibited loss of contact inhibition of growth after confluence when tested with focus formation assay. Allowing growth beyond confluence, SH, SM and SL clones continued to divide and form multilayered foci on culture dishes for up to 20 days (Figure 4.7 (A)). Few foci were found in WT, EV and AS clones where the single layers of cells started to detach from culture dishes after confluence (10 days). Interestingly, numbers of foci formed by WNT5A overexpressing clones were correlated with the level of WNT5A expression (Figure 4.7 (B)) suggesting that WNT5A could promote loss of contact inhibition in enamel epithelium in a dose dependent manner.

Next, we analyzed anchorage-independent growth in these clones by soft agar assay. S clones formed visible multiple colonies within 2 weeks after seeding (Figure 4.8 (A)), whereas WT, EV and AS clones did not form colonies visible by bare eyes even after 4 weeks of culture. Similar to what we found in foci formation, the numbers of colonies formed in soft agar by WNT5A overexpressing clones corresponded to their level of WNT5A expression (Figure 4.8 (B)).

Finally, we determined if WNT5A overexpression could affect the tumorigenicity of LS-8 cells when subcutaneously transplanted into nude mice. SH cells formed palpable tumors in the mice within 4 days after injection (Figure 4.9 (A)). Interestingly, EV cells also exhibited tumor formation at 18 days after injection. After monitoring for up to 48 days, all mice in the experiment developed tumors; however SH cells appeared to develop tumors earlier than EV control cells. On average, mice injected with SH cells

exhibited tumors within 18 days post implantation whereas the EV controls developed tumor in 32 days. SH cells also showed a faster tumor growth rate compared to the EV group when total tumor volume was calculated (Figure 4.9 (B)). Histology of the tumors from both groups appeared to be similar (Figure 4.10 (a) and (c)). They both were composed of a mixture of epithelium and mesenchyme. Duct-like or follicular structures formed by cuboidal epithelium were found randomly distributed in the tumor with mesenchymal cells making up the majority of the tumor. Most of the mesenchymal cells were fibroblast-like, poorly differentiate and were in active mitosis as shown by dense chromatin condensation. No calcifying areas or tooth forming structures were observed in any of the tumors.

We also performed RT-PCR to confirm the origin of the tumors. For tumors formed by SH cells, PCR using T7 forward and *Wnt5a* reverse primers generated the products approximately 1150 bp of the transfected and integrated *Wnt5a* plasmid (Figure 4.11 (A)). For tumors formed by EV group, PCR using T7 forward and BGH reverse primers yielded the approximately 250 bp bands of integrated pcDNA3.1 V5-HisA Empty vectors (Figure 4.11 (B)). The detection of these PCR products indicated that tumors formed in nude mice were developed from injected enamel epithelium and not from the indigenous mouse cell populations.

WNT5A increased cell migration and actin polymerization of enamel epithelium. Since WNT5A activation has been shown to promote cell migration during normal embryonic development as well as tumor metastasis in several types of cancer (188), we examined whether or not WNT5A could induce actin filament rearrangement to regulate cell migration in enamel epithelium. Scratch wound was created on 90% of

confluent monolayer of cell in culture medium containing 2.5% FBS to stimulate cell migration. At 17 h after scratching (Figure 4.12, middle row), the highest numbers of cells migrating into the created wounds were from S clones, moderate numbers from WT and EV and fewest numbers from AS clones. At 24 h (Figure 4.12, lower row), cells from S clones were able to close the wound. Although additional cells from WT and EV migrated to the space when compared to the results at 17 h, they did not close the wound. Interestingly, only small numbers of cell from AS clones migrated into the created wound suggesting that WNT5A regulated enamel epithelium cell migration.

Since the change in stress fiber formation is a necessary process in cell migration (109-111), we decided to evaluate the role of WNT5A in this process by actin filament staining. Cells were plated on fibronectin coated coverslips. Actin cytoskeleton was stained using FITC phalloidin (Figure 4.13). Consistent with the results of our cell migration assay, stress fiber formation density was greatly increased in the cells from S clones (Figure 4.13, middle row), but largely decreased in the cells from AS clones (Figure 4.13, lower row), when they were compared to EV cells (Figure 4.13, upper row). Interestingly, filopodia and lamellipodia, structures important for cell migration, were generally seen in WNT5A overexpressing cells (Figure 4.13, arrows in middle row), while WNT5A underexpressing cells appeared to form clusters with migratory structures rarely being seen (Figure 4.13, lower row). These results confirm that WNT5A induces enamel epithelium cell migration and this process involves actin cytoskeleton reorganization.

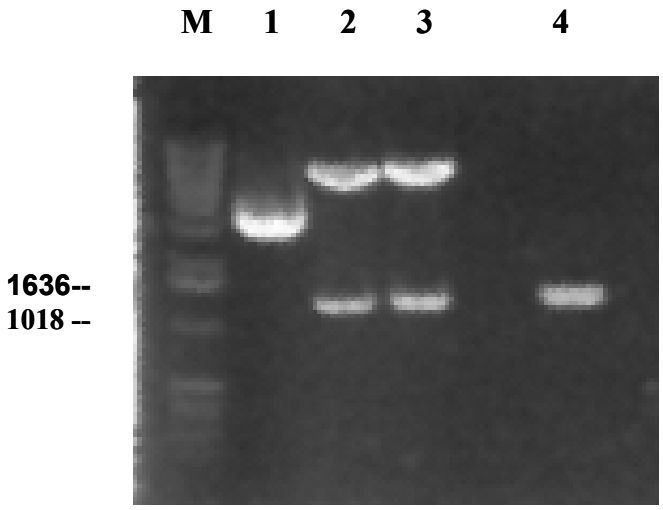


Figure 4.1 *Wnt5a* PCR product and restriction enzyme analysis of pcDNA3.1 vectors harboring *Wnt5a* cDNA. The full length-*Wnt5a* amplicon from mouse kidney is 1140 bp (lane 4). pcDNA3.1/*Wnt5a* digested with *HindIII* and *XhoI* demonstrated that the correct size of full length-*Wnt5a* was inserted into the vectors. Uncut pcDNA3.1 vector harboring *Wnt5a* cDNA is seen in lane 1. The sense construct is shown in lane 2 and the antisense construct appears in lane 3. Orientation of all inserts was verified by DNA sequencing.

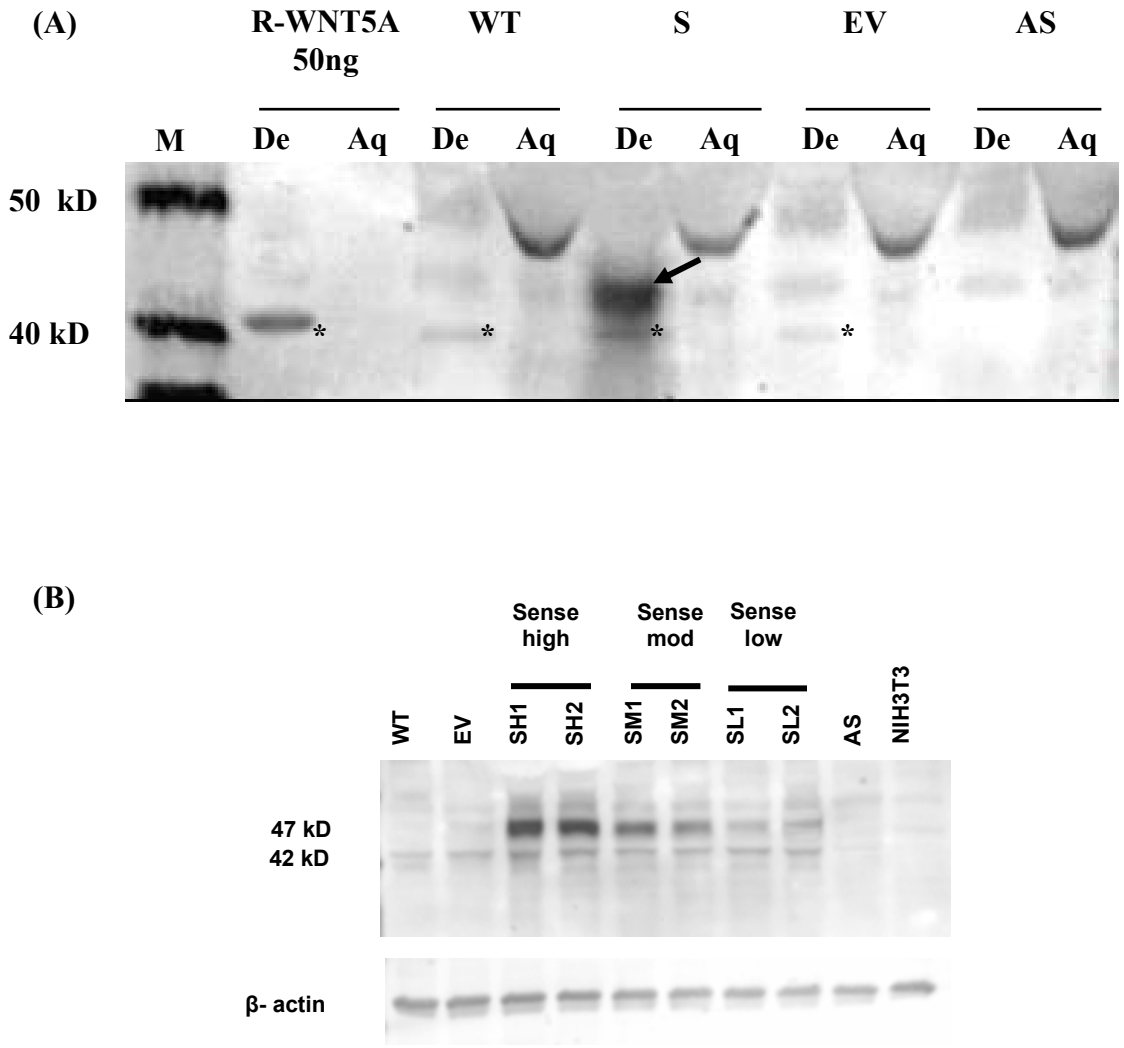


Figure 4.2 Level of WNT5A protein secretion from different transfection. (A) Secreted WNT5A in conditioned medium was partitioned in detergent phase (De) but not aqueous phase (Aq) after Triton X-114 phase separation. The 42 kD band of endogenous WNT5A (*) was faintly visible in Wild type (WT), overexpressing (S) and empty vector (EV) transfections. The 47 kD band of WNT5A fusion protein (arrow) was only detected in S transfected clones while WNT5A production was suppressed in AS transfections. (B) Stable clones established from S transfection were selected and categorized according to level of WNT5A collected from ECM and cell surface into high (SH), moderate (SM) and low (SL) overexpressing WNT5A clones.

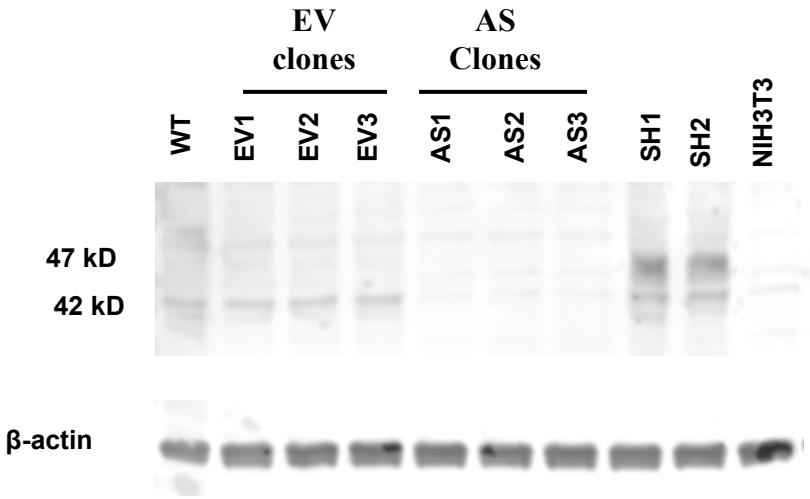


Figure 4.3 Level of WNT5A expression in EV and AS clones. WNT5A bound to ECM and cell surface was collected from EV and AS clones. The endogenous WNT5A expression (42 kD) in EV clones was comparable to WT, while it was suppressed in AS stable clones. Cell lysate from NIH3T3 cells was used as a negative control. Three independent clones from each S group, together with EV and AS clones were selected for further experiments.

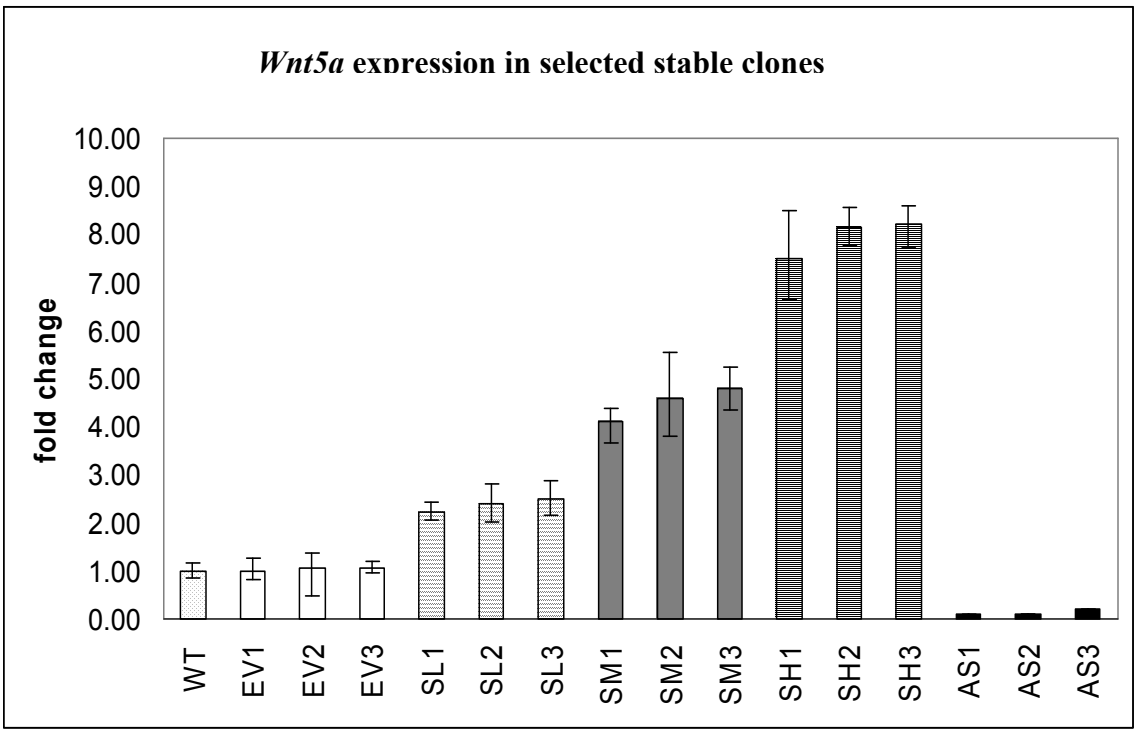


Figure 4.4 Quantification of *Wnt5a* expression in selected stable clones by Real-time PCR. Selected stable clones from S transfection showed an increase in *Wnt5a* expression compared with WT and EV clones (2.37, 4.5 and 7.97 fold increase in SL, SM and SH respectively). *Wnt5a* expression in stable clones from AS transfection was decreased by 0.13 fold.

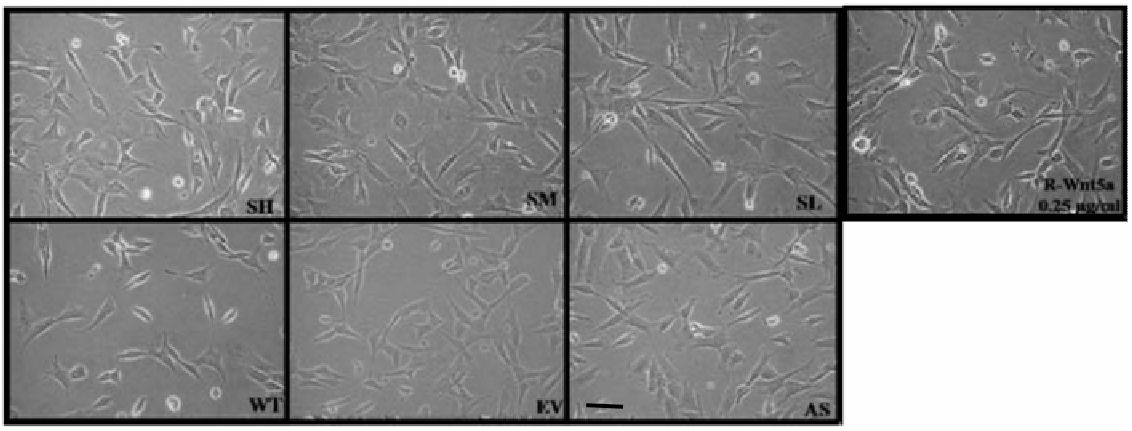
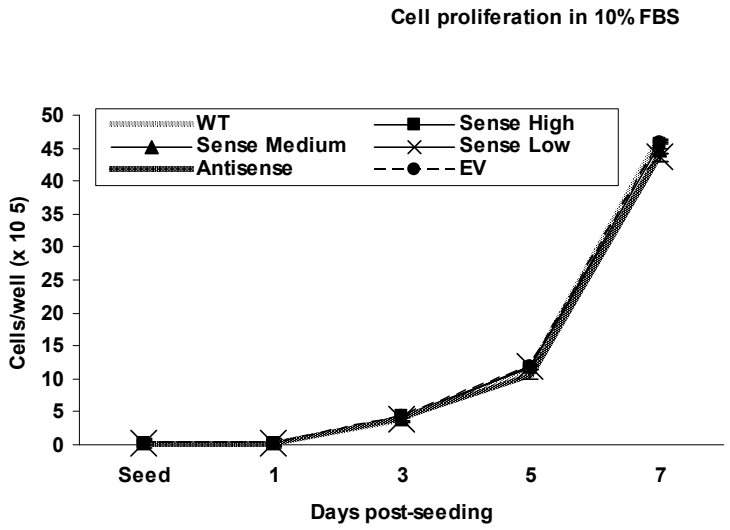


Figure 4.5 Cell morphology of stable clones. No significantly different morphology was observed among WT, EV, S and AS clones. When recombinant WNT5A (0.25 μg/ml) was added to the culture medium, WNT5A treated LS-8 showed similar phenotype to WT. (Magnification 200x), (Bar, 100 μm)

(A)



(B)

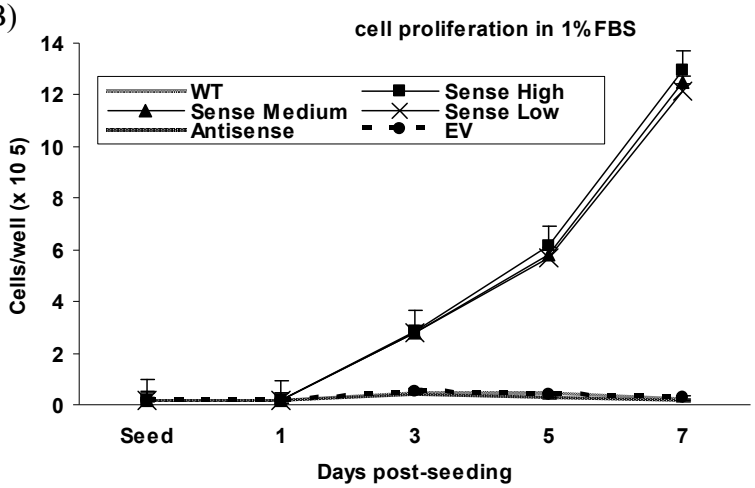
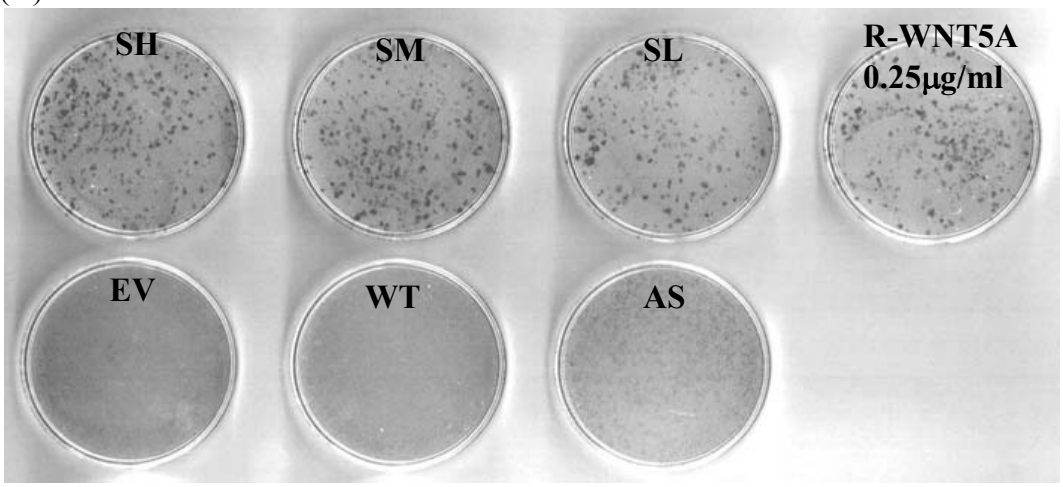


Figure 4.6 Cell proliferation assays of stable clones. (A), 1 x 10^5 cells from each clone were plated and supplemented with medium containing 10% FBS. Proliferation rates were similar among all groups. (B), When the same number of cells were cultured in 1% FBS medium, sense clones had a dramatically decreased proliferation rate but still survived in serum reduced conditions up to 7 days of culture. WT, EV and AS clones could not survive under these conditions.

(A)



(B)

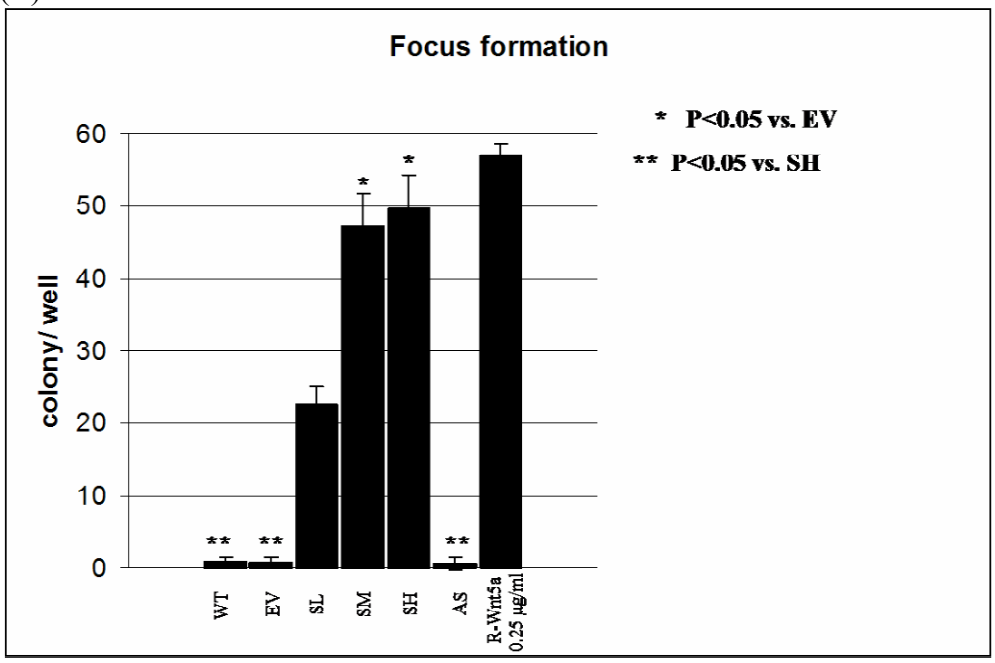
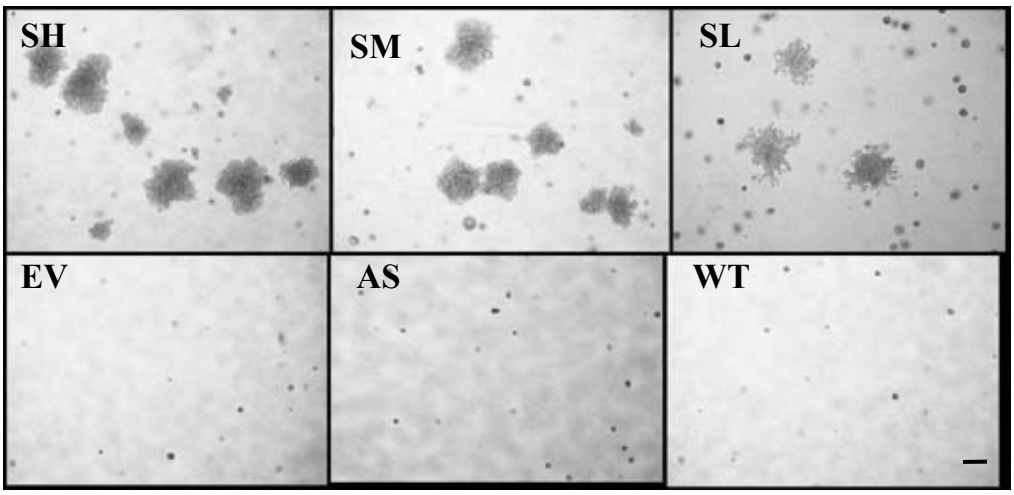


Figure 4.7 WNT5A promoted loss of contact inhibition in enamel epithelium. (A), SH, SM and SL formed multilayered foci on culture dishes after being cultured for 3 weeks while few foci formations were found in WT, EV or AS clones. (B) Numbers of foci formation were correlated with the level of WNT5A expression in overexpressing clones suggesting a dose dependent effect.

(A)



(B)

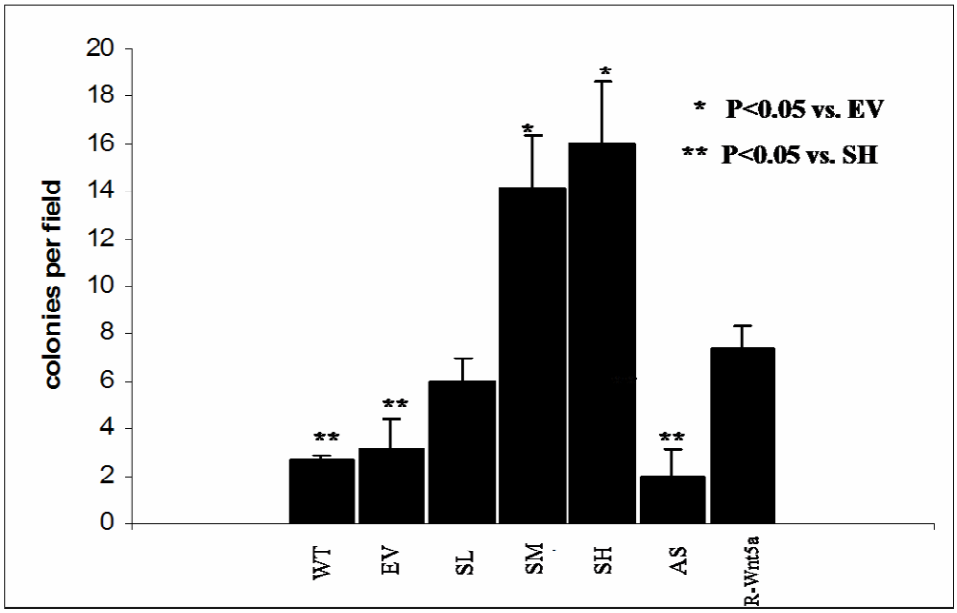
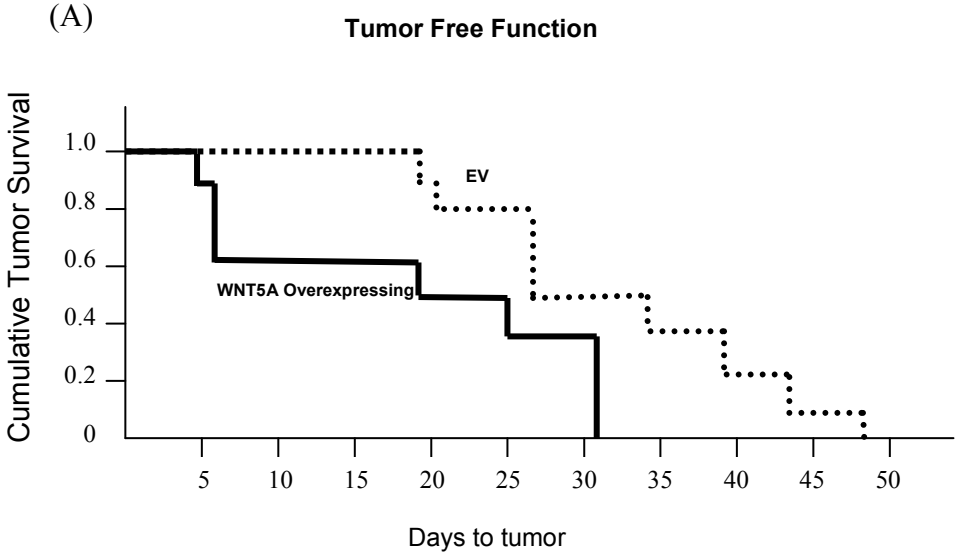


Figure 4.8 WNT5A promoted anchorage-independent growth in enamel epithelium. (A), S clones formed visible colonies in soft agar within 2 weeks after seeding, whereas WT, EV and AS clones failed to form visible colonies by bare eyes. (B), The number of colonies formed by S clones corresponded to the level of WNT5A expression. (Magnification 40x), (Bar, 200 μm)

(A)



(B)

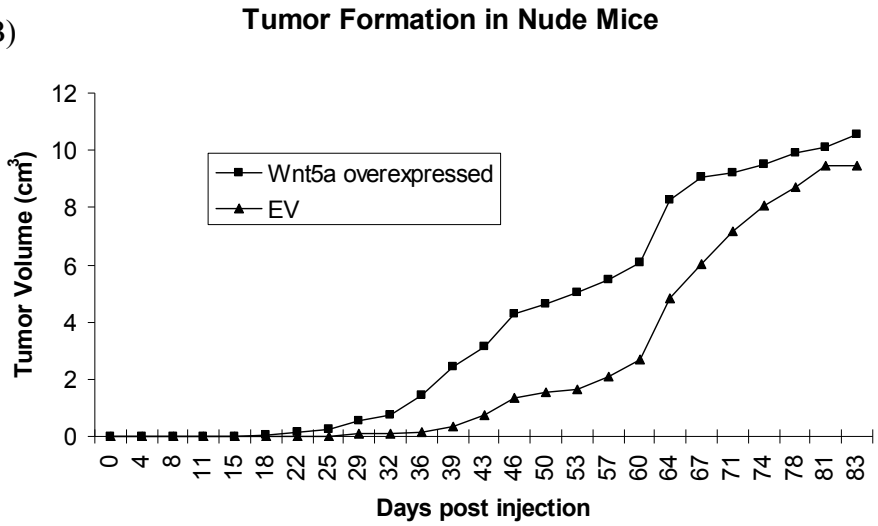


Figure 4.9 Tumor formation in nude mice. WNT5A overexpressing (SH) or EV-LS-8 cells ($2x10^6$) were subcutaneously transplanted into nude mice (*n*=8 per group). (A), SH cells formed tumors within 4 days after injection. EV groups also exhibited tumor growth at 18 days. On average, SH cells exhibited tumors within 18 days post implantation compared to 32 days in EV group. (B), SH groups also showed faster tumor growth rates when collective total tumor volume was calculated. Tumor formation was monitored for up to 83 days.

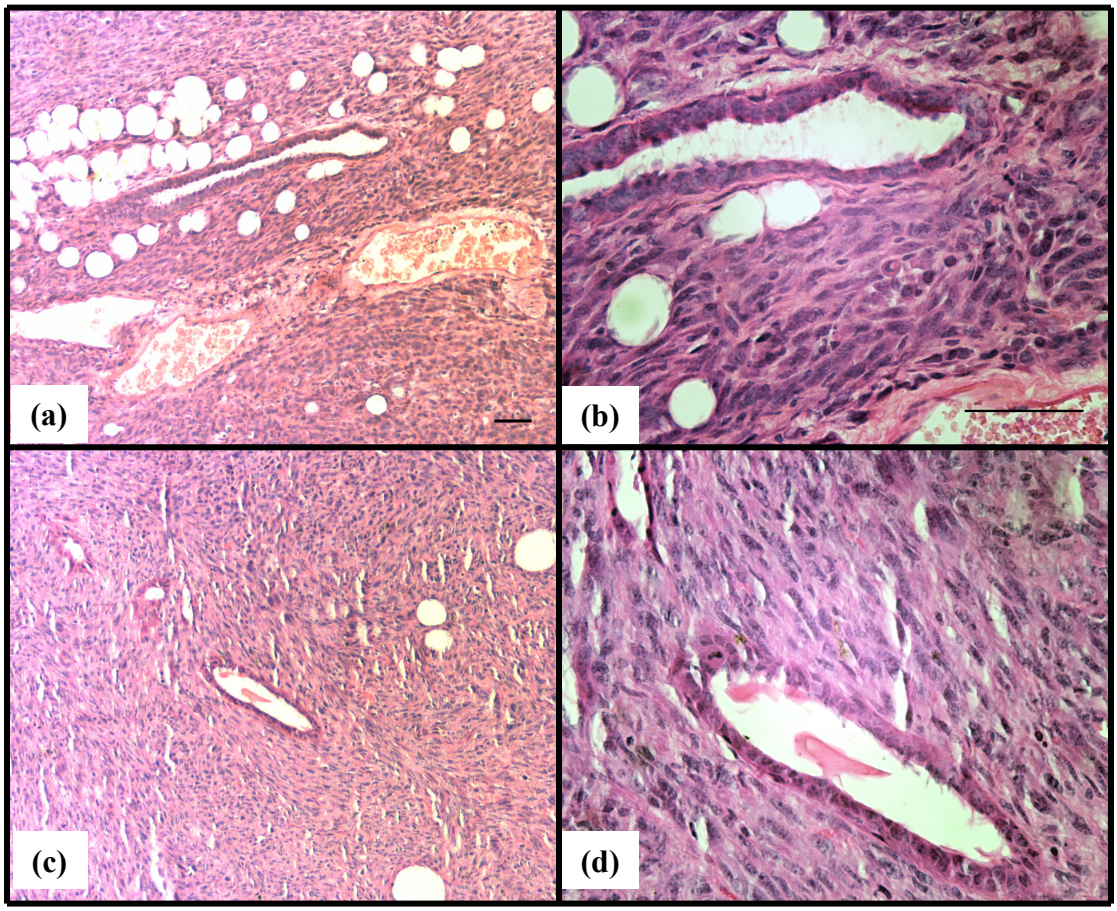


Figure 4.10 H&E staining of tumors formed in nude mice. Tumor histology appeared similar in both groups. The majority of the tumor mass was comprised of mesenchyme with epithelium that randomly formed duct-like structure. Chromatin condensation was found in most of the mesenchymal cells indicating that cells were in active mitosis. (Tumor formed by SH cells under 100x (a) and 400x (b). Tumor formed by EV cells under 100x (c) and 400x (d) magnification.) (Bar, 50 μm)

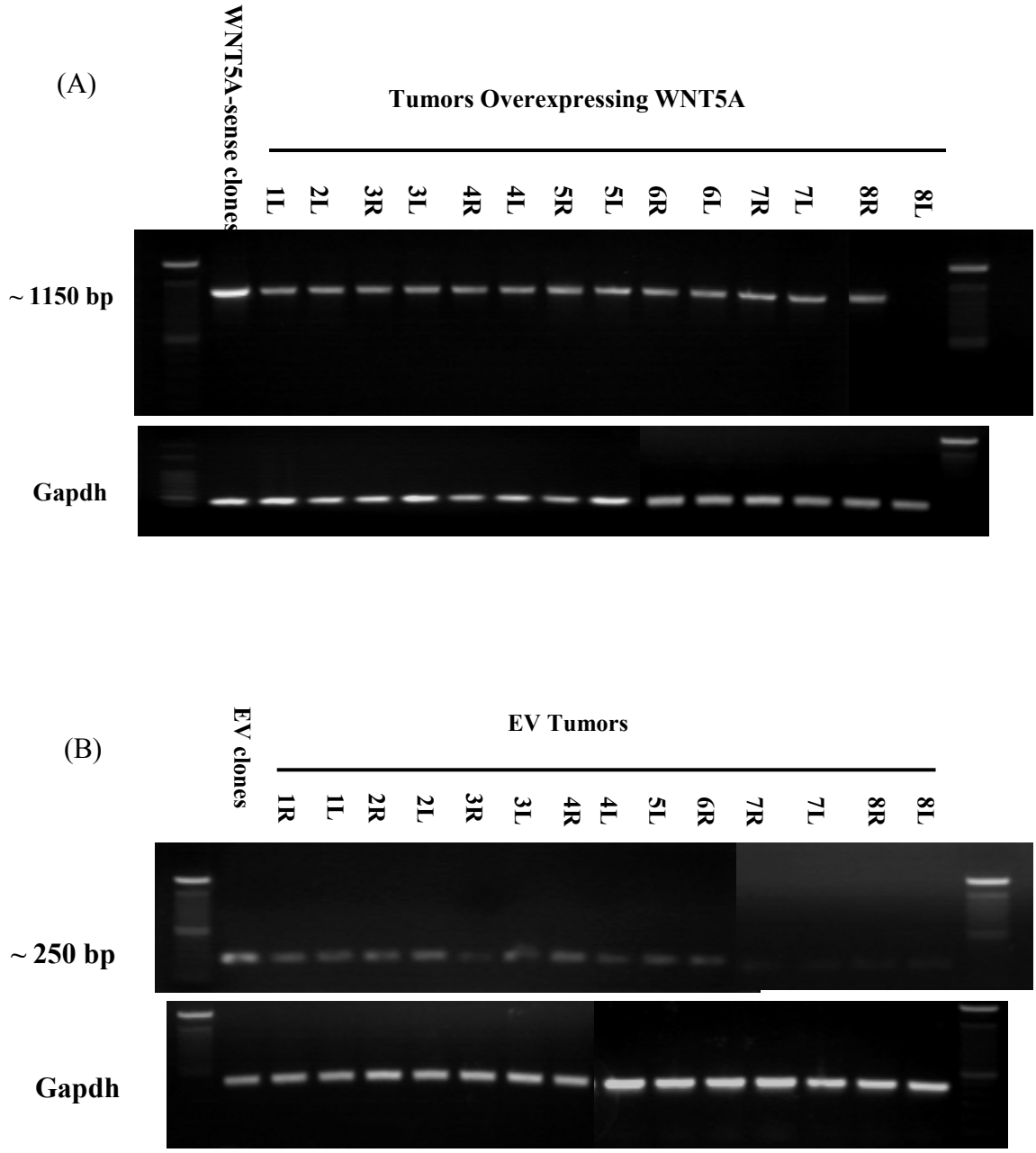
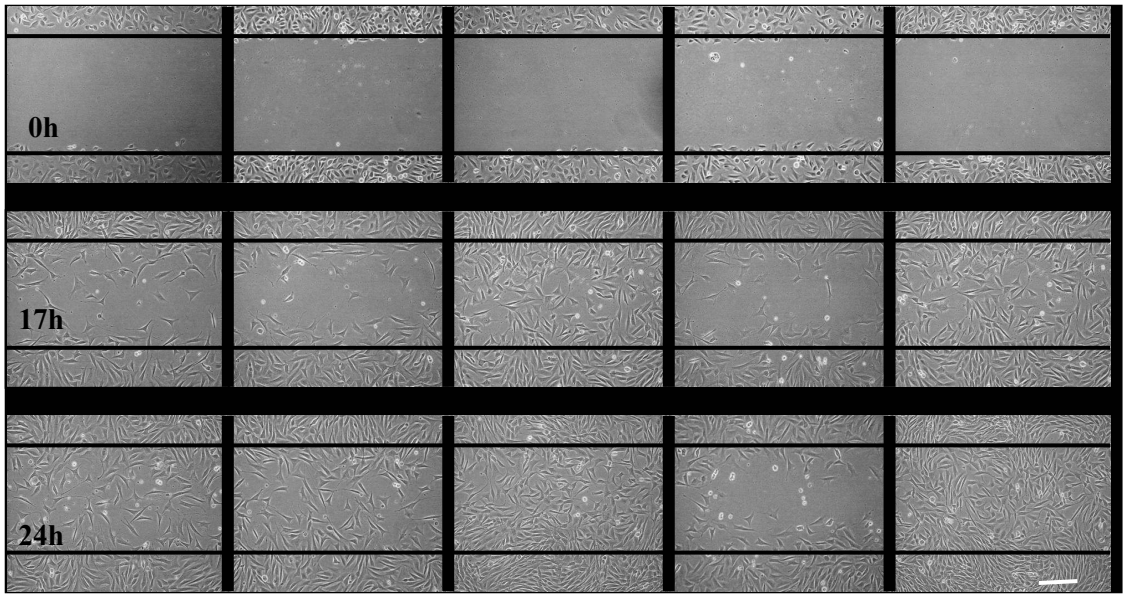


Figure 4.11 RT-PCR of the tumors formed in nude mice. (A), Bands of integrated *Wnt5a* plasmid approximately 1150 bp were detected in tumors overexpressing WNT5A using T7 forward and *Wnt5a* reverse primers. (B), Tumors formed in the EV group yielded the approximately 250 bp band of integrated pcDNA3.1 V5-HisA Empty vectors when evaluated using PCR with T7 forward and BGH reverse primers

(A)



WT **EV** **S** **AS** **R-WNT5A 0.25µg/ml**

(B)

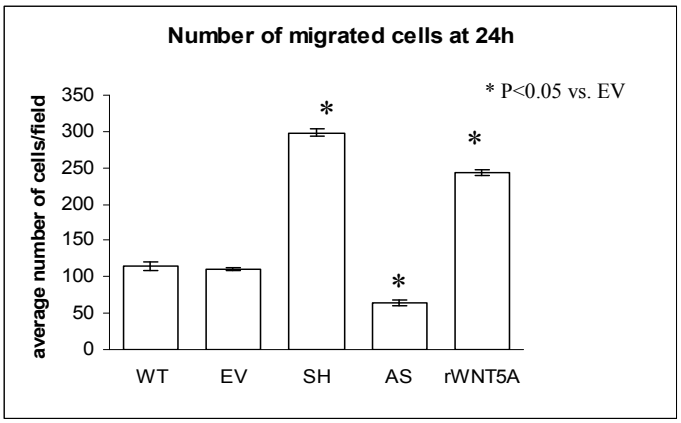


Figure 4.12 WNT5A increased cell migration in enamel epithelium cells. $2x10^6$ of cells were seeded onto the fibronectin coated 60-mm dish and supplemented with 2.5% FBS in DMEM to create a confluent monolayer. (A), A scratch was made and cell migration into the space was monitored at 17 and 24 h. WNT5A overexpressing cells (S) showed enhanced migration compared to EV control. In contrast, WNT5A underexpressing cells (AS) had greatly decreased cell migration. (B), Number of cells migrated into the space were quantified from five random fields. (Magnification 100x), (Bar, 100 µm)

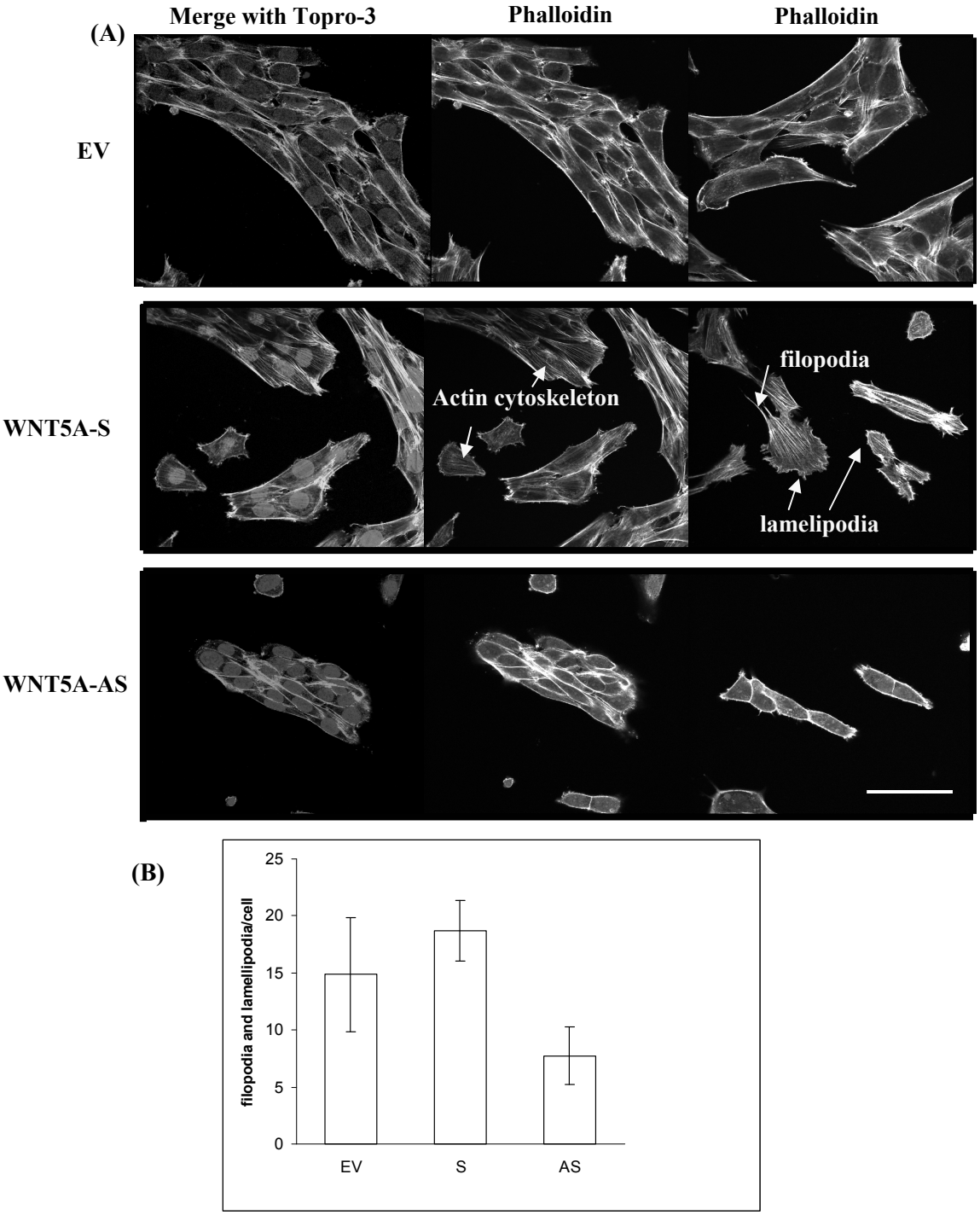


Figure 4.13 Effect of WNT5A on the morphology and actin cytoskeleton of enamel epithelium. (A), Actin cytoskeleton was stained using FITC phalloidin (bright) and examined using confocal microscopy. WNT5A overexpressing (S) cells showed a greatly increased density of stress fiber formation when compared to EV and AS cells. Filopodia and lamellipodia (arrows), were generally seen in WNT5A-S. (B), Number of filopodia/lamellipodia were counted and shown as mean±SD value from 20 cells/group. (Magnification 600x), (Bar, 20 μm)

DISCUSSION

One major difficulty in studying the molecular pathology of odontogenic tumors is the lack of appropriate cell lines and animal models. Attempts by multiple investigators show that primary cultured ameloblastoma cells have a very limited life span and required immortalizing procedures either by papillomavirus or SV40 large T antigen (256, 257). Additionally, relatively normal cell origins are widely used as the models to study an early event during tumorigenesis (214, 215). For the cell origin of odontogenic tumors, LS-8 cell exhibits several characteristics making it suitable for these studies (251, 252, 258). LS-8 cells are derived form new born mouse enamel organ (251). The remnants of enamel epithelium after completion of tooth formation are believed to give rise to odontogenic tumors (32, 40, 41). Until now, LS-8 cells are the most widely use *in vitro* model to study enamel epithelium biology and gene regulation of ameloblast development (251-253,258-261).

Since LS-8 cells are immortalized by SV40 large T antigen, a viral antigen known to transform growth of several human cell types (262), the interpretation of our results should be done with some caution. It has been reported that ectopic expression of SV40 small T antigen, but not large T antigen, was required in oncogenic RAS cell growth transformation (263, 264) and expression of large T antigen alone was not sufficient for cell transformation (262, 265). Interestingly, we observed in *in vivo* tumor formation assays where LS-8 cell populations had already acquired some growth transformation properties. This discrepancy might be due to the different transforming susceptibility of the SV40 large T antigen in each cell type (262). Though LS-8 and EV cells form few colonies in soft agar and tumors in nude mice, statistically significant differences were

observed with S cells that showing robust activities in all cell growth transformation assays. In the *in vivo* tumor formation assay, SH cells formed tumors 2 weeks earlier and exhibited faster tumor growth rates compared with EV cells. These phenomena could be partly explained by the possible function of WNT5A as the oncogenic enhancer. LS-8 cells potentially obtained some tumorigenicity from SV40 large T antigen; however, our results clearly show that WNT5A enhances tumorigenicity of these cells.

The role of WNT5A in tumorigenesis, whether it functions as tumor suppressor, oncogenic transformer or oncogenic enhancer is still debatable. WNT5A was categorized as non-transformed WNT due to the inability to transform mammary epithelial cells (212, 213), but its transforming property has rarely been tested in other cell models. In fact, WNT signaling outcomes are largely dependent on receptor availability (131, 188). Given that the expressions of *Lrp5/6, Fzd2, -4, -5* and *Ror2*, the known receptors for WNT5A (131, 140, 146), are detected in LS-8 cells by RT-PCR (data not shown), the effects of WNT5A on these LS-8 cells are highly anticipated. More recently the line of evidence leans towards the fact that the major role of WNT5A in cancer is to promote the aggressiveness of cancer cells rather than cancer initiation (188). WNT5A increased cell aggressiveness and cell motility when it was expressed in gastric cancer cell and melanoma cell (168, 200). Our observations from cell growth transformation assays also suggest that WNT5A functions as an enhancer to increase the oncogenic properties in LS-8 cells. Whether or not WNT5A can function as an oncogenic transformer is still inconclusive. More informative cell and animal models are needed to ultimately answer this question.

It is notable that histology of the tumors formed in both groups appears to be similar with the bulk of the cells being mesenchymal even though the cells being injected were epithelial. RT-PCR showed that these tumors developed from the injected enamel epithelium cells since the integrated empty vector and *Wnt5a* plasmids were detected from tumor RNA. Although the reasons are not presently known, it is possible that the LS-8 cells could have undergone EMT. This change in cell phenotype is found in some tumorigenic cells (231, 238). Additionally, *WNT5A* has been associated with the loss of E-cadherin and EMT expression in squamous cell carcinoma (238). Another possible explanation is based on the fact that enamel epitheliums are able to induce proliferation and differentiation of surrounding mesenchyme (odontoblast during tooth development). LS-8 cells might induce improper proliferation and differentiation of surrounding mesenchyme and secreted WNT5A from LS-8 cells could function in a paracrine manner to help accelerate this process. Although there was no area of tooth forming-like structure and no exact match when histology of nude mice tumors was compared to known mouse or human odontogenic tumors, these results were not altogether surprising. Since the environment at the inoculating sites was considerably different from the jaws, we did not expect the similarity of our tumors to any odontogenic tumor. However, since our tumors are composed of several duct-like structures, the closest comparison would be adenomatoid odontogenic tumor, which is an epithelial tumor that contains duct-like structure. Injection of LS-8 cells into the jaw area should be considered in the future experiment to create a potentially more appropriate environment for odontogenic tumor development.

The function of WNT5A in cell migration is another controversial subject evaluated by our studies. WNT5A has been shown to suppress migration and invasion of breast epithelial cells (232, 241). On the other hand, WNT5A was found to increase cell migration and invasion in melanoma cell, fibroblast and breast cancer (168, 227, 228). In our study, we found that WNT5A drastically promoted cell migration in LS-8 cells when tested by the scratch wound assay. This effect was due to increased cell migration rather than cell proliferation since the experiment was done in cultured medium containing 2.5% FBS and WNT5A had no effect on LS-8 cells when tested with the cell proliferation assay. Next, we also investigated if WNT5A increased enamel epithelium cell migration via a complex process of actin reorganization. Cells overexpressing WNT5A exhibited actin rearrangement and formed protruded membrane structures at the edges of the cells when cells were stained with phalloidin. These structures resemble lamellipodia and filopodia that produce the force for actively migrating cells. When endogenous WNT5A was suppressed in enamel epithelium, AS cells failed to form significant actin reorganization and membrane protrusion was rarely seen. Moreover, we observed different cell morphology between these cells after the cells were grown on fibronectin coated coverslips. S cells were more spread out while AS cells appeared to form cell clusters. Therefore, our data indicate a role for WNT5A in regulating enamel epithelium cell motility by increasing actin reorganization and extension that generates the force for cell movement.

In summary, our results demonstrate that WNT5A overexpression has multiple effects on enamel epithelium cells. WNT5A has the effect of promoting cell survival,

instilling a loss of contact inhibition and loss of anchorage dependence, and allowing an increase in cell migration, which are all important characteristics of tumorigenic cells.

CHAPTER V

STUDY III

WNT5A promotes tumorigenic behaviors of enamel epithelium via the non-canonical WNT5A/Rho/JNK pathway

Specific aims

1. To study the effect of WNT5A on the canonical WNT signaling pathway in enamel epithelium by TCF luciferase reporter assay.
2. To study the effect of WNT5A on the non-canonical WNT signaling pathway in enamel epithelium.

INTRODUCTION

WNT5A is known to initiate multiple downstream signaling pathways, including, WNT5A/PCP, WNT5A/calcium and WNT5A antagonized WNT/β-catenin pathways (188). These WNT5A pathways are complex and closely cross-talk to others. Studies show that WNT5A signals play essential roles, not only during embryogenic development but also during tumorigenesis as well as cancer progression (186, 248, 267). Our previous data in Chapter IV demonstrated that WNT5A is important for regulating enamel epithelium tumorigenicity and motility. In the present study, we attempted to identify the downstream signaling mechanisms involved in these processes. The specific inhibitors for the known effector proteins in WNT5A signaling pathways were used in *in vitro* cell growth transformation and wound healing assays. The inhibitor approach allowed us to evaluate reversed in the tumorigenic phenotype of cells overexpressing WNT5A (SH). Finally, the involvement of WNT5A/Rho/JNK pathway in modulating tumorigenic behaviors of enamel epithelium was also demonstrated by immunohistochemical and Western blot analyses.

EXPERIMENTAL PROCEDURES

Cell culture, transient transfection and luciferase assays. A series of experiments were conducted to elucidate whether the canonical or non-canonical WNT pathways were involved in WNT5A signaling. A luciferase assay for the canonical WNT signaling pathway was performed as previously described with modification (131, 190). LS-8 cells were cultured in DMEM containing 10% FBS at a density of $1x10^5$ cells in a 6-well plate. Cells were transiently transfected with a TCF reporter plasmid (0.3 μg/well) (Topflash), β-galactosidase reporter plasmid (0.2 μg/well) (pCMVβ, Clontech) together with pcDNA3.1/*Wnt5a* (0.75 μg/well) and/or pcDNA3.1/*Wnt1* (0.75 μg/well) using Fugene 6 transfection reagent (Roche). The total amount of transfected DNA (2 μg/well) was kept constant by adding pcDNA3.1/V5 empty vector. In order to determine the dose independent effect, luciferase assays were performed in LS-8 cells treated with various doses of recombinant WNT5A (0-250 ng/ml) (R&D system). *Lef-1* cDNA and Fopflash reporter constructs were used as positive and negative controls respectively. At 48 h post-transfection, luciferase assays were performed using the Luciferase Reporter Assay System (Promega). Relative luciferase units were measured and normalized against β-galactosidase activity. Data were presented as the mean $\pm$ SD. Each assay was performed at least three times.

Inhibitor treatment and Western blot analysis. LS-8, EV, SH and AS clones were cultured in DMEM containing 10% FBS and then treated with inhibitors specific to each effector protein known for WNT5A signaling pathways. Inhibitors used were as follows; JNK inhibitor (SP600125), RHOA kinase (ROCK) inhibitor (Y27632), CAMKII inhibitor (KN93) and PKC isoform inhibitors (Go6983 and GF109203X). All inhibitors

were obtained from Sigma-Aldrich. Dimethyl sulfoxide (DMSO) was used as vehicle and the final concentration of DMSO was maintained at 20 μl/ml. Inhibitor efficiency was verified by Western blot analysis. At 24 h after inhibitor treatment, cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (Sigma-Aldrich) containing the P1860 protease inhibitor cocktail (Sigma-Aldrich) and the P2850 phosphatase inhibitor cocktail (Sigma-Aldrich). Supernatant was collected and dissolved in SDS sample buffer (100mM Tris-HCl, pH 8.8, 0.01% bromophenol blue, 36% glycerol, and 4% SDS) in the presence of 10mM DTT, separated on SDS-PAGE gels (Invitrogen) and transferred to nitrocellulose membrane (Biorad). Antibodies used were as follows; anti- phospho-JNK (Thr183/Tyr185), anti-total JNK, anti-phospho-PKC (pan), anti-total PKC isoforms, anti-total CAMKII (pan), anti- β-actin, and anti-rabbit conjugated with HRP antibodies were obtained from cell signaling and were used in 1:1000 dilution. Anti-phospho-CAMKII (Thr286) (1:1000) was from Chemicon. The immunoreactivity was visualized using the ECL Plus kit (GE Healthcare). The final concentration of inhibitors that inhibited kinase activity of targeted proteins, but did not cause cell death was maintained in all further experiments in this study. The final concentrations of each inhibitor were as follows; SP600125 and Y27632 at 20 μM, KN93 at 25 μM, Go6983 and GF109203X at 1 μM.

Cell proliferation assay. LS-8 , EV and SH clones were seeded at a density of $1x10^5$ cell/well and maintained in DMEM containing 10% FBS before adding inhibitors for an additional 24 h incubation. Every two days, cells were supplemented with fresh medium containing inhibitors. Cells were trypsinized and counted at day 1, 3, 5 and 7. Experiments were performed in triplicate.

Growth transformation assays. Serum independence of cell growth was determined. Briefly, LS-8 , EV and SH clones were seeded in triplicate at a density of $1x10^5$ cell/well and maintained in DMEM containing 10% FBS and cells were allowed to adhere to the culture dish. Then, the culture medium was changed to 1% FBS containing inhibitors followed by 24 h of incubation. Cells were supplemented with fresh medium (1% FBS) containing inhibitors every two days. Cells were trypsinized and counted at day 1, 3, 5 and 7 to determine cell proliferation and viability.

Focus formation assay was performed as previously described with modification (249, 254). Cells were seeded in triplicate of $1x\ 10^5$ cell/ 60-mm cultured dish. Culture medium containing inhibitors was replaced twice weekly and cells were maintained in this cultured condition for 3 weeks. After that, cells were fixed with 3:1 (v/v) methanol: acetic acid stained with 0.4% crystal violet in 20% ethanol and photographed. Stained foci were counted for quantification of transforming activity.

To determine the anchorage-independent growth, soft agar assays were performed as previously describe with modification (249, 254). Cells were cultured in medium containing 10% FBS and the monolayer of cells was trypsinized to obtain single-cell suspensions. A total of 5×10^3 cells were suspended in DMEM containing 0.3% agar and were then seeded onto a 0.5% agar base in six-well plates. Inhibitors were added to both the agar containing the cells and the feeding medium. Completed medium with inhibitors was replaced twice weekly. Four weeks after the initial seeding, colony growth was assayed by counting the number of colonies (>50 μm in diameter) in five random three-dimensional fields in each well under 4x objective lens and photographed. To confirm

the reproducibility of the results, three independent experiments were performed for each assay.

Wound healing assay. Cells were grown on fibronectin (Sigma-Aldrich) coated 60-mm dish supplemented with completed medium (10% FBS) to create a monolayer of cells. After the cells reached 90% confluence, the medium was switched to 2.5% FBS supplemented with inhibitors and a wound was created by scratching a line across the monolayer of cells with a pipette tip (255)**.** At 17 and 24 h after the scratch was made, cell migration into the created wound was monitored. The number of cells migrated into the space were counted in five random fields, photographed and fixed with 3:1 (v/v) methanol: acetic acid, and stained with 0.4% crystal violet in 20% ethanol. To confirm the reproducibility of the results, five independent experiments were performed in duplicate.

Immunohistochemistry. First occurrence, multicystic/solid ameloblastoma tumors were collected from UNC Oral Pathology. Immunohistochemical analysis was performed on 4 μm paraffin-embedded sample sections using Elite Vectastain kit (Vector Laboratories). Deparaffinized sections were preincubated with normal serum to block nonspecific binding and then incubated with an anti-WNT5A antibody (1:200) (R&D systems) or anti-phospho-JNK antibody (1:200) (Santa Cruz) at 4 ^{o}C overnight. A biotinylated rabbit anti-goat and goat anti-mouse antibodies were used as the secondary antibodies for anti-WNT5A and anti-phospho-JNK antibodies respectively. Immunoreaction was visualized by using DAB substrate kit for peroxidase (Vector Laboratories, Burlingame, CA). Sections were counterstained with hematoxylin and evaluated for immunoreactivity.

JNK expression and activation assay. EV, S and AS clones were cultured in DMEM containing 10% FBS. EV clones were either treated with recombinant WNT5A (300ng/ml) (R&D systems) alone for 1 h, pretreated with SP600125 for 1 h followed by recombinant WNT5A for additional 1 h of incubation or treated with UV radiation ($300J/M^2$) for 1 h. Cells were then lysed in RIPA buffer (Sigma-Aldrich) containing the P1860 protease inhibitor cocktails (Sigma-Aldrich) and the P2850 phosphatase inhibitor cocktail (Sigma-Aldrich). The supernatant was collected and dissolved in SDS sample buffer containing 10 mM DTT, separated on SDS-PAGE gels (Invitrogen) and transferred to nitrocellulose membrane (Biorad). Western blot analyses were done using primary antibody against total JNK, phosphorylated JNK (Thr183/Tyr185) and β-actin. Anti-rabbit conjugated with HRP antibody was used as secondary antibody. The immunoreactivity was visualized using ECL Plus kit (GE Healthcare).

Statistical analysis. All statistical analyses were performed using Sigma stat software. Data are expressed as mean$\pm$SD. Statistical differences were determined by one-way ANOVA followed by a Tukey-Kramer multiple comparison test at P=0.05.

RESULTS

WNT5A antagonized WNT canonical pathway in enamel epithelium. It is well established that WNT5A can initiate several downstream signaling pathways and the effect largely depends on the receptor contexts at the cell surface (131). Studies show that WNT5A can antagonize the canonical WNT/β-catenin pathway in several cell types (131, 139, 189, 190). WNT/β-catenin pathway is known to be critical in regulating normal tooth formation; therefore we investigated if WNT5A could inhibit the canonical WNT/β-catenin pathway in enamel epithelium by monitoring the activation of Topflash, the widely used reporter gene for canonical WNT signaling.

When LS-8 cells were transiently cotransfected with the Topflash reporter and *Wnt1* plasmid, the WNT/β-catenin pathway activator, the luciferase activity of the reporter gene was increased by 1.6 fold (Figure 5.1 (A)). When LS-8 cells were cotransfected with *Wnt5a* plasmid (0.75 μg/well) or treated with recombinant WNT5A (50 ng/ml), the luciferase activity was decreased by 0.46 and 0.42 fold, respectively. Cotransfection of *Wnt5a* together with *Wnt1* in LS-8 cells resulted in greater reduction of luciferase activity by 0.24 fold. A similar result was found when *Wnt5a* plasmid transfection was substituted with recombinant WNT5A treatment, the luciferase activity was suppressed by 0.32 fold. Moreover, when LS-8 cells were treated with different doses of recombinant WNT5A (0-250 ng/ml), the luciferase inductions were inhibited in a dose dependent manner (Figure 5.1 (B)). Our results corroborate previous studies in other cell types (131, 139, 190), suggesting that WNT5A antagonizes the canonical WNT/β-catenin pathway in enamel epithelium.

JNK inhibitor and ROCK inhibitor treatment reversed the tumorigenic phenotype of enamel epithelium overexpressing WNT5A. WNT5A is a known ligand for different signaling pathways (188). Studies indicate that WNT5A triggers intracellular calcium flux leading to the activation of calcium dependent effectors such as CAMKII, PKC and NFAT (166, 175, 177). It is also known that WNT5A/DVL activation can lead to increased phosphorylation of JNK (183,184) and involves RHO GTPases and ROCK activation (186, 187). In order to identify downstream signals triggered by WNT5A in enamel epithelium several inhibitors specific for each effector protein in the pathways that are known to be activated by WNT5A were used to treat cells overexpressing WNT5A in this study. Dosage use for each inhibitor was determined by Western blot analysis (Figure 5.2) and cytotoxicity (data not shown). The working dose was the minimum concentration of inhibitors that inhibit kinase activity of targeted proteins, but did not cause cell death. Working concentrations of each inhibitor were as follows; CAMKII inhibitor (KN93) 25 μM (Figure 5.2 (A)), PKC inhibitors (Go6983 and GF109203X) 1 μM each (Figure 5.2 (B)), ROCK inhibitor (Y27632) 20 μM (Figure 5.2 (C)), and JNK inhibitor (SP600125) 20 μM (Figure 5.2 (D)). DMSO was used as a vehicle and the final concentration of DMSO was maintained at 20 μl/ml for each inhibitor.

In vitro cell growth transformation was performed, together with inhibitor treatment, on three independent clones overexpressing WNT5A (SH). First, we evaluated the morphology of these cells. When seeded on culture dishes, no change in cell morphology was observed at 24 h after inhibitor treatment. All inhibitor treated SH cells

showed similar phenotypes to DMSO treatment alone, untreated SH and WT cells (Figure 5.3).

Inhibitor addition to SH cells did not affect cell proliferation rate when cells were maintained in completed medium containing 10% FBS (Figure 5.4). In our previous experiment, SH cells survived and proliferated in serum reduced condition that contained 1% FBS for up to 7 days (Figure 5.5, left upper row). Interestingly, only JNK inhibitor (Figure 5.5, right lower row) and ROCK inhibitor (Figure 5.5, left lower row) treatment suppressed SH cell survival and proliferation when cells were cultured in the starved condition. Other inhibitors had no effect on SH cell survival (Figure 5.5).

Additionally, when tested with the focus formation assay, treatment with JNK or ROCK inhibitors significantly reduced the numbers of colonies formed by SH cells (Figure 5.6). CAMKII inhibitor treated groups also showed lower numbers of colony formation but this difference was not statistically significant (P=0.075), whereas PKC inhibitor treated cells showed similar numbers of colonies formed when compared to DMSO treated group (Figure 5.6). The same results were demonstrated when we analyzed anchorage-independent growth in these clones by soft agar assay (Figure 5.7). Only treatment with JNK or ROCK inhibitors significantly decreased the numbers of colonies formed by SH cells (Figure 5.7) in soft agar when compared to DMSO treated and EV controls. Taken together with our *in vitro* analyses, we concluded that JNK and ROCK pathways were associated with the WNT5A induced tumorigenicity of enamel epithelium.

Previous studies show that activation of JNK and ROCK by WNT5A involves regulation of cytoskeleton and subsequent cell migration (186, 187). We therefore

investigated the effect of JNK and ROCK inhibitors on SH cell migration. At 24 h after the scratch, more SH cells migrated and filled up the created space compared with EV control. As we expected, treatment with either JNK or ROCK inhibitors greatly reduced the numbers of SH cells that migrated to the created space at 24 h (Figure 5.8, middle and right lanes) compared with the vehicle treatment control cells (Figure 5.8, left lane). Our results indicated that WNT5A regulated enamel epithelium cell migration via the activation of JNK and ROCK pathways.

Expression of phospho-JNK in ameloblastoma. Since our inhibitor experiments suggested the involvement of the non-canonical WNT5A/JNK pathway in tumorigenicity of enamel epithelium, we expected to find the activation of JNK in human ameloblastoma samples that were found positive for WNT5A expression from our previous experiment. Immunohistochemical analysis was performed on all human ameloblastomas (n=52) to detect phosphorylated forms of JNK. When using serial sections, 88.5% (46/52) of samples were found positive for both WNT5A and phospho-JNK and 3.8% (2/52) were negative for both. However, 7.7% (4/52) were negative for WNT5A but positive for phospho-JNK (Table 5.1). Strong phospho-JNK immunoreaction (Figure 5.9, (b), (d) and (f)) was detected in the epithelial component of ameloblastomas showing a similar expression pattern to WNT5A (Figure 5.9, (a) and (c)). These results provide the first connection between WNT5A and activation of JNK in ameloblastoma tumorigenicity and suggesting that the WNT5A/JNK pathway could be responsible for certain tumorigenic behaviors of enamel epithelium.

WNT5A activates JNK phosphorylation in enamel epithelium. It has been shown that WNT5A regulates JNK phosphorylation and subsequently changes cell

behaviors such as cell migration in several cell types (146, 227, 229, 267). For further confirmation, we performed Western blot to check if JNK was activated in our enamel epithelium overexpressing WNT5A and that exhibited several tumorigenic properties. Treatment of UV radiation (300 J/M^2) is known to activate JNK in several cell types (268, 269) and was used for the positive control in the present study (Figure 5.10, lane 1). When using a specific antibody against phosphorylated JNK1 (P-p46) and JNK2 (P-p54) at Thr183/Tyr185 (P-Thr183/P-Tyr185), increased phosphorylation of JNK isoforms was detected in UV activated- EV, SH and SL cells (Figure 5.10, lane 1, 2 and 3) compared with untreated EV control cells (Figure 5.10, lane 7). Moreover, treatment of EV cells with recombinant WNT5A (300 ng/ml) for 1 h resulted in activation of JNK (Figure 5.10, lane 5) and JNK phosphorylation was inhibited when cells were preincubated with JNK inhibitor (20 μM) for 1 h before recombinant WNT5A addition (Figure 5.10 lane 6). Interestingly, a similar result was found in AS clones where phosphorylation of JNK1 and JNK2 was decreased (Figure 5.10, lane 8) compared with EV cells. Taken together, our results provide evidence that WNT5A induced tumorigenic behavior of enamel epithelium and this process involves activation of the JNK pathway.

Table 5.1 Immunohistochemical analysis of phosphorylated JNK expression in human ameloblastoma in relation to WNT5A expression.

	WNT5A positive	WNT5A negative	Total
P-JNK positive	46 (88.5%)	4 (7.7%)	50
P-JNK negative	0 (0%)	2 (3.8%)	2
Total	46	6	52

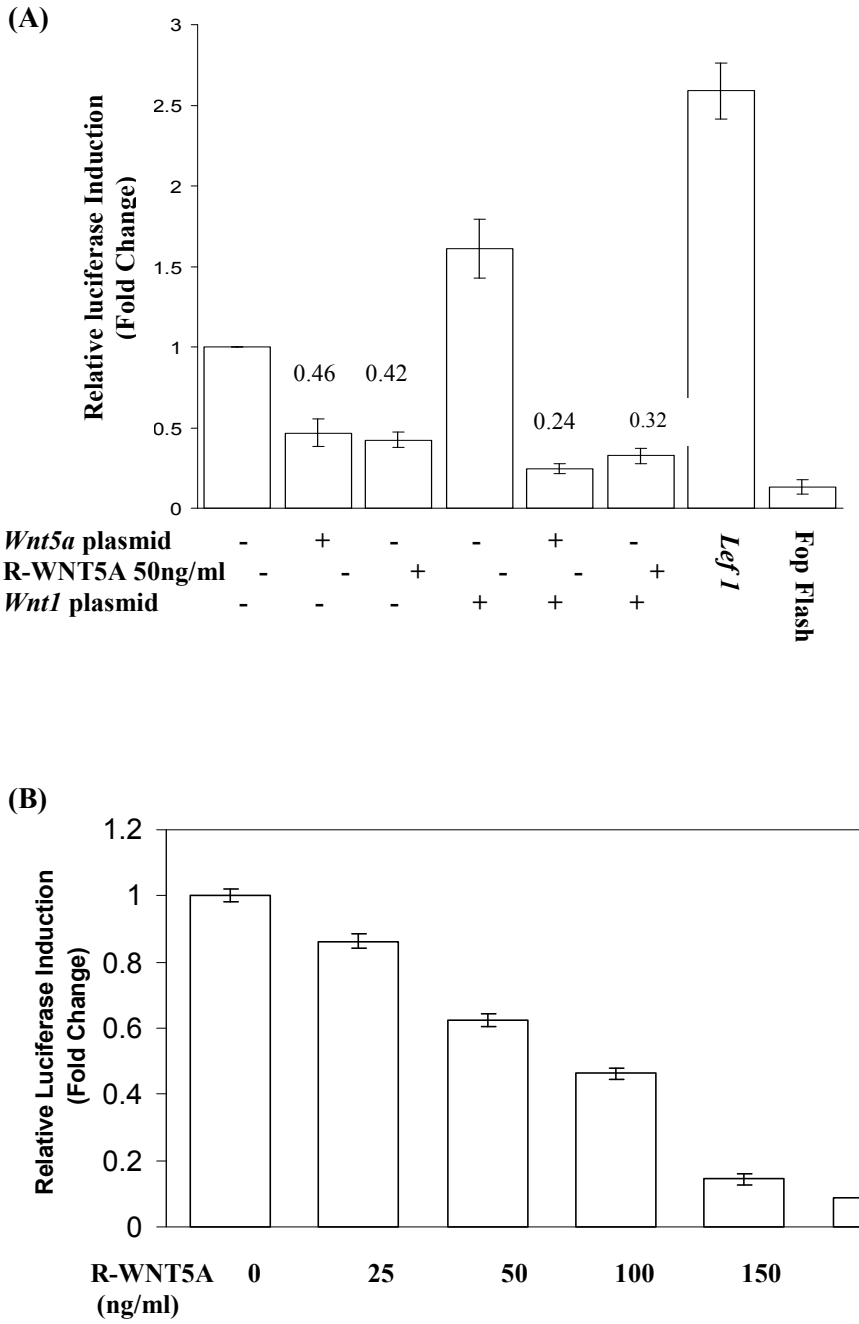


Figure 5.1 WNT5A antagonized Wnt canonical pathway in LS-8 cells. (A) When *Wnt5a* plasmid was cotransfected or recombinant WNT5A (50 ng/ml) was added to LS-8 cells, the luciferase activity was decreased by 0.46 and 0.42 fold, respectively. Cotransfection of *Wnt5a* plasmid together with *Wnt1* resulted in greater reduction of luciferase activity to 0.24 fold. (B) When different doses of recombinant WNT5A were added (0-250 ng/ml), the luciferase induction was inhibited in a dose dependent manner.

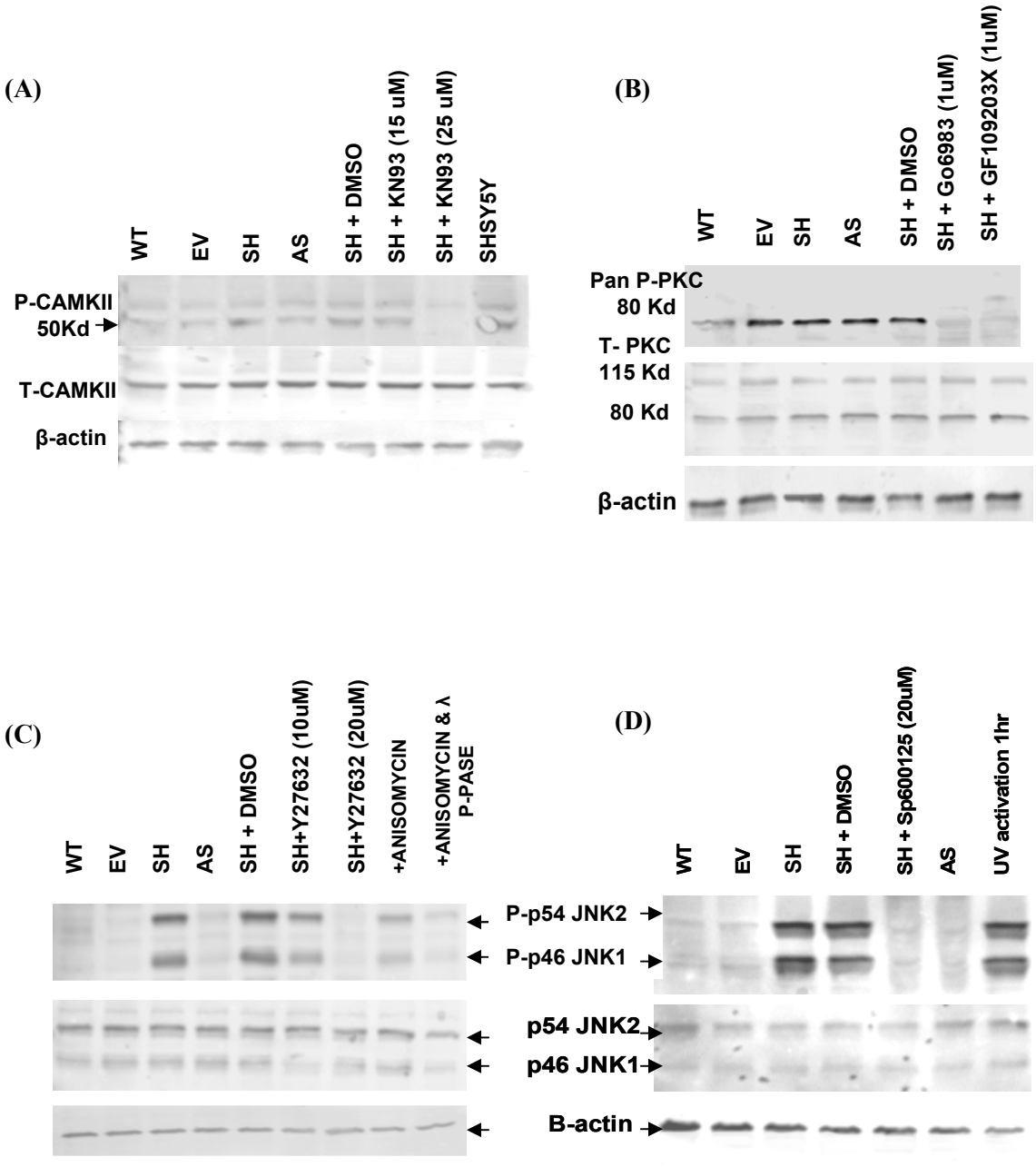


Figure 5.2 Working concentration for each inhibitor determined by Western blot. (A) CAMKII inhibitor (KN93) 25 μM, (B) PKC inhibitors (Go6983 and GF109203X) 1 μM each, (C) ROCK inhibitor (Y27632) 20 μM and (D) JNK inhibitor (SP600125) 20 μM were the minimum concentrations that inhibit kinase activity of the targeted proteins, without causing cell death.

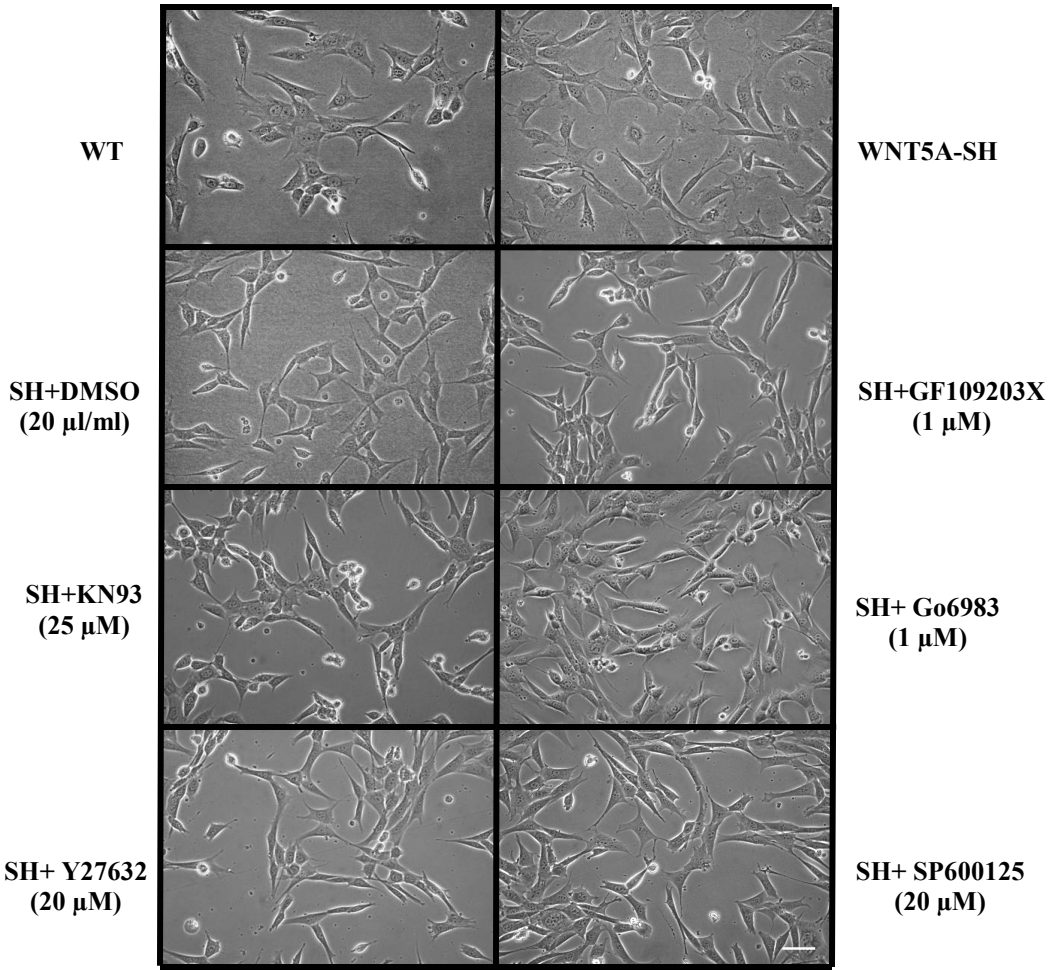


Figure 5.3 Morphology of enamel epithelium cells overexpressing WNT5A after 24 h of inhibitor addition. No cell morphological change was observed after inhibitors were added to cells overexpressing WNT5A (SH). All inhibitor treated SH cells showed similar phenotypes to DMSO treatment alone, untreated SH and WT cells. (Magnification 200x), (Bar, 100 µm)

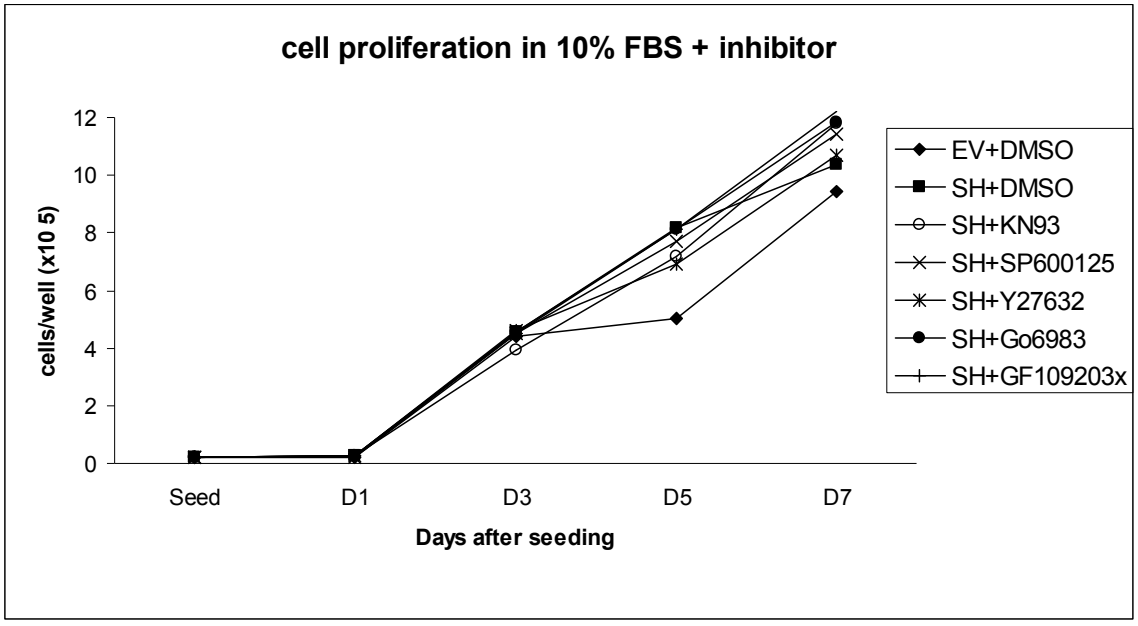


Figure 5.4 Proliferation of cells overexpressing WNT5A in 10% FBS after inhibitor addition. Inhibitor addition to SH cells showed no effect on the cell proliferation rate when cells were cultured in completed medium containing 10% FBS

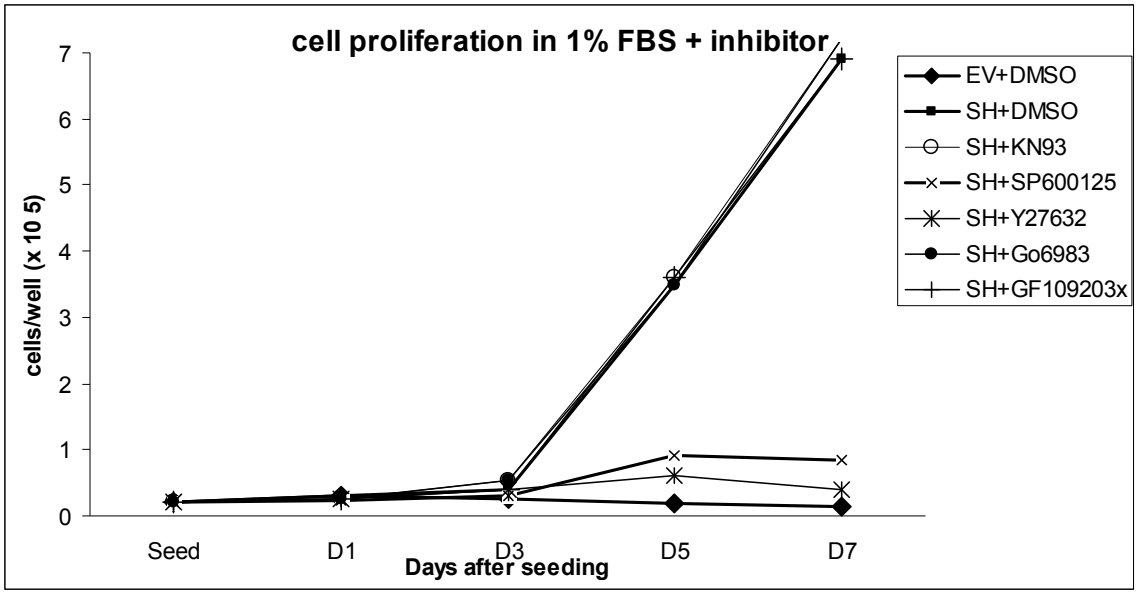


Figure 5.5 JNK and ROCK inhibitors suppressed survival of cells overexpressing WNT5A in 1% FBS. WNT5A-SH cells survived in serum reduced condition for up to 7 days. When inhibitors were added, only JNK inhibitor (SP600125) and ROCK inhibitor (Y27632) suppressed SH cell survival but other inhibitors had no effect on SH cell survival.

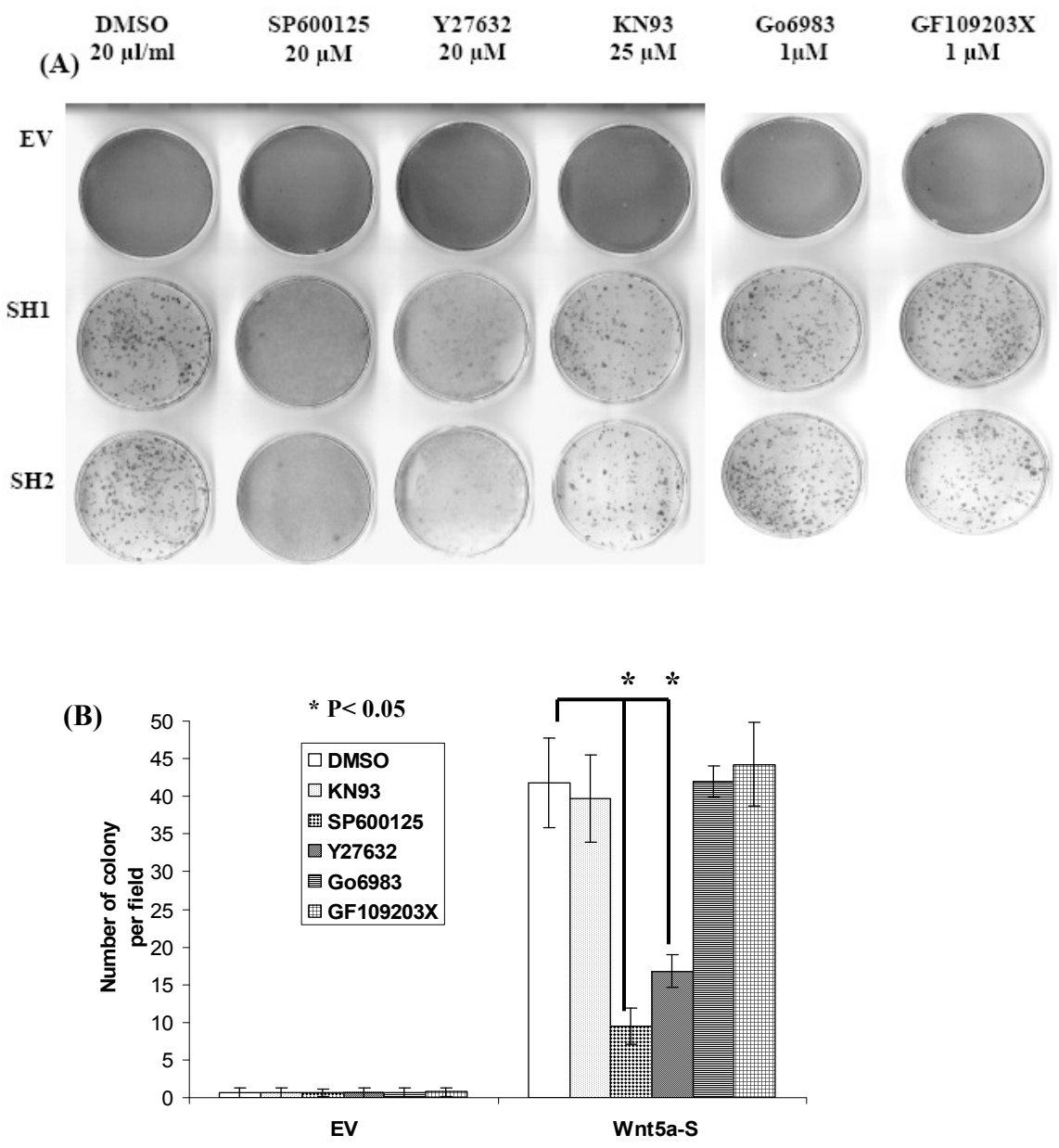


Figure 5.6 JNK and ROCK inhibitors decreased loss of contact inhibition in cells overexpressing WNT5A. JNK and ROCK inhibitors significantly reduced the numbers of colonies formed by SH cells in the focus formation assay. CAMKII inhibitor (KN93) also lowered numbers of formed colonies but the difference was not statistically significant (P=0.075), whereas PKC inhibitors (Go6983 and GF109203X) showed similar numbers of colonies when compared to DMSO treated group.

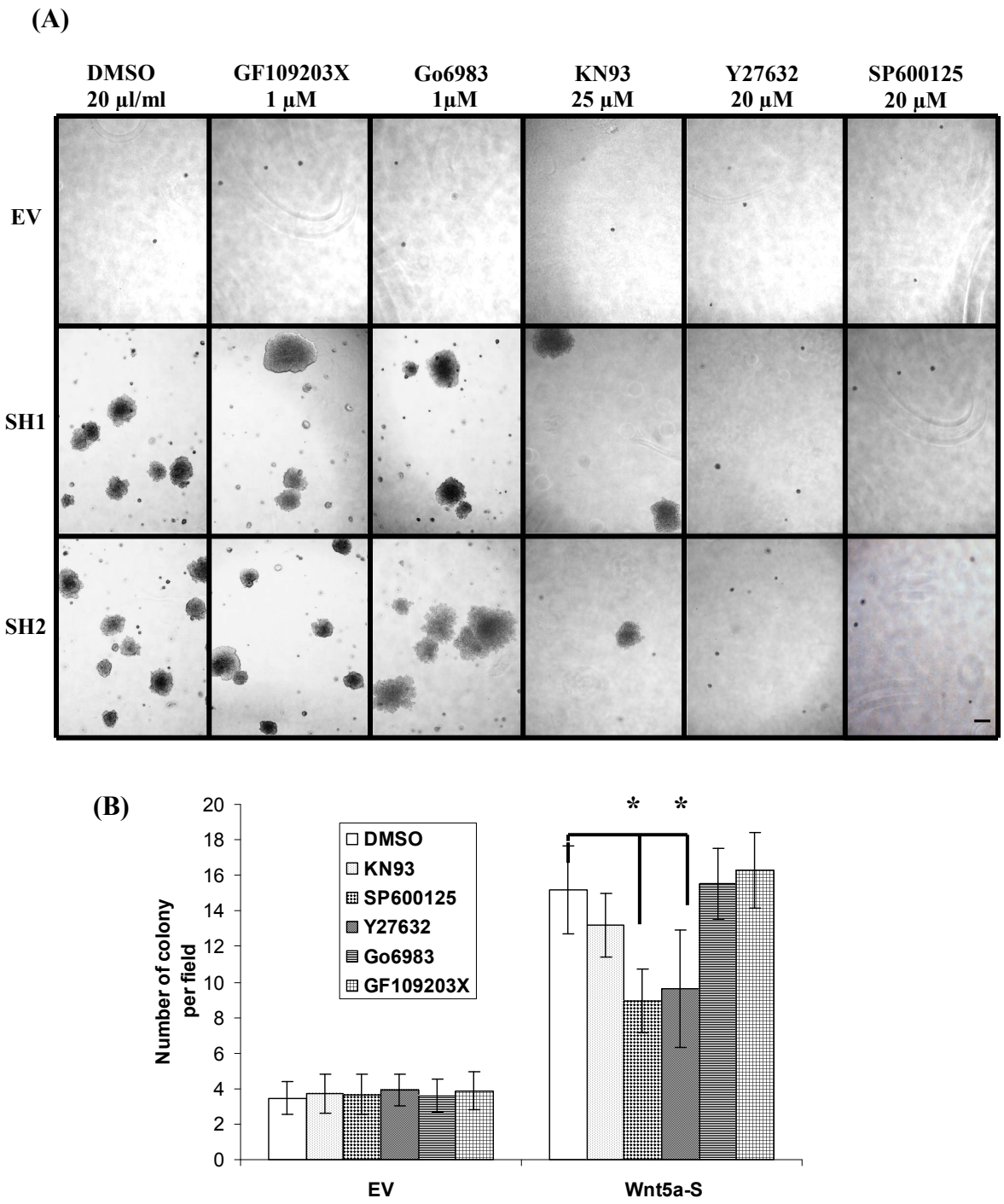


Figure 5.7 JNK and ROCK inhibitors decreased anchorage-independent growth in cells overexpressing WNT5A. JNK and ROCK inhibitors significantly reduced the numbers of colonies formed by SH cells in soft agar assay. CAMKII inhibitor (KN93) showed lesser colony formation, whereas PKC inhibitors (Go6983 and GF109203X) had similar numbers of colonies compared to the DMSO treated group. (Magnification 40x), (Bar, 200 µm)

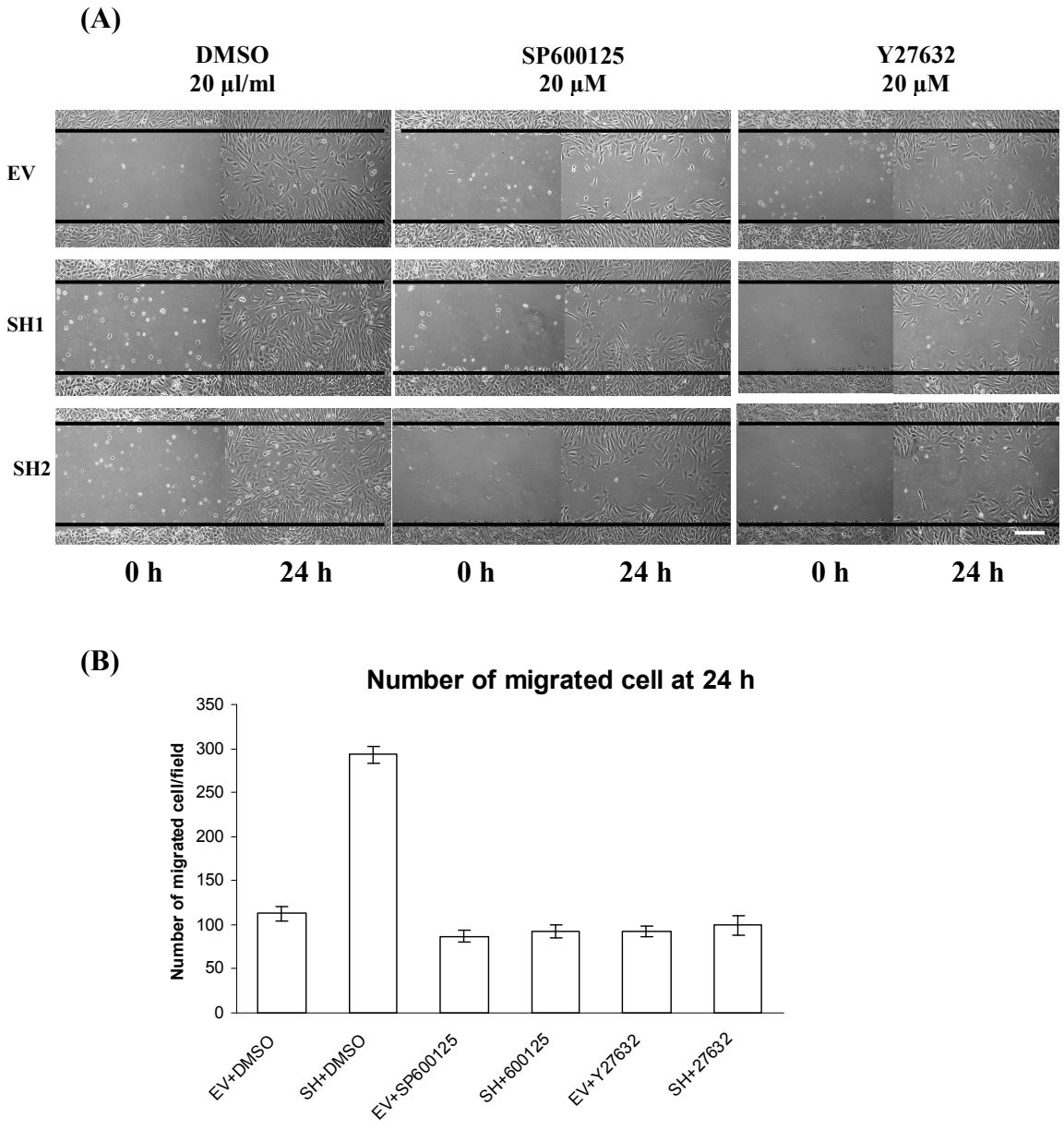


Figure 5.8 JNK and ROCK inhibitors suppressed cell migration in cells overexpressing WNT5A. (A), 2x10 ^{6} of cells were seeded onto the fibronectin coated 60-mm dish and supplemented with 2.5% FBS. 24 h after the scratch, SH cells migrated into most of the space. The addition of JNK or ROCK inhibitors to the SH cells greatly impaired SH cell migration compared to DMSO treatment alone. (B), Number of migrated cells into the space were counted in five random fields and shown as mean$\pm$SD (Magnification 100x), (Bar, 100 µm)

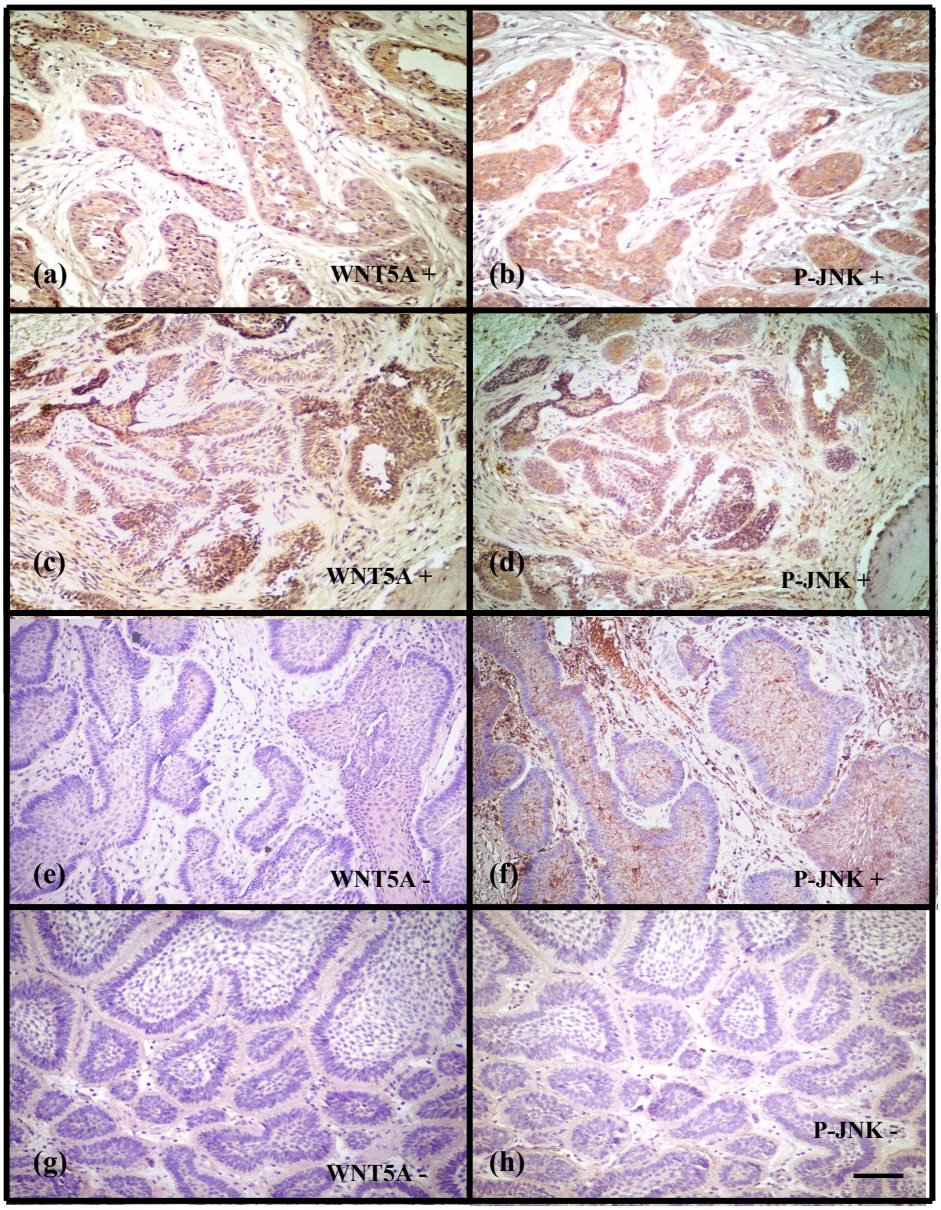


Figure 5.9 Immunohistochemical analysis of WNT5A and phosphorylated JNK in ameloblastomas. 88.5% of ameloblastoma samples were found positive for WNT5A and phospho-JNK ((a) and (b)), (c) and (d)), and 3.8% was negative for both ((g) and (h)). 7.7% was negative for WNT5A but positive for phospho-JNK ((e) and (f)). Both WNT5A and phospho-JNK showed similar patterns of immunoreaction with strong expression detected in the epithelium component of the ameloblastomas. (Magnification 100x), (Bar, 100 µm), (*n*=52)

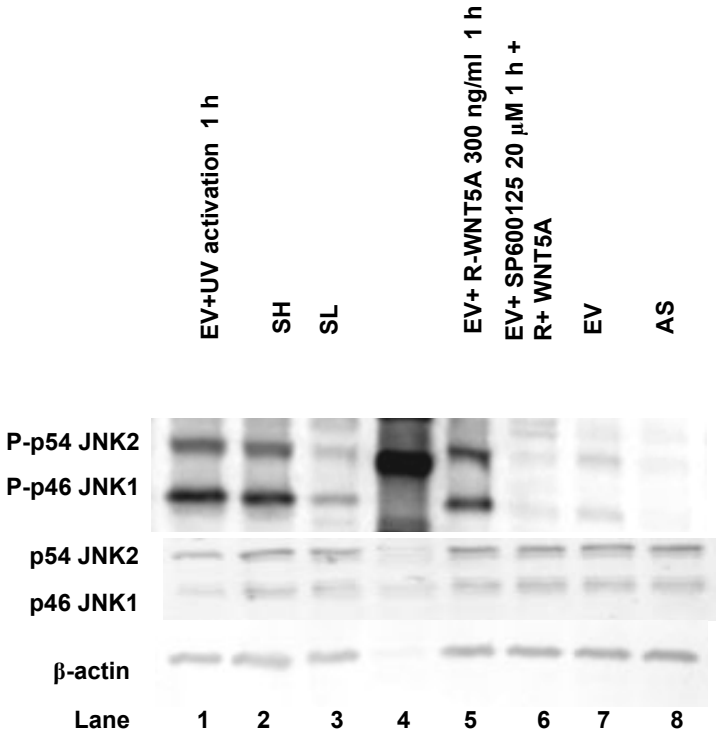


Figure 5.10 WNT5A activates JNK phosphorylation in enamel epithelium cells. Phosphorylated JNK1 (P-p46) and JNK2 (P-p54) were increased in UV activated-EV, SH and SL (lane 1, 2 and 3) compared to EV alone (lane 7). Treatment of EV cells with recombinant WNT5A (300 ng/ml) for 1 h resulted in activation of JNK (lane 5) and JNK phosphorylation was diminished when JNK inhibitor (SP600125, 20μM) was added before recombinant WNT5A incubation (lane 6). Phosphorylation of JNK1 and JNK2 was also decreased in AS clones (lane 8) compared with EV cells.

DISCUSSION

Our previous data indicated that WNT5A is important for mediating enamel epithelium tumorigenicity and motility. In these experiments, we investigated the signaling mechanisms involved in these processes. Studies show there are multiple ways that the non-canonical WNT5A pathway can interact with the canonical WNT/β-catenin and the outcomes can be varied depending on cell surface receptors and intracellular context (131, 188). Our results indicate that WNT5A antagonizes the WNT/β-catenin pathway in enamel epithelium that could have implications during tooth development. Studies show that WNT/β-catenin signaling operates at multiple stages of tooth morphogenesis and promotes a cascade of several downstream signaling events (202, 205, 270). Although the canonical WNT signaling pathway is indispensable during odontogenesis, WNT/β-catenin signaling must be tightly regulated for establishing normal tooth formation. Studies show that gain of function mutation in β-catenin causes abnormal dental invaginations and ectopic tooth formation (270). WNT5A might function as a regulator or fine tuner of WNT/β-catenin signaling during tooth development since its antagonized effect appears to be in a dose dependent manner. This hypothesis clearly needs further investigation.

Our data suggest the involvement of WNT5A/JNK and WNT5A/Rho pathways in enamel epithelium tumorigenicity and further indicate that these two pathways are closely related. The Rho family of small GTPases are known to activate upstream of JNK in variety of cell systems (271-274). However, studies show that activation of RHO GTPases is not required for JNK phosphorylation in WNT5A signaling (275). RHO GTPases-independent activation of JNK via ROR2 can antagonize WNT/β-catenin

signaling (131, 146). Recent studies show that activation of JNK via RHOA results in increased cell migration and invasion (267) providing a potential key pathway for WNT5A in mediating cancer aggressiveness. Our study supports these previous observations. When LS-8 cells overexpressing WNT5A were treated with specific inhibitors for each known effector in the WNT5A signaling pathway, similar results were observed with JNK and ROCK inhibitors treatment which suppressed cell proliferation in reduced serum. Decrease in the loss of contact inhibition and loss of anchorage dependence in SH cells were seen when cells were treated with JNK and ROCK inhibitors. Our observations also suggest that WNT5A activation through the JNK and ROCK pathway is crucial for enamel epithelium cell migration. Reduced expression of WNT5A greatly impaired enamel epithelium cell migration and specific JNK or ROCK inhibitors were able to diminish the WNT5A-enhancement of cell migration in SH cells. Although we have not tested the effect of JNK and ROCK inhibitors on actin rearrangement, our results were in line with other studies that shown the increased activation of JNK and RHO GTPases coincided with WNT5A increased cell migration (222, 227, 229, 267). Interestingly, when we blocked the Rho pathway with a ROCK inhibitor the level of JNK phosphorylation was also decreased, suggesting that JNK was downstream of RHO and ROCK in enamel epithelium tumorigenesis. Whether there was WNT5A/Rho independence JNK activation involved remains unknown.

Although activation of WNT/calcium pathway is known to play major roles in tumor progression and metastasis in many types of cancer (175, 179, 231), our study showed different results. We observed no significant change when PKC or CAMKII inhibitors were added in the system. However, inhibition of CAMKII seems to partially

relieve the effect of WNT5A since we observed a decrease in colony formation in soft agar and focus formation assays. In the case of PKC, using either individual or a combination (data not shown) of specific PKC isoform inhibitors showed no effect on SH cells. Furthermore, the level of phospho-PKC and phospho-CAMKII appeared unchanged after WNT5A was over or underexpressed in enamel epithelium cells. These data indicate that the WNT5A/calcium pathway is not a major player in WNT5A activation in enamel epithelium cells.

We also demonstrated that the expression of WNT5A coincided with expression of phospho-JNK in 92.3% of our samples. This data supports the involvement of WNT5A/JNK activation in enamel epithelium tumorigenicity. However, 7.7% of samples were found positive for only phospho-JNK but not WNT5A suggesting the existence of the non-WNT5A activation of JNK that could play a role in ameloblastoma tumorigenicity.

Finally, we confirmed the activation of JNK by WNT5A in enamel epithelium cells. The level of phospho-JNK was dramatically increased when WNT5A was overexpressed in enamel epithelium cells and similar results were observed when cells were treated with recombinant WNT5A. Taken together, our results provide the first demonstration that WNT5A is able to mediate enamel epithelium tumorigenic behaviors in multiple ways and WNT5A/RHO/JNK is a key pathway in these processes.

CHAPTER VI

CONCLUDING REMARKS

CONCLUSIONS

Despite extensive investigations over decades, the molecular determinants involved in the initiation, progression and maintenance of odontogenic tumors remains poorly understood. It is highly likely that deviance in several critical signaling pathways, including the Wnt signals, could play important roles in odontogenic tumorigenesis and maintenance. Detection of WNT5A expression in ameloblastomas has been suggested by other studies (81), but for the first time our study reports the molecular consequences of Wnt5a signaling activation in enamel epithelial cell.

In this study, we clearly demonstrate that WNT5A expression was intense in both the epithelial component of ameloblastoma and in the cervical loops during normal human tooth formation. These data suggest the importance of WNT5A during normal and diseased enamel epithelium development and provides further support for the assumption that enamel epithelium remnants from cervical loop are the cell of origin for odontogenic tumors.

We have also shown that overexpression of WNT5A in enamel epithelium cells promoted *in vitro* tumorigenic characteristics of cell growth, including growth factor independence, loss of anchorage dependence, and loss of contact inhibition compared with epithelial cells expressing lower levels of WNT5A. Interestingly, when EV cells and cells overexpressing WNT5A were tested for *in vivo* tumor formation, both of them exhibited tumor formation. However, tumors initiated from cells overexpressing WNT5A formed tumors faster than the control cells. Collectively the results of these

experiments indicate that WNT5A functions as an oncogenic enhancer in enamel epithelium.

Thirdly, overexpression of WNT5A enhanced cell migration, one of its major putative functions, in enamel epithelium compared with LS-8 and EV cells. Increased cell motility coincided with increased actin reorganization and filopodia/lamellipodia formation, the events important for cell movement. These findings indicate that WNT5A can regulate cell migration of enamel epithelium cells.

Finally, inhibition of JNK and Rho pathways resulted in suppression of tumorigenic growth properties of enamel epithelium cells overexpressing WNT5A. In addition, JNK and ROCK inhibitors blocked the WNT5A induced increase in cell migration observed in enamel epithelium. Increases in the level of phosphorylated JNK was observed in enamel epithelium clones overexpressing WNT5A and JNK phosphorylation was activated when enamel epithelium cells were treated with recombinant WNT5A. These data indicate that WNT5A activates Rho/JNK downstream pathway and subsequently modulates enamel epithelium tumorigenicity.

Based on the results that we observed from this study, it is evident that WNT5A potentially modulates aggressive behaviors of enamel epithelial cells via the non canonical Wnt5a/Rho/JNK pathway. Furthermore our findings suggest that WNT5A could also play an important role during enamel epithelium development since it is differentially expressed in enamel knots and cervical loop areas, the signaling centers that control adjacent cell development during tooth formation. Aberrance of such signal could result in uncontrolled cell growth which we demonstrate in WNT5A induced enamel epithelium tumorigenicity.

Although our study provides the possible explanation of how WNT5A modulates enamel epithelium aggressive behaviors, several aspects still need further investigation. For example, it is unclear whether WNT5A has transforming ability in enamel epithelium. A more appropriate cell culture and/or animal model is needed to answer this question.

Obviously, the invasive properties of a tumor, such as angiogenesis and ECM degradation, are potentially influenced by WNT5A and they have not been addressed in the present study. Interestingly we have shown that cells that highly express WNT5A are more robust and are able to survive serum deficiency, a feature that could certainly enhance continued tumor growth as seen in ameloblastomas. Development of an ameloblastoma cell line will help provide more insight as to the roles of WNT5A in odontogenic tumor progression.

Although the Wnt5a signaling in enamel epithelium can be partly explained by our findings, much additional work is clearly needed to elucidate the cascade of WNT5A mediating tumorigenic behavior of these cells. Identifying the downstream targets of these pathways is of particular interest as they could provide novel therapeutic opportunities. Targeted inhibition of WNT5A signaling, especially the Rho/JNK pathway, could possibly serve an effective adjunctive therapeutic approach to augment or replace radical resection of the jaws that is currently the standard treatment of ameloblastomas. These surgeries are frequently associated with marked facial deformity making the development of non-surgical approaches highly desirable. The high recurrence rate of ameloblastomas, even after radical surgery, also make novel non-surgical approaches important.

Importantly, the putative function of WNT5A during normal tooth development needs further investigation. Tooth explant culture using WNT5A bead implantation or *Wnt5a* cre/lox mouse models will provide powerful tools to elucidate the role of WNT5A in tooth development and might provide insights for enhancing dental stem cell regeneration as occurs in the dental cervical loop.

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