Review

Molecular Cancer Therapeutics

Hey Factors at the Crossroad of Tumorigenesis and Clinical Therapeutic Modulation of Hey for Anticancer Treatment

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Abstract

Hairy and Enhancer-of-split related with YRPW motif (Hey) transcription factors are important regulators of stem cell embryogenesis. Clinical relevance shows that they are also highly expressed in malignant carcinoma. Recent studies have highlighted functions for the Hey factors in tumor metastasis, the maintenance of cancer cell self-renewal, as well as proliferation and the promotion of tumor angiogenesis. Pathways which regulate Hey gene expression, such as Notch and TGFβ signaling, are frequently aberrant in numerous cancers. In addition, Hey factors control downstream targets via recruitment of histone deacetylases (HDAC). Targeting these signaling pathways or HDACs may reverse tumor progression and provide clinical benefit for cancer patients. Thus, some small molecular inhibitors or monoclonal antibodies of each of these signaling pathways have been studied in clinical trials. This review focuses on the involvement of Hey proteins in malignant carcinoma progression and provides valuable therapeutic information for anticancer treatment. *Mol Cancer Ther; 1–14. ©2017 AACR.*

Introduction

Hairy and Enhancer-of-split related with YRPW motif factors (Hey1/2/L) belong to the basic helix-loop-helix Orange (bHLH-O) family which is also known as Hairy and Enhancer-of-split related protein (Hesr), Hairy-related transcriptional factor (HRT), Hes-related repressor protein (HERP), and cardiovascular helix-loop-helix factors (CHF; refs. 1–5). All three Hey genes have been found in developmental tissue, and abnormal expression of these proteins promotes abnormalities in stem cells, even leading to organ defects. Hey proteins can maintain an undifferentiated state of precursor cells by transcriptionally repressing cell fate regulators such as achaete-scute homolog 1 (6). In the developing heart, Hey proteins regulate cardiomyocyte precursor cell differentiation as well as epithelial–mesenchymal transition (EMT) of endocardium cells (7, 8). Since we recognized that cancer cells can monitor and utilize similar physiologic strategies to normal cells and promote tumor progression, for instance, cancer cells can initiate cellular plasticity and/or activate similar signaling pathways as mesenchymal cells, stem cells, or precursor cells do, we have started to realize the significant role played by Hey factors in tumor progression (9, 10). Hey proteins are found to be selectively expressed in malignant tumor tissues, and numerous studies have been undertaken to explain the molecular mechanism governing the Hey proteins in tumorigenesis. The most outstanding feature is that many signaling pathways can potentially confer EMT via Hey factors in malignant carcinomas. In addition, Hey factors not only regulate differentiation, self-renewal, and proliferation of cancer cells, but contribute to tumor neovasculature as well. Accumulating evidence indicates Hey factors lay at the crossroad of tumor progression. However, there are currently very few review articles illustrating the roles of the Hey family in tumorigenesis. The current review explores the functional significance of the Hey family in initiating these processes. We also describe the signaling pathways involved in the control of Hey expression. Small molecular inhibitors or monoclonal antibodies to each of these signaling pathways show promising antitumor or antiangiogenic effect in clinical trials. Here, these promising avenues for cancer treatment are also discussed.

Structure of the Hey family proteins

Hey family members are highly conserved and resemble their homologs, the Hairy and Enhancer of Split (Hes) family, in the four domain structures: basic, helix-loop-helix (HLH), Orange, and two C-terminal motifs. Hey proteins are directly connected to the E-box DNA sequence (CANNTG) via the glycine-rich basic domain (11, 12). The bHLH-O domain serves as a platform for cofactor interaction (3, 13). Despite extensive homology with the Hes family, Hey proteins also have significant features that distinguish them from Hes proteins, namely, the YRPW motif (YHSW for HeyL) and GTEIGAF (GTEVGAF for Hey2) peptides (ref. 1; Fig. 1). Hey proteins have been regarded as transcription inhibitors in the past. They have since been shown to act as transcription activators as well as inhibitors (Table 1). Strikingly,

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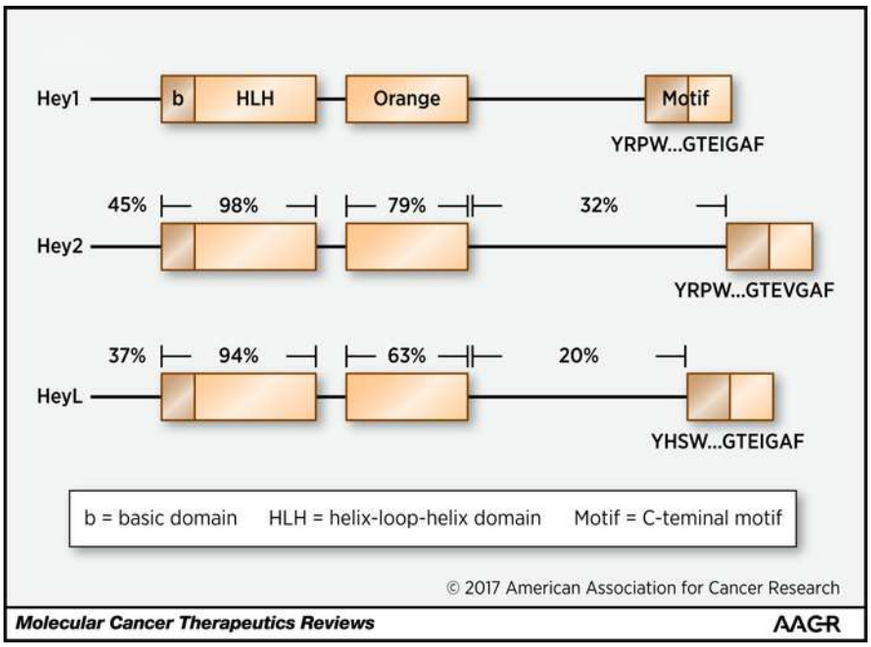

Figure 1
Domain structures with percentage identity of Hey2 and HeyL with Hey1. The bHLH domains show the highest conservation among other domains. The Orange domain shows less conservation. Individual Hey proteins potentially recruit selective cofactors via Orange domain and C-terminal motif.

their function seems to be regulated at multiple levels. For instance, nonsynonymous single-nucleotide polymorphism (SNP) naturally occurs at codon 94 of Hey1, which leads to a substitution of a leucine residue by methionine (L94M) in the helix 2 domain. The L94M-mutant Hey1 transforms from an androgen receptor corepressor to androgen receptor coactivator without changing its intrinsic repressive domains (14). The phosphorylation of the Serine-68 residue of Hey1 prevents its enhancement of p53 transcriptional activation but confers p53-activating chemotherapy resistance, whereas wild-type Hey1 stimulates p53 and alters the sensitivity to p53-activating chemotherapy drugs. Interestingly, such posttranscriptional regulation is also observed within Hey2 (15). The dynamic regulation of Hey proteins at pretranscriptional levels, posttranscriptional levels, or their own characteristic structure could partly explain why Hey proteins eliminate one target molecule in certain cancers but activate the same molecule or its analogues in others. It should be noted that the specificity for protein interactions and target molecules of different Hey variants is differential between certain cell types. L94M Hey1 variant strongly interacts with Hey2, whereas Hey1 forms an unstable homodimer with Hey2 (14). There is a potential, unknown Hey1 variant enhancing matrix metallopeptidase 9 (MMP9) expression in osteosarcoma, whereas wild-type Hey1 is unable to bind to the MMP9 promoter itself (16).

Table 1. Summary of target genes, cytokine, and transcriptional factors of Hey

Targets	Hey proteins	Comment	References
P53	Hey1, Hey2, HeyL	Activation	14, 15
MMP9	Hey1	Activation	16
Snai1	Hey1, Hey2, HeyL	Activation	26, 30
IL6	Hey1	Activation	37
Twist1	Hey1, Hey2	Repression	34
Snai2	Hey1, Hey2	Repression	34
Runx2	Hey1	Repression	40, 41
Col2a1	Hey1	Repression	42
VEGFR2	Hey1, Hey2	Repression	51, 58, 60, 61

Hey proteins in malignant carcinomas

The levels of Hey factors are strikingly elevated in high-grade glioma, malignant osteosarcoma, high-grade esophageal squamous cell carcinoma, aggressive pancreatic adenocarcinoma rhabdomyosarcoma, as well as colorectal carcinoma (17–23). In these malignant carcinomas, aberrant Hey expression has been associated with poor prognosis, overall survival (OS), tumor grade, chemotherapy resistance, lymphatic metastasis, and vascular proliferative properties (17, 23–25). Taken together, these studies suggest that elevated levels of Hey proteins contribute to tumor progression, and to a certain extent, this is a result of their regulation of the behavior of cancer cells as well as remodeling of the tumor microenvironment (Fig. 2).

The roles of Hey proteins in cancer metastasis

It was first observed that Hey-induced EMT was required in the developing heart (26–29). Subsequently, Hey proteins were found to be involved in tumor metastasis progression. *In vitro*, Hey1 knockdown inhibited the invasive phenotype of osteosarcoma via downregulation of MMP9 (16). Furthermore, the transfection of Hey1 antisense oligonucleotides blocked EMT through E-cadherin expression, and Smad3 inhibition repressed the Hey1-induced EMT phenotype even with the presence of TGFβ (30). Strikingly, interaction between Hey1 and Smad3 has been observed *in vitro* (31), suggesting a Hey1–Smad3 complex transcriptionally represses E-cadherin. However, in the absence of activated Smad3, Hey1 does not influence EMT promotion, but only acts as a Snai1-initiated EMT marker (30, 32). On the other hand, Snai1, known as an E-cadherin repressor, potentially contributes to this repression process. Snai1 is reduced in Hey1/HeyL double knockouts and Hey2 knockout AV canals, and Snai1 can form a complex with Smad3 to occupy the E-cadherin promoter (26, 33). All these observations hint that Hey1 interacts with Smad3 and may inhibit E-cadherin directly or in a Snai1-Smad3-Hey1 manner. In other situations, Hey proteins promote mesenchymal–epithelial

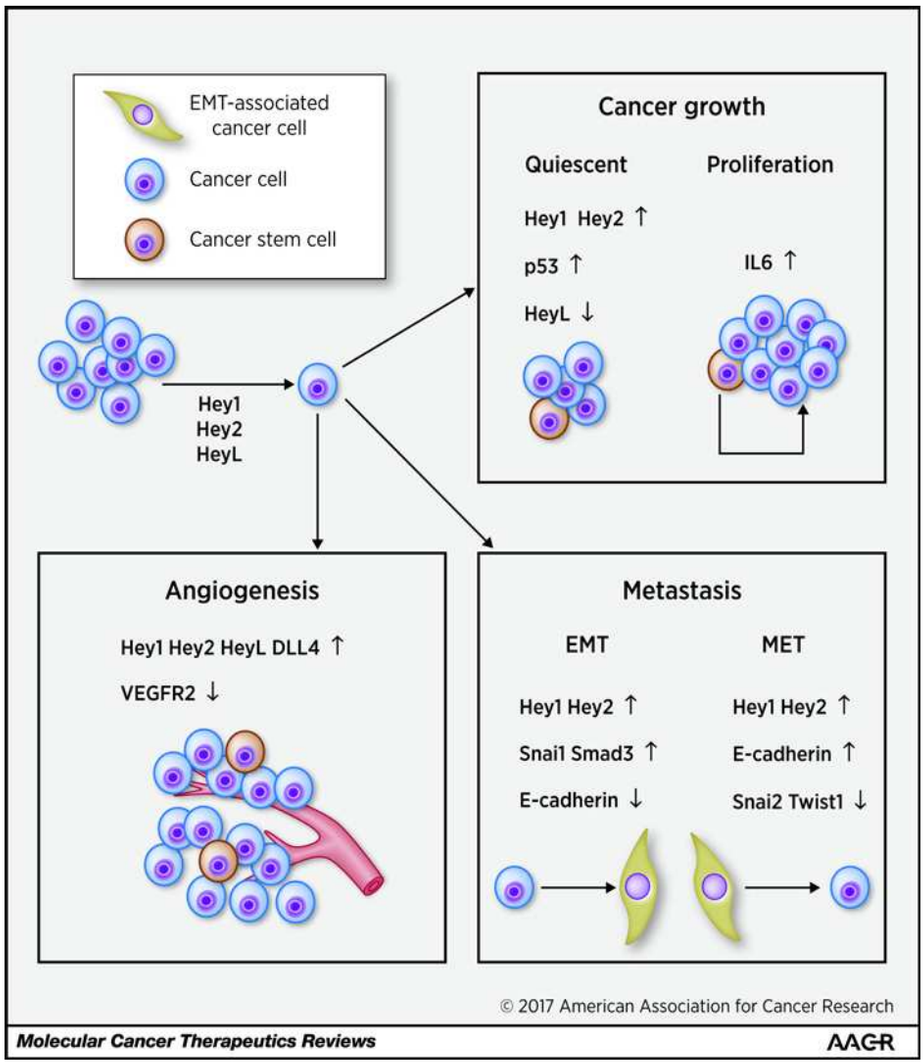

Figure 2
Hey proteins in tumorigenesis. Via activating or inactivating cytokines and other transcriptional factors, Hey proteins show their regulation on tumor progression including cancer metastasis, cancer cells' quiescence maintenance as well as cancer neovasculature.

transition (MET). Upon Notch4 induction, Hey proteins promote melanoma MET and are important in promoting metastatic colonization because Hey1 and Hey2 can eliminate Snai2 as well as Twist1 expression via binding to their promoters (34). The different stimuli have a potential influence on Hey function, as TGFβ-induced Hey1 promotes EMT and Notch-induced Hey proteins regulate MET or transition irrelevant due to lack of Smad3. However, it is more complex than first thought. Forced expression of Hey proteins has no impact on Snai2 or E-cadherin expression in other cell lines (35, 36). Does the paradox happen in different cell types? Evidence from the previous section indicates the nonsynonymous SNP of Hey genes in different cell types will affect different Hey variants' DNA-binding ability as well as protein-interaction specificity. Based on this, we presume that Hey variants affect Snai1/Snai2 expression transcriptionally to mediate EMT/MET in different cell lines. More intensive research is required to fully characterize Hey variants and the posttranscriptional modification of Hey. Also, Hey1 participates in metastatic microenvironment remodeling. Tumor-derived Jagged1 enhances osteoblast secretion of IL6 via Hey1 activation, and, in turn, IL6 confers a proliferative advantage to cancer cells (37). Epithelium-derived Jagged1 activates Hey1 which then promotes metastatic lymphoma cell chemotherapy resistance as well as progression in the tumor perivascular niche (38).

Hey proteins can regulate the differentiation, self-renewal, and proliferation of cancer cells

Hey proteins were identified as one of a few genes specifically expressed during embryogenesis (1, 39). Following this discovery, the potential capacity of the Hey family in sustaining cell quiescence was recognized (6, 40–42). Cancers monitor the quiescence strategy to keep their nondivide state and contribute to tumor progression (10, 43). The upregulation of Hey1 is likely to inhibit differentiation because rhabdomyosarcoma cells with an shRNA antagonizing Hey1 display differentiation morphology changes and the expression of differentiation marker myogenin (22). The introduction of Hey1 into proliferating osteosarcoma increases p53 expression and makes tumor cells stay in a nondividing state through p53-dependent reversible cell-cycle arrest (14). In the context of quiescence, elevated Hey family expression can reflect the undifferentiating property of malignant cancer cells. In

addition, the ability of Hey proteins in maintaining self-renewal was investigated. The expression of Hey1 and Hey2 is remarkably higher in cancer stem cells (CSC), also referred as tumor-initiating cells (TIC), than that in non-CSCs (44). Hey1 is supposed to maintain CSCs self-renewal capacity as the silencing of Hey1 dampens malignant tumor-initiating ability as well as tumor growth *in vivo* and reduces cancer cell sphere formation *in vitro* (45, 46). In hepatocellular carcinoma, Hey1 upregulation upon c-Met/FRA1 signaling increases the number and the size of TICs spheroids which represent the self-renewal ability of these cells (47). Furthermore, Hey proteins have an effect on cancer proliferation. Hey2 overexpression increases hepatocellular carcinoma cell viability and proliferation (48). HeyL can promote breast cancer initiation through interaction with TGFβ-activated Smad3 (31). Interestingly, HeyL promotes p53-induced cell-cycle arrest which results in suppression of cancer cell proliferation and induction of cancer cell apoptosis in hepatocellular carcinoma (49). The same study also reported that 75% of hepatocellular carcinoma tissues had inaction of HeyL, suggesting that HeyL is a potential tumor suppressor in hepatocellular carcinoma. This is an interesting observation, despite that it was a single study and demonstrated in a small cohort (n = 80), this will require further validation on a larger scale. However, the fact that HeyL differs in one of the key motifs, namely the YHSW motif, from Hey1 and Hey2 which both have the YRPW motif, may be one of the reasons why it acts differently from other Hey proteins. While YRPW appears to be a good target (16), YHSW, at least in hepatocellular carcinoma, may not be the case. This is clearly a fascinating area to explore, both in research and in clinical settings.

Balance between HeyL and Hey1/Hey2 regulates cancer neovasculature

Genetic studies have highlighted the great influence of Hey proteins in angiogenesis during development or pathologic conditions (27, 50–52). Angiogenesis actively requires a strict hierarchy between sprouting and vascular tubes (53). Previous research suggests a factor acting upstream of Hey, Delta-like 4 (DLL4), is capable of controlling this hierarchy, as the inhibition of DLL4 leads to a hyper-sprouting phenotype following exposure to proangiogenic factors (54–56). Much evidence, however, supports that DLL4's control on vascular sprouting is via its downstream factors, Hey1/2. It is acknowledged that epithelium with higher VEGFR2 emerges at the tip position and sustained VEGFR2 pathway activation results in excessive sprouting (57–59). Strikingly, Hey1, as well as Hey2, can suppress VEGFR2 expression and eliminate the increased frequency of epithelial cells at the tip position (58, 60, 61). When activated by the bone morphogenetic protein (BMP)/Activin receptor-like kinase (ALK) pathway, Hey1 as well as Hey2 abrogate the hyper-sprouting phenotype and induces tube formation (58, 62). In tumors, the coordinated balance between VEGFR2 and DLL4/Hey is tightly required for tumor cell expansion (63). DLL4/Hey2 overexpression leads to tumor growth by promoting low-density and mature tumor vessels through downregulation of VEGFR2 levels (64). DLL4/Hey blockage leads to VEGFR2 upregulation, which restrains tumor progression by producing hyper-sprouting, thin, fragile, and nonfunctional tumor vessels (56, 65–67). Interestingly, Jagged1-associated Hey upregulation seems to have little effect on low-density and mature tumor vessel phenotype, and Jagged1 promotes tumor-spouting angiogenesis through distinct mechanisms (54, 68, 69). In contrast, HeyL potentially promotes neovascularization. Studies reveal that breast tumor–derived vascular samples exhibit at least 20-fold higher levels of HeyL than normal breast vasculature. The elevated level of HeyL potentially promotes neovasculature by forcing vascular endothelial cells to undergo proliferation and ceasing apoptosis (25). Taken together, this evidence highlights the complexity of Hey in angiogenesis, and drugs targeting DLL4, Jagged1, and ALK1 are promising.

Notch-Hey signaling pathway

The mature heterodimeric Notch receptors are cleaved at two sites once the five ligands (Delta-like 1, 3 and 4, and Jagged 1 and 2) bind to the four membrane-bound Notch receptors (Notch 1, 2, 3, and 4), firstly by a disintegrin and metalloproteinase domain-containing protein 10/17 (ADAM10/17) and secondly by γ-secretase to release the Notch intracellular domain (NICD). In the nucleus, NICD interacts with the CBF1/Suppressor of Hairless/Lag1 (CSL) and recruits coactivators, allowing for transcriptional activation of Hey genes (4, 70, 71). Intriguingly, Notch receptors or Notch ligands show little selectivity for the induction of individual Hey proteins. Aberrant Notch-Hey axis shows great relevance to cancer biology. The Notch-Hey1 signaling pathway is over activated in invasive breast cell lines. Upon Notch inhibition via γ-secretase inhibitors (GSI), their migration and invasion capacity is reduced and this is accompanied by downregulation of Hey proteins (32, 72). The disruption of Notch-Hey1 in stroma bone cells decreases Jagged1-mediated breast tumor growth and bone metastasis (37). In osteosarcoma as well as rhabdomyosarcoma, Notch-Hey inhibition reverses tumor cell proliferative and relieves tumor burden (20, 22). GSI treatment also contributes to depletion of breast CSCs (44). Furthermore, a nonfunctional vascular network which results in tumor growth inhibition emerges when the DLL4-Notch-Hey1/2 pathway is blocked by DLL4 antibodies (56, 67). Thus, because GSIs, anti-Notch receptors, as well as anti-DLL4 are effective in Notch-Hey pathway inhibition, they have been developed into promising preclinical drugs (as summarized in Table 2).

γ-secretase inhibitors

Various preclinical trials show that GSIs have strong antitumor effects (73, 74). When treated with MK-0752 in phase I studies, clinical benefits such as complete response (CR) and prolonged stable disease (SD) were observed (75–78). However, patients present no objective responses to monotherapy of RO-4929097 in phase II clinical trials of solid tumors (79–82). Clinical indication of GSIs is still controversial, as a portion of cancer patients experienced SD during RO-4929097 or MK-0752 therapy, 1 advanced thyroid cancer patient achieved CR, and 71.4% (5/7) desmoid tumor patients had a partial response (PR) when they received another GSI, PF-0308414 (83). The most prominent and dose-limiting toxicity of GSIs is gastrointestinal (GI) events including diarrhea, vomiting, and nausea. This GI toxicity is likely based on the mechanism that inhibition of Notch signaling abrogates the undifferentiated state of intestinal crypt progenitor cells and results in differentiation into goblet cells (84). To reverse GI events, some investigators use glucocorticoid or tamoxifen therapy which potentially protects the intestine from goblet cell metaplasia (85, 86). Besides, the adverse events are scheduled dependent. Once-per-week dosing schedule of MK-0752 shows less severe GI events as well as fatigue than intermittent dosing for

Table 2. Selected therapeutic inhibitors of Notch signaling, TGFβ signaling, and HDACs

Mechanism of action	Agent	Biology targeted	Clinical benefits	Disease	Stage	NCT number
Notch						
Pan-Notch inhibitor	RO-4929097	Antitumor	SD, PD	Metastatic colorectal cancer	Phase II	NCT01116687
			SD, PD	Recurrent ovarian cancer	Phase II	NCT01175343
			SD	Pretreated pancreatic adenocarcinoma	Phase II	NCT01232829
			PR, SD	Metastatic melanoma	Phase II	NCT01120275
	MK-0752	Antitumor	CR, SD	Advanced solid tumors	Phase I	NCT00106145
			SD	Children CNS malignancies	Phase I	NCT00572182
	LY900009	Antitumor	SD	Advanced cancers	Phase I	NCT01158404
	PF-0308414	Antitumor	CR, PR, SD	Advanced solid tumors	Phase I	NCT00878189
Notch1-specific antibody	OMP-52M51	Antitumor	PR, SD	Solid tumors	Phase I	NCT01778439
Notch2/3-specific antibody	OMP-59R5	Antitumor	SD, PD	Untreated metastatic pancreatic cancer	Phase I	NCT01647828
			PR, SD	Untreated small-cell lung cancer	Phase I	NCT01859741
DLL4-specific antibody	REGN421	Angiogenesis targeting	PR, SD	Advanced solid tumors	Phase I	NCT00871559
	OMP-21M18	Angiogenesis targeting	PR	Pretreated solid tumors	Phase I	NCT00744562
TGFβ						
ALK1-specific antibody	ACE-041	Angiogenesis targeting	PR, SD	Advanced solid tumors	Phase I	NCT00996957
	PF-03446962	Angiogenesis targeting	PR, SD	Pretreated advanced solid tumors	Phase I	NCT00557856
			SD	Pretreated urothelial cancer	Phase II	NCT01620970
			SD	Advanced solid tumors	Phase I	NCT01337050
ALK4/5/7 antibody	LY2157299	Antitumor	CR, PR, SD	Advanced cancer and glioma	Phase I	Unavailable[a]
			SD	Advanced solid tumors	Phase I	NCT01722825
HDAC						
Pan-HDAC inhibitors	Vorinostat	Antitumor		Cutaneous T-cell lymphoma	Approved	
	Belinostat	Antitumor		Peripheral T-cell lymphoma	Approved	
	Panobinostat	Antitumor		Multiple myeloma	Approved	
	Romidepsin	Antitumor		Cutaneous T-cell lymphoma	Approved	

Abbreviation: CNS, central nervous system
[a]Reference 121.

3 to 7 days or continuous daily dosing group and once-per-week group also achieved substantial Notch signaling inhibition (75). With glucocorticoid therapy and intermittent schedule, cancer patients are more tolerant to higher GSIs exposure and may associate with better outcomes. However, it is worth considering that GSIs have an off-target effect as γ-secretase cleaves more than 90 substrates (87). Strikingly, two types of GSIs reduce Notch1 but not Notch4 activity, suggesting some GSIs are receptors specific (88). In addition, different GSIs enjoy quite inequivalent pharmacokinetics. LY900009 is cleared by oxidation and amide hydrolysis, and its renal clearance is low, while semagacestat, an analogue of LY900009, mostly depends on renal clearance (89, 90). RO4929097 is cleared by auto-induction of cytochrome P450 family 3 subfamily A polypeptide 4 (CYP3A4), indicating that combination RO4929097 therapy with antitumor agents metabolized by CYP3A4 might show limit clinical utility (91). Furthermore, intravenous GSIs are under development (ref. 92; chemical structures of the unproved GSIs are available in Supplementary Data: Supplementary Figs. S1–S4).

Anti-Notch receptor antibodies

As GSIs are pan-Notch inhibitors, several antibodies were launched to block Notch receptors more specifically by binding with their extracellular-negative regulator region or ligand-binding domain. Preclinical data show antitumor, antiangiogenesis effects and decreasing CSCs frequency following treatment with these receptor-specific antibodies (93–95). Based on the success of Notch-specific antibodies, OMP-52M51 (anti-Notch1) and OMP-59R5 (anti-Notch2/3) have been studied in clinical trials. In a phase I study in solid tumors, the best response to OMP-52M51 was 2 patients with adenoid cystic carcinomas: one achieved PR; the other had SD for 290 days and SD was also observed in other tumors (96). Untreated metastatic pancreatic cancer patients only present SD, whereas 75% (6/8) of untreated extensive-stage small-cell lung cancer patients achieve PR to OMP-59R5 monotherapy (97, 98). Anti-Notch receptor antibodies are attractive, as they still function even in Notch receptors carrying mutations, and some of these tumors carrying mutations may be highly sensitive to these antibodies (93).

Anti-DLL4 monoclonal antibodies

Considering the great importance that DLL4 exerts on tumor vessel formation, targeting DLL4 was used to target tumor angiogenesis in preclinical studies, and several DLL4-blocking monoclonal antibodies have also been used to target Notch-mediated tumor angiogenesis in clinical trials (64, 99). SD and PR were noted in 41% of patients with advanced solid tumors when treated with REGN421 (Enoticumab), a DLL4 monoclonal antibody, in a phase I trial (100). OMP-21M18 (Demcizumab), another anti-DLL4 monoclonal antibody, showed antitumor effect, and 40% of patients with solid tumors responded with a reduction in tumor size (101). Although promising and well tolerated, severe adverse events, including hemangiomas, bleeding episodes, increased levels of troponin I, and ventricular dysfunction, were observed. In addition, targeting Jagged1 seems to exhibit an alternative therapeutic strategy which requires further clinical data (102, 103).

Agents in preclinical stage

Other agents blocking Notch signaling are also under development. Soluble decoys, which are Notch receptor extracellular domains or Notch ligands fused with or without human IgG, compete with endogenous ligands and inhibit Notch signaling activation. Notch1 decoys consisting of certain

EGF-like repeats can interrupt Jagged-class–induced Notch uniquely and show antiangiogenesis as well as antitumor effects with limited adverse events *in vivo* (68). Soluble DLL1 or Jagged1 decoys can also block Notch signaling successfully (104, 105). Also, cell-permeable peptides interact with NICD-CSL and form a transcriptionally repressive complex which halts leukemia cell proliferation (106, 107).

TGFβ-Hey signaling pathway

Recent evidence has documented that TGFβ signaling induces Hey protein in a Notch-independent manner or through canonical Notch. Upon TGFβ1 activation, initiation of Hey is conducted by Smad3/Smad4 complex binding to Hey promoters at Smad-binding element core repeat (SCR) positions, and Hey gene activation is still observed when canonical Notch is abrogated by GSI (30). BMP9 protein activates Smad1/5/8 and directly stimulates Hey expression via a noncanonical Notch signaling pathway when it binds to TGFβ type I receptor-ALK1 receptor (58, 62, 108). On the other hand, activation of the Hey family can be enhanced by synergy between TGF-β/BMP and Notch signaling. Smads physically interact with Notch-dependent NICD and synergistically activate transcription of Hey1, Hey2, and HeyL, when Smads are activated by BMP-ALK5/6 or TGFβ1 treatment (30, 109, 110). As with the Notch pathway, TGFβ signaling is often elevated in tumors and contributes to tumor progression. Subsequent studies have indicated a crucial role for TGFβ in EMT initiation, and tumors break free from their neighboring tissue to undergo metastasis through TGFβ-induced EMT (111). Smad3 is of significant importance for Hey1-induced EMT as Smad3 is an integral molecule for repressing E-cadherin. In addition to metastasis, TGFβ pathway activation has been linked to tumor angiogenesis. Upon BMP9 treatment, the ALK1-Hey signaling pathway forces epithelial cells to remain in a stalk cell state, resulting in tube induction and mature vessel phenotype (58). If the ALK1-Hey signaling pathway is abrogated through addition of the ALK1 inhibitor, K02288, a hyper-sprouting phenotype is induced *in vitro* and angiogenesis is disrupted *in vivo* (112). Thus, TGFβ receptor inhibitors, which are potentially antitumor as well as antiangiogenesis drugs, have been applied in preclinical trials (as summarized in Table 2).

ALK1 blockers

Several ALK1 inhibitors have been studied in clinical trials. ACE-041 (Dalantercept), another ALK1 blocker, was tested in squamous cell carcinoma, non–small cell lung cancer, and intestinal adenocarcinoma and displayed antitumor activity in phase II clinical trial (113). No responses or PR to PF-03446962, an antibody targeting ALK1, was observed in hepatocellular carcinoma, urothelial cancer, colorectal cancer, malignant pleural mesothelioma, and other solid tumors (114–116). Three patients with metastatic hepatocellular carcinoma, metastatic clear cell renal carcinoma, and KRAS-mutant non–small cell lung cancer showed PR to PF-03446962 in another phase I trial (117). SD was observed among these four studies. Although only a very small part of patients have PR to anti-ALK1, further research is required into anti-ALK1. PR and SD were observed in portions of patients who still had lesions and cancer progression following VEGFR tyrosine kinase inhibitor (TKI) treatment. The combination of VEGFR TKI and ACE-041 results in a promising antiangiogenesis effect with marked tumor vascular disruption in xenograft models (118).

ALK5 inhibitor

LY2157299 (galunisertib), a small molecular inhibitor targeting the TGFβ receptor I, was originally developed as an ALK5 inhibitor and proved to complement ALK4/7 inhibitors (119). LY2157299 exerts an anti-invasive effect rather than antiproliferative effect on hepatocellular carcinoma cells via repression of Smad2 and Smad3 phosphorylation (120). A total of 24.3% of patients with glioma had either CR or PR to LY2157299, and 26.7% showed SD to LY2157299 in a phase I trial (121). Interestingly, 80% of low-grade glioma patients with isocitrate dehydrogenase mutation received clinical benefits in this study, when given LY2157299 treatment. In addition, LY2157299 is well tolerated and safe without adverse cardiac events. However, LY2157299 shows limited antitumor effects in pancreatic tumors (122).

Hey mediates histone deacetylases

The mechanisms through which Hey factors regulate their downstream effectors might also provide promising strategies for anticancer treatment. Hey factors are known to repress the expression of their target genes through recruitment of cofactors (123). Through Hey-mediated transcriptional repression, cancer cells maintain their undifferentiation state. Hey1 transcriptionally represses myogenin expression to sustain embryonal rhabdomyosarcoma cells in an immature state (22). Heterodimers between Hes1 and Hey factors potentially silence achaete-scute homolog 1, which results in the maintenance of an undifferentiated state of tumors (124–126). Histone deacetylases (HDAC) are potentially involved in the repressive effects of Hey factors, as treatment with trichostatin A, a pan class I and II HDAC inhibitor, partially abrogates the repressive effect of Hey factors (127–129). It has been suggested that Hey factors can use their bHLH domain to recruit the mSin3-NCoR-HDAC1 complex or associate with SIRT1, a member of NAD^+-dependent HDACs, and induce transcriptional repression (11, 127). Further research indicates that Hey-HDACs complexes reduced target gene expression by downregulation of histone H3 lysine 27 acetylation (H3K27ac), which represents active transcription (130). Conversely, the inhibition of HDACs can lead to accumulation of acetylated histones and results in active transcription of target genes which are expected to cause tumor differentiation and induction of apoptosis (131, 132). Because the expression of HDACs is required for tumor cell survival and maintenance of an undifferentiated state, HDAC inhibitors might provide a new antitumor strategy. However, the application of HDAC inhibitors remains paradoxical and should be studied in different types of cancer. The silencing of HDAC1 and/or HDAC2 can give rise to hematologic malignancy initiation (133). Knockout of HDAC3 impairs genome stability as well as integrity and results in hepatocellular cancer (134).

HDAC inhibitors

Five HDAC inhibitors have been approved for T-cell lymphoma treatment, vorinostat (MK0683), belinostat (PXD-101), panobinostat (LBH-589), and romidepsin (FK-228), by the FDA, and chidamide (CS055/HBI-8000) approved in China (ref. 135; as summarized in Table 2). These highlight the impact of HDAC inhibitors as antitumor agents. A great number of HDAC

inhibitors are currently in testing in different phases of trials, either combined with other antitumor chemotherapeutics or as monotherapies. A phase II study indicates that entinostat (SNDX-275/MS-275), an inhibitor of HDAC 1 and 3, brings clinical benefits (PR, CR, and SD) to 24% of Hodgkin lymphoma patients, and the median progression-free survival (PFS) as well as OS of these patients was 5.5 months and 25.1 months, respectively (136). Entinostat also shows antitumor effect in several clinical trials (137, 138). In estrogen receptor–positive breast cancer, the combination of exemestane with entinostat improves median PFS to 4.3 months and median OS to 28.1 months, whereas median PFS and OS is 2.3 and 19.8 months, respectively, in the exemestane plus placebo group (139). Other HDAC inhibitors, such as ITF2357, CHR-3996, and JNJ-26481585, have been studied and show promising antitumor effect (140–142).

Combination of therapies

The combination of therapies targeting TGFβ, HDACs, and Notch pathways requires thorough investigation regarding their cross-talk in specific cancer settings. For example, Notch and TGFβ have synergetic carcinogenic effects in lung carcinoma, head and neck squamous, esophageal adenocarcinoma, renal cell carcinoma, thyroid carcinoma, and breast cancer (31, 143–146). Because both TGFβ and Notch signaling can activate Hey, the simultaneous inhibition of both pathways might result in better outcomes than blockade of either individually. Interestingly, inhibition of both Notch and TGFβ cannot increase the synergetic effects on inhibition of cancer cell migration, but additional blockage of Notch attenuates cancer cell proliferation in TGFβ-treated cells (145). This highlights that combination therapies may affect more than one angle. Besides, combination of ALK1 inhibitors and GSI shows promise in targeting tumor angiogenesis, as inhibition of both signaling pathways further abolishes angiogenesis when compared with the inhibition of each alone (58). However, there is little clinical trial data about the combination of Notch and TGFβ inhibitors, and further insightful studies are required. In another instance, targeting both Hey levels and Hey activity concomitantly might prove advantageous in cancer treatment. As an example, Hey proteins exert their influence on tumor cells by recruiting HDACs; when combining GSI and vorinostat, glioma and melanoma cells show a decreased viability (147).

Another strategy is to combine molecular-targeted drugs with classical chemotherapies. The combination of GSIs, HDAC inhibitors, or TGFβ inhibitors with cytotoxic agents results in a more effective therapy since the inhibition of these pathways has been observed to enhance cancer cell lines sensitive to chemotherapy (148–150). Some clinical trials have also established the efficacy of combination therapies. For example, when combined GSIs with cytotoxic chemotherapy, clinical benefits, such as PR and prolonged SD, are observed in solid cancer patients (73, 151, 152). Encouraging antitumor activity is noticed in a Notch2/3-specific antibody study. Treatment OMP-59R5 with etoposide/cisplatin or nab-paclitaxel/gemcitabine shows 100% (3/3) PR in small cell lung cancer and 35% (9/26) PR and 35% (9/26) SD in untreated metastasis pancreatic cancer, respectively (97, 98). HDAC inhibitors in combination with classical chemotherapy also lead to a stronger antitumor effect. For instance, 64% thymoma and thymic carcinoma patients show objective response to belinostat in combination with cisplatin, doxorubicin and cyclophosphamide and vorinostat combined with fludarabine, mitoxantrone, and dexamethasone results in a 77.8% overall response rate in relapsed or refractory mantle cell lymphoma (153, 154). However, the combination of HDAC inhibitors with chemotherapy may lead to unacceptable toxicity and on times is less efficient (155–157).

Perspective in selectively targeting Hey proteins and bHLH factors

Because different tumors tend to upregulate Hey proteins via distinct pathways, targeting Hey proteins directly may bring about a higher response rate than blocking these pathways individually. Besides, targeting Hey proteins themselves may result in fewer side effects because the target genes of Notch, TGFβ, and HDAC signaling pathways will be unaffected. To target Hey, we have to understand the mechanism of action of Hey. There are two possible mechanisms of transcriptional regulation mediated by Hey proteins. The first mechanism is E-box–dependent transcriptional regulation. Hey proteins bind to E-box via basic domain and form functional complex with other cofactors via HLH domain. A domain located between amino acids 47 and 122 is necessary (11, 158). The second mechanism is E-box independent. Hey interacts with DNA-binding proteins via HLH-O domain and performs as a cofactor. The critical domains locate in amino acids 47 to 76 and 111 to 291, which stride over bHLH and Orange domains (61, 159). Based on these, some small molecular inhibitors to antagonize Hey–DNA interaction and Hey–cofactor interaction might be promising. Dimer inhibitors from natural compounds were reported to disrupt the Hey homolog Hes1 dimerization (160). It is still possible to isolate small-molecule inhibitors targeting Hey. In addition, mutagenesis of Hes1 amino acid sequence in the basic domain does not decrease its dimerization-forming ability, but abrogates its transcriptional function (161, 162). Thus, we may construct high structural compatible Hey-dominant–negative peptides which can form inert complexes with Hey and block the three critical functional domains of Hey to disrupt their protein–protein and DNA–protein interfaces. The most successful example is designing stabilized, cell-permeable peptides which bind with NICD–CSL complex and prevent mastermind-like-1 interfacing to antagonize leukemia proliferation (107).

Human bHLH transcription factors contain over 200 members and can be divided into five classes based on phylogenetic analysis (163). Hey transcriptional factors belong to clade B, and other transcriptional factors, such as Twist1-2 (clade A), MyoD (clade C), Max (clade D), Myc (clade E), and hypoxia-induced factor (HIF, clade E), are also bHLH factors. From the mechanistic inhibitory action of bHLH, the bHLH inhibitors can be summarized into the following groups: preventing dimerization, preventing DNA binding, and preventing bHLH factors expression (164). For example, some small-molecule inhibitors were isolated to specifically inhibit Myc-Max dimerization and block the binding of Myc-Max to DNA without affecting other structure-like bHLH factors dimerization (165, 166). By using Myc bHLH-Zip domain fragments, researchers also discovered local conformational changes and formation of hydrophobic cavities at the specific peptide sequences of the fragments upon binding with these small-molecule inhibitors (167). This makes it possible to design specific inhibitors simply through protein sequence analysis because the small-molecule binding sites have certain peptide

sequence criteria. Also, HIF dimer inhibitors as well as HIF DNA-binding inhibitors have been reported (168, 169). In addition, dominant negative peptides mimicking the HLH domain show a significant impact on E2A dimerization (170). Peptides of MyoD which have a high affinity for Id1 can interrupt MyoD–Id1 interaction and exhibit antitumor effects *in vitro* (171).

Conclusion

Hey proteins, a subfamily of mammalian bHLH-O transcriptional factors, have been highly investigated in several research studies since they have been found to be overexpressed in aggressive tumors. Previous work has focused on their transcriptional repressive roles in the maintenance of the undifferentiated state. More recently, studies reveal novel characteristics of Hey proteins in the regulation of cancer metastasis and their influence on angiogenesis. This article offers insight into the significant roles of Hey proteins in tumorigenesis. Alternatively, therapeutic agents able to reverse aberrant Notch, TGFβ, and HDACs levels have been evaluated in clinical trials, but treatment-associated toxicities are also observed. Targeting Hey factors may represent an opportunity for higher response rates but fewer side effects than treatment with GSIs, TGFβ blockers, and HDAC inhibitors. Attention should be drawn to the Hey family in drug design, and studies must be carried out to analyze outcomes using Hey-specific inhibitors in the future.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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