Hedgehog signaling, epithelial-to-mesenchymal transition and miRNA (Review)

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Abstract. SHH, IHH, and DHH are lipid-modified secreted proteins binding to Patched receptors, and CDON, BOC or GAS1 co-receptors. In the absence of Hedgehog signaling, GLI1 is transcriptionally repressed, GLI2 is phosphorylated by GSK3 and CK1 for the FBXW11 (ßTRCP2)-mediated degradation, and GLI3 is processed to a cleaved repressor. In the presence of Hedgehog signaling, Smoothened is relieved from Patched-mediated suppression due to the Hedgehog-dependent internalization of Patched, which leads to MAP3K10 (MST) activation and SUFU inactivation for the stabilization and nuclear accumulation of GLI family members. GLI activators then upregulate *CCND1*, *CCND2* for cell cycle acceleration, *FOXA2*, *FOXC2*, *FOXE1*, *FOXF1*, *FOXL1*, *FOXP3*, *POU3F1*, *RUNX2*, *SOX13*, *TBX2* for cell fate determination, *JAG2*, *INHBC*, and *INHBE* for stem cell signaling regulation. Hedgehog signals also upregulate *SFRP1* in mesenchymal cells for WNT signaling regulation. Epithelial-to-mesenchymal transition (EMT) during embryogenesis, adult tissue homeostasis and carcinogenesis is characterized by class switch from E-cadherin to N-cadherin. SNAI1 (Snail), SNAI2 (Slug), SNAI3, ZEB1, ZEB2 (SIP1), KLF8, TWIST1, and TWIST2 are EMT regulators repressing *CDH1* gene encoding E-cadherin. Hedgehog signals induce JAG2 upregulation for Notch-CSL-mediated *SNAI1* upregulation, and also induce TGFß1 secretion for *ZEB1* and *ZEB2* upregulation via TGFß receptor and NF-κB. TGFß-mediated downregulation of miR-141, miR-200a, miR-200b, miR-200c, miR-205, and miR-429 results in upregulation of ZEB1 and ZEB2 proteins. Hedgehog signaling activation indirectly leads to EMT through FGF, Notch, TGFß signaling cascades, and miRNA regulatory networks. miRNAs targeted to stem cell signaling components or EMT regulators are potent drug targets; however, off-target effects should be strictly controlled before clinical application of synthetic miRNA. Peptide mimetic and RNA aptamer could also be utilized as Hedgehog signaling inhibitors or EMT suppressors.

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Contents

1. Introduction

Hedgehog family members are key regulators of embryogenesis, adult tissue homeostasis, and carcinogenesis (1-5). Genetic alterations and aberrant expression of Hedgehog signaling molecules in medulloblastoma, glioma, basal cell carcinoma, lung cancer, esophageal cancer, gastric cancer, pancreatic cancer, prostate cancer, and ovarian cancer have been reported, and reviewed elsewhere.

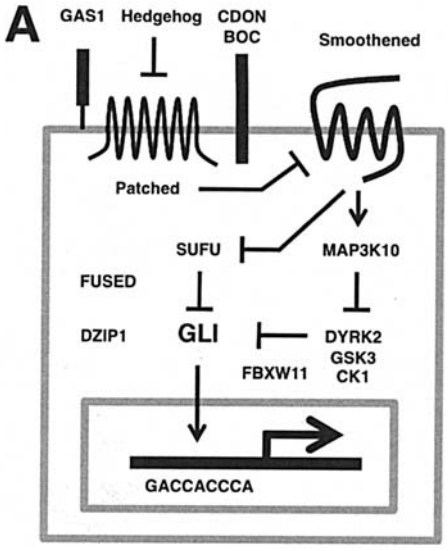
Hedgehog signaling cascade cross talks with WNT, Notch, EGF/FGF, and TGFß/Activin/Nodal/BMP signaling cascades to constitute the stem cell signaling network (6-9). Deregulation of the stem cell signaling network due to the accumulation of germline mutation, SNP, chronic inflammation, epigenetic change, and genetic alteration leads to carcinogenesis (10).

Epithelial cells undergo fibroblastoid morphological changes associated with increased motility or invasiveness due to decreased cell-cell adhesion (11-14). Fibroblastoid morphological changes of epithelial cells are known as epithelial-to-mesenchymal transition (EMT).

Regulatory mechanisms of EMT are hot issues in life science, especially in the fields of developmental biology and oncology. Herein recent advances in the Hedgehog research will be reviewed with the emphasis on EMT and microRNA (miRNA).

2. Hedgehog signaling pathway

Sonic Hedgehog (SHH), Indian Hedgehog (IHH), and Desert Hedgehog (DHH) are mammalian Hedgehog family ligands,



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Function of target genes	Gene
HH signaling cascade	*GLI1*
	PTCH1
Cell cycle regulation	*CCND1*
	CCND2
Cell - fate determination	*FOXA2*
	FOXC2
	FOXE1
	FOXF1
	FOXL1
	FOXP3
	POU3F1
	RUNX2
	SOX13
	TBX2
Stem cell signaling network	*JAG2*
	INHBC
	INHBE
	SFRP1 *

Figure 1. Hedgehog signaling pathway. (A) Schematic representation of Hedgehog signaling cascade. Patched family members are Hedgehog receptors, while CDON, BOC and GAS1 are Hedgehog co-receptors. In the absence of Hedgehog signaling, GLI1 is transcriptionally repressed, GLI2 is phosphorylated by GSK3 and CK1 for the FBXW11-mediated degradation, and GLI3 is processed to a cleaved repressor. In the presence of Hedgehog signaling, Smoothened is relieved from Patched-mediated suppression to induce MAP3K10 activation and SUFU inactivation. GLI activators then bind to the GACCACCCA motif for the transcriptional upregulation of target genes. (B) List of representative Hedgehog target genes.

consisting of N-terminal signal peptide, Hedgehog core domain, and C-terminal processing domain (15-17). Hedgehog precursors are autoprocessed to cut off the C-terminal processing domain for cholesteroylation, and then further processed by Hedgehog acyltransferase (HHAT) to cut off the N-terminal signal peptide for palmitoylation (18,19). Mature Hedgehog proteins with lipid modifications are then transported to the cell surface for packaging into lipoprotein particles depending on Dipatched 1 (DISP1), or for multimerization via lipophilic tails (19,20). Mature Hedgehog proteins secreted from producing cells induce concentration-dependent effects on target cells expressing Hedgehog receptors.

Patched family members, PTCH1 and PTCH2, are Hedgehog receptors distantly related to Dispatch family members with multi-transmembrane domains and a sterol-sensing domain (21,22). PTCH1 and PTCH2 do not directly transduce Hedgehog signals to the intracellular signaling cascade, but indirectly through the Smoothened seven-transmembrane-type receptor (23). Patched family members, inhibiting Smoothened function, are rapidly internalized upon Hedgehog-binding (24). Due to the release of Smoothened from Patched-dependent suppression, Hedgehog-binding to Patched family receptors indirectly activates the Smoothened-GLI signaling cascade (Fig. 1A).

CDON and BOC are transmembrane proteins with extracellular immunoglobulin-like (Ig-like) and fibronectin type III (FNIII) domains, which enhance Hedgehog signaling activity as co-receptors (25). GAS1 is a GPI-anchored cell surface protein binding to Hedgehog ligands for the potentiation of Hedgehog signaling (26). On the other hand, HHIP1/HHIP is a Hedgehog-bindng protein to compete Patched receptors for Hedgehog-binding (27,28).

GLI1 gene was initially cloned as an oncogene amplified in malignant glioma, and then characterized as a transcription factor functioning as a Hedgehog signaling effector (29,30). GLI1, GLI2, and GLI3 are human homologs of *Drosophila* Cubitus interruptus. In the absence of Hedgehog signaling, GLI1 is transcriptionally repressed, GLI2 is phosphorylated by GSK3 and CK1 for the FBXW11 (ßTRCP2)-mediated degradation, and GLI3 is processed to a cleaved repressor (30,31). In the presence of Hedgehog signaling, Smoothened induces MAP3K10 (MST) activation and SUFU inactivation for the stabilization and nuclear accumulation of GLI family members, respectively (32,33). GLI1 functions as transcriptional activator of Hedgehog target genes, while GLI2 and GLI3 as transcriptional activator or repressor in a context-dependent manner (30).

Hedgehog signaling activation leads to transcriptional activation of target genes through GLI-binding to the GACC ACCCA motif (34-36). *GLI1*, *PTCH1* and *HHIP1* are upregulated by Hedgehog signaling, but *CDON*, *BOC* and *GAS1* are downregulated. Hedgehog-dependent *GLI1* upregulation constitutes a positive feedback loop, while Hedgehog-dependent regulation of *PTCH1*, *HHIP1*, *CDON*, *BOC*, and *GAS1* constitutes a negative feedback network. Hedgehog signals induce transient upregulation of target genes through the combination of positive and negative feedback mechanisms.

Hedgehog signals upregulate *CCND1* and *CCND2* for cell cycle acceleration, *FOXA2*, *FOXC2*, *FOXE1*, *FOXF1*, *FOXL1*, *FOXP3*, *POU3F1*, *RUNX2*, *SOX13*, and *TBX2* for cell fate determination. Hedgehog signals also upregulate *JAG2*, and *INHBC/E* to regulate Notch, and Activin signaling cascades, respectively. In addition, Hedgehog signals upregulate *SFRP1* at least in mesenchymal cells without its promoter CpG hypermethylation to inhibit canonical WNT signaling cascade in epithelial cells (Fig. 1B).

3. Epithelial-to-mesenchymal transition (EMT)

Epithelial cells are tightly held together with uniform neighboring cells to move as a sheet en block, while mesen-

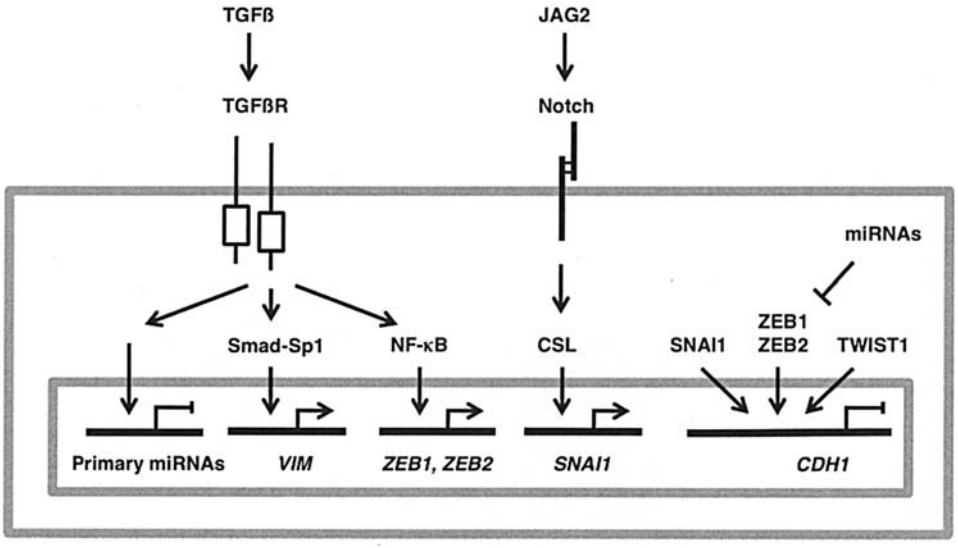

Figure 2. Hedgehog and EMT. Hedgehog signals induce JAG2 upregulation for Notch-CSL-mediated *SNAI1* upregulation, and also induce TGFß1 secretion for *ZEB1* and *ZEB2* upregulation via TGFß receptor and NF-κB. TGFß-mediated downregulation of miR-141, miR-200a, miR-200b, miR-200c, miR-205, and miR-429 results in upregulation of ZEB1 and ZEB2 proteins. Hedgehog signaling activation indirectly leads to EMT through Notch, TGFß signaling cascades, and miRNA regulatory networks.

chymal cells are loosely connected with diverse neighboring cells to move individually. EMT, allowing cells to dissociate from epithelial tissue, is necessary for gastrulation movements and neural crest formation during embryogenesis, and also for invasion and metastasis during carcinogenesis (11-14).

E-cadherin, occludin and cytokeratin are downregulated during EMT, while N-cadherin, vimentin, fibronectin, SNAI1/SAIL, SNAI2/SLUG, ZEB2/SIP1, and TWIST1 are upregulated (14). E-cadherin and N-cadherin are representative adhesion molecules expressed on epithelial cells and mesenchymal cells, respectively. E-cadherin at the adherens junction is implicated in the stable cell-cell contact of epithelial cells, while N-cadherin in the weak intercellular contact of mesenchymal cells (37,38). Class switch from E-cadherin to N-cadherin results in the loss of epithelial phenotype and the acquisition of mesenchymal phenotype. Transcriptional repression of *CDH1* gene or functional repression of E-cadherin protein is the critical step for EMT.

Zinc-finger domain proteins SNAI1, SNAI2, SNAI3, ZEB1, ZEB2, KLF8 as well as basic helix-loop-helix (bHLH) domain proteins TWIST1 and TWIST2, bind to the proximal promoter region of the *CDH1* gene for the EMT induction through E-cadherin repression (11-14,39-42). We previously reported *SNAI1* expression in neuroblastoma, diffuse type gastric cancer, and *SNAI2* expression in embryonic stem cells, leiomyosarcoma, neuroblastoma, and glioblastoma (41). Rosivatz *et al* reported preferential upregulation of *ZEB2* in intestinal type gastric cancer, and those of *SNAI1* and *TWIST1* in diffuse type gastric cancer (39). Alves *et al* reported co-expression of *ZEB2* and *SNAI2* in intestinal type gastric cancer, and that of *SNAI1* and *SNAI2* in diffuse type gastric cancer (42). Upregulation of EMT regulators is associated with more malignant phenotypes in a variety of human cancer, such as gastric cancer, pancreatic cancer, breast cancer, and ovarian cancer.

4. Hedgehog and EMT

Hedgehog signaling cascade cross-talks with WNT, EGF/FGF, and TGFß/Activin/Nodal/BMP signaling cascades, which are implicated in EMT through E-cadherin repression (6-14). In this section, direct and indirect mechanisms of EMT regulation by the Hedgehog signaling cascade will be reviewed (Fig. 2).

Upregulation of *SNAI1* and *PTCH1* mRNAs is induced 3 h after GLI1 expression in RK3E cells by using the 'tet-on' system, and that of Snai1 protein 12 h after GLI1 expression (43). Although these facts indicate that the Hedgehog signaling cascade induces the SNAI1 upregulation, there is no evidence for the direct transcriptional activation of *SNAI1* by the Hedgehog signaling cascade.

On the other hand, Hedgehog signals induce *JAG2* upregulation (Fig. 1B), and TGFß1 secretion to promote motility and invasiveness of cancer cells (44). JAG2 signal induces processing of Notch receptor to Notch intracellular domain (NICD). NICD is then associated with CSL transcription factor in the nucleus to induce *SNAI1* upregulation (45). TGFß1 signal activates TGFß receptor for the NF-κB-mediated transcriptional upregulation of *ZEB1* and *ZEB2* (46), and also for the SMAD-Sp1-mediated transcriptional upregulation of mesenchymal markers, such as Vimentin (VIM). Together these facts indicate that the Hedgehog signals indirectly induce EMT through the upregulation of multiple EMT regulators via Notch and TGFß signaling cascades (Fig. 2).

5. MicroRNA (miRNA)

Primary miRNAs are processed by Drosha/DGCR8 complex to give rise to precursor miRNAs, which are then processed by Dicer to produce mature miRNAs. Most target mRNAs with partial complementarity to miRNA are repressed

through translational downregulation and deadenylation, while several target mRNAs are activated (47-50). Mechanisms of miRNA-induced translational or transcriptional regulation as well as clinical application of miRNA are the frontier of medical science in the post-genome era.

Zebrafish miR-214 binds to the 3'-UTR of Sufu to downregulate Sufu (51). Because Sufu is implicated in the nuclear trafficking of Gli activator and repressor, miR-214-induced downregulation of Sufu results in maximal activation of Gli in the presence of Hedgehog and complete repression of in the absence of Hedgehog. It is noteworthy that human miR-214, up-regulated in ovarian cancer, binds to the 3'-UTR of PTEN to downregulate PTEN for the activation of PI3K-AKT signaling cascade (52), which is also able to induce EMT. Effects of human ortholog of zebrafish miR-214 on the Hedhehog-GLI signaling cascade as well as EMT remain to be elucidated.

TGFß downregulates the expression of human miR-141, miR-200a, miR-200b, miR-200c, miR-205, and miR-429, which are targeted to *ZEB1* and *ZEB2* mRNAs (53). TGFß-induced downregulation of miRNAs mentioned above synergizes with NF-κB sinaling to induce upregulation of ZEB1 and ZEB2 (Fig. 2).

SNAI, ZEB and TWIST family members repress the *CDH1* gene to induce EMT, but also regulate the transcription of other target genes. TWIST1 is upregulated in human breast cancer, gastric cancer, esophageal cancer, and prostate cancer. TWIST1 directly activates the transcription of miR-10b primary miRNA, located within the *HOXD* gene cluster (54). *HOXD10* mRNA is the target of miR-10b in human breast cancer, and HOXD10 is implicated in the suppression of RhoC-mediated cell motility. TWIST1 promotes invasion and metastasis through miR-10b-induced *HOXD10* repression.

6. Perspectives

KAAD-cyclopamine, SANT1-4, and Cur61414 are small-molecule Hedgehog signaling inhibitors targeting Smoothened (55). Other therapeutic devices targeted to the Hedgehog signaling cascade and EMT regulators will be described in the last section.

miRNAs targeted to mRNAs, encoding stem cell signaling components or EMT regulators, are potent drug targets. miRNAs inducing proliferative, anti-apoptotic, pro-angiogenic, or pro-metastatic effects on tumor cells could be down-regulated for cancer therapy, while those inducing pro-apoptotic, anti-angiogenic, or anti-metastatic effects could be applied for synthetic miRNA (48,49). Because the off-target effects are a serious problem associated with miRNA and siRNA technologies, great care should be taken before clinical application of these technologies.

RNA aptamer is a short RNA oligonucleotide with a stable three-dimensional structure (49). RNA aptamers binding to extracellular region of PTCH1 could be utilized for drug delivery to cancer cells with Hedgehog signaling activation. RNA apatamers binding to cytoplasmic region of SMO, and those binding to Fused or GLI1 could be utilized as Hedgehog signaling inhibitors.

Peptide mimetics, resembling WNT and FGF family members, have been developed (56,57). Because WNT5A transduces signals through ROR1 or ROR2 to activate the non-canonical signaling cascade for the induction of EMT partly through SNAI1 upregulation (58-61), WNT5A mimetic is able to suppress invasion and metastasis of cancer cells. Peptide mimetic, resembling core region of mature Hedgehog signaling domain, could be developed as a novel Hedgehog antagonist.

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