bHLH-Orange Transcription Factors in Development and Cancer

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Abstract: Basic helix-loop-helix (bHLH) proteins are a large superfamily of transcription factors that play critical roles in many physiological processes including cellular differentiation, cell cycle arrest and apoptosis. Based on structural and phylogenetic analysis, mammalian bHLH-Orange (bHLH-O) proteins, which constitute the repressor family of bHLH factors, can be grouped into four subfamilies: Hes, Hey, Helt and Stra13/Dec. In addition to the bHLH domain that mediates DNA-binding and protein dimerization, all members of this family are characterized by a distinctive motif called the "Orange domain" which is present exclusively in these factors. Genetic studies using targeted mutagenesis in mice have revealed essential roles for many bHLH-O genes in embryonic development, cell fate decisions, differentiation of a number of cell types and in apoptosis. Furthermore, growing evidence of crosstalk between bHLH-O proteins with the tumor suppressors p53 and hypoxia-inducible factor, have started to shed light on their possible roles in oncogenesis. Consistently, deregulated expression of several bHLH-O factors is associated with various human cancers. Here, we review the structure and biological functions of bHLH-O factors, and discuss recent studies that suggest a potential role for these factors in tumorigenesis and tumor progression.

Keywords: bHLH-Orange, differentiation, knockout, p53, Hypoxia inducible factor-1, cancer

Introduction

In 1992, the first group of mammalian Hes (Hairy and Enhancer-of-Split) proteins were cloned based on their similarity in the bHLH domain to the *Drosophila* Hairy and Enhancer-of-Split [E(Spl)] gene products (Sasai et al. 1992). Members of Hairy and E(spl) subfamily share many common structural features such as a proline residue in basic motif, and the highly conserved Trp-Arg-Pro-Trp (WRPW) tetrapeptide in the C-terminus. In addition, a ~35 amino acid motif termed 'Orange domain' or Helix III/IV located just C-terminal of the bHLH domain provides an additional protein-protein interaction interface (Dawson et al. 1995). Similar to Hairy and E(Spl) members, Hes proteins also contain a basic domain capable of DNA binding, an adjacent helix-loop-helix domain for homo- or hetero-dimerization, and the 'Orange' domain. In the following decade, several additional bHLH proteins were identified that exhibited significant sequence homology to Hairy and E(Spl) in the bHLH and Orange domains. Since all these bHLH factors share two conserved domains i.e., a bHLH domain and an Orange domain, they are referred as the bHLH-Orange (bHLH-O) factors (Fig. 1A). Based on the sequence similarity with Hairy and E(Spl), the bHLH-O family was initially grouped into four subfamilies: Hairy, E(spl), Hey, and Stra13/Dec (Davis and Turner, 2001). Recently, several independent groups characterized Helt, a new member of the bHLH-O family. To include the newest members of the bHLH-O family, we have organized the mammalian bHLH-O factors into four subfamilies based on structural and phylogenetic analysis: Hes, Hey, Helt, and Stra13/Dec (Fig. 1). Numerous studies have recently been conducted to investigate the biological functions of bHLH-O proteins. Studies on the role of bHLH-O proteins in human cancers have also been initiated.

In this review, we describe the structure, transcriptional properties, and biological functions of the four mammalian bHLH-O factor subfamilies. We also discuss recent studies suggesting a potential role for these factors in carcinogenesis.

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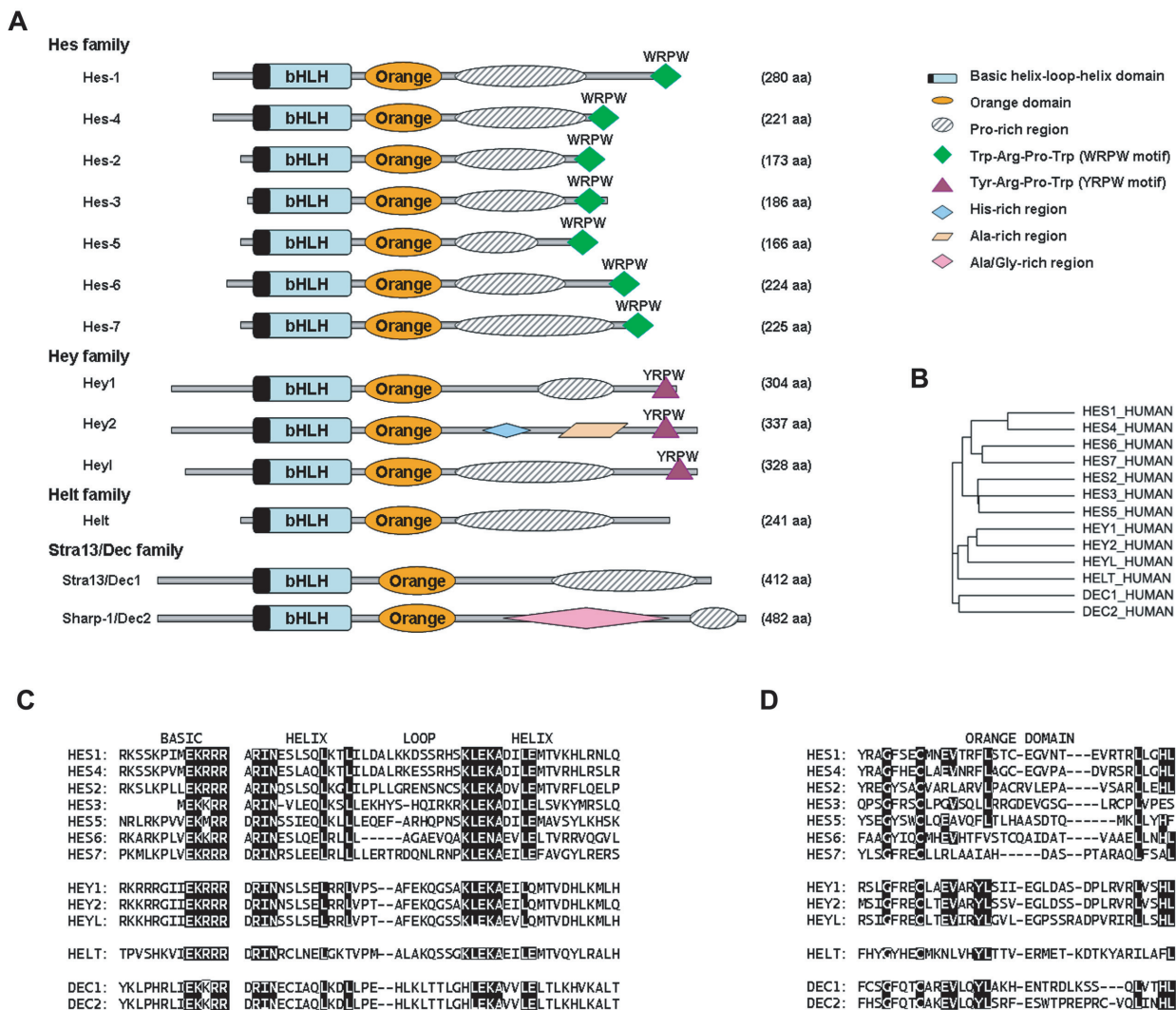
Figure 1. Structure and phylogenetic analysis of mammalian bHLH-O factors. **A.** Schematic diagram showing the domain structure of 13 bHLH-O factors. **B.** Phylogenetic analysis of human bHLH-O factors. The tree was created using the ClustalW method with full-length amino acid sequence of bHLH-O proteins. **C** and **D.** Amino acid sequence comparison of the bHLH domain (C), and Orange domain (D) of bHLH-O factors. Conserved residues are highlighted.

Subfamilies of bHLH-O Factors

The Hes subfamily

Seven Hes proteins have been identified in human and mouse, termed Hes1–7 (Akazawa et al. 1992; Sasai et al. 1992; Bae et al. 2000; Bessho et al. 2001a; Koyano-Nakagawa et al. 2000; Pissarra et al. 2000; Vasiliauskas and Stern, 2000). The amino acid sequence of each family member exhibits significant homology within the bHLH domain and the Orange domain (Fig. 1). Based on their sequence homology to the *Drosophila* Hairy or E(spl) proteins, the Hes family can be further divided into two subgroups (Davis and Turner, 2001). Hes 1 and Hes 4 exhibit greater similarity to Hairy proteins, whereas Hes 2, 3, 5, 6, 7 are more similar to E(spl) proteins. Hes proteins contain a proline-rich region between the Orange domain and the WRPW motif. While the function of this region in Hes is not clear, a truncated E(spl) protein lacking the region between the Orange domain and the WRPW motif failed to suppress bristle development, suggesting an essential role for this region in E(spl) function (Giebel and Campos-Ortega, 1997). The C-terminus of Hes proteins contain the WRPW motif, which is crucial for transcriptional repression (Fisher et al. 1996). The corepressor transducin-like enhancer of split (TLE), which is the mammalian counterpart of the *Drosophila* corepressor Groucho, interacts with Hes proteins through the WRPW motif (Fisher et al.

1996; Grbavec and Stifani, 1996). This interaction is required for transcriptional repression mediated by Hes proteins, and occurs by recruitment of the histone deacetylase (HDAC) complex on Hes-bound target gene promoters resulting in inhibition of transcription (Chen et al. 1999; Yao et al. 2001).

Most bHLH factors bind to a specific sequence known as the E-box (CANNTG). However, the best characterized binding site for Hes1 is an alternate sequence, CACNAG (known as N-box) (Sasai et al. 1992) or sequences similar to N-box (CACGCG, also known as class C site). Hes1 negatively regulates its own expression through four N-box sites located in its promoter (Takebayashi et al. 1994). In addition, Hes1 represses CD4 expression by binding to a N-box in the CD4 promoter (Kim and Siu, 1998). In hepatoma-derived Hep G2 cells, Hes1 binds to the class C site in the acid alpha-glucosidase promoter and inhibits its activity (Yan et al. 2001). Moreover, mouse achaete-scute homolog-1 (Mash-1), an important Hes1 target gene that plays a critical role in neurogenesis, can be negatively regulated by Hes1 through a variant class C site (CACGCA) (Chen et al. 1997). Hes 2, 3, 6 can bind to both the N-box and the E-box *in vitro* (Sasai et al. 1992; Ishibashi et al. 1993; Hirata et al. 2000; Cossins et al. 2002). However, Hes 5 and 7 preferentially bind to the N-box (Akazawa et al. 1992; Chen et al. 2005), while Hes 2 appears to preferentially bind to class B E-box sequences (CACGTG) (Ishibashi et al. 1993). In contrast to the well-defined targets of Hes1, the *in vivo* targets of other Hes proteins are not very clear.

The Hey subfamily

The Hey family contains three members: Hey1, Hey2 and HeyL (Leimeister et al. 1999). Since the Hey genes were independently cloned by several laboratories, they are known by several different names: Hey (Hairy/E(spl)-related with YRPW) (Leimeister et al. 1999), HRT (Hairy-related transcription factor) (Nakagawa et al. 1999), Hesr (Hairy/E(spl)-related protein) (Kokubo et al. 1999), Herp (Hes-related repressor protein) (Iso et al. 2001a; Iso et al. 2001b), and CHF (Cardiovascular helix-loop-helix factor) (Chin et al. 2000).

As indicated by their names, Hey proteins are highly related to Hes proteins, and share about ~56% homology with Hes1 in the bHLH domain. The three Hey proteins exhibit extensive homology (>90%) within the bHLH domain. The Orange domain is highly conserved, and shares significant similarity to the Orange domain in Hes proteins. However, despite these similarities in the bHLH and Orange domains with Hes proteins, Hey proteins have two structural characteristics that distinguish them from the Hes subfamily: (1) The basic region of Hey proteins has a conserved glycine residue in place of the proline residue that is present in Hes factors at the corresponding position. The role of the glycine residue is not clear, but replacement of glycine with proline in CHF2 (Hey1) did not exhibit any significant effect on repression of the myogenin promoter (Sun J et al. 2001). In contrast to Hes proteins that have the capacity to bind both the N-box and E-box, Hey proteins bind primarily to the E-box with either class A (CAGGTG) or class B (CACGTG) sites. (2) Instead of the WRPW motif present in Hes proteins, Hey proteins have a YRPW motif or close variants. However, unlike the WRPW motif in Hes that recruits TLE, there is no evidence that the YXXW motif recruits the TLE corepressor. In addition, deletion of the YRPW motif in Hey1 does not abolish its transcriptional repression activity, suggesting different roles for the YRPW motif of Hey1 and WRPW motif of Hes1 (Sun J et al. 2001). Though Hey proteins do not recruit TLE as a corepressor, the bHLH domain and Orange domain can recruit the HDAC corepressor complex to repress transcription of target genes (Nakagawa et al. 2000; Iso et al. 2001b).

The Stra13/DEC subfamily

The Stra13 subfamily contains two members, which have also been given several different names. The first member was called Stimulated with retinoic acid 13 (Stra13) (Boudjelal et al. 1997) or Clast5 (Seimiya et al. 2002) in mouse, Differentially Expressed in Chondrocytes (Dec1) (Shen et al. 1997), E47 interacting protein 1 (Eip1) (Dear et al. 1997) or Cytokine response gene 8 (CR-8) (Beadling et al. 2001) in human, and Enhancer-of-Split and Hairy-related protein-2 (Sharp-2) in rat (Rossner et al. 1997). Similarly, the other member was called Sharp-1 in rat (Rossner et al. 1997) and mouse (Azmi and Taneja, 2002) and Dec2 in human (Fujimoto et al. 2001).

Members of this subfamily exhibit ~43% homology with the bHLH domain in Hes1. The two subfamily members Stra13/Dec1 and

Sharp-1/Dec2 share the highest homology within bHLH domain (96%) and an overall homology of 50% over the entire length of the two proteins (Azmi et al. 2003). Similar to Hes proteins, Stra13 retains a proline residue in the basic domain (Boudjelal et al. 1997). The proline residue is however displaced 2 residues closer to the N-terminus compared to Hes proteins, which may contribute to its altered DNA binding preferences. Stra13/Dec1 and Sharp-1/Dec2 bind with high affinity to the class B sites CACGTG (St-Pierre et al. 2002; Zawel et al. 2002; Azmi et al. 2003). Strikingly, in contrast to Hes and Hey family members, Stra13/Dec family members have neither the WRPW nor the YRPW motif in the C-terminus. However, both Stra13 and Sharp-1 recruit the corepressor HDAC1 (Sun and Taneja, 2000; Fujimoto et al. 2007) and thereby act as transcriptional repressors. Stra13 also interacts with the basal transcription machinery (Boudjelal et al. 1997) resulting in HDAC1 independent repression of some target genes (Sun and Taneja, 2000). In addition, both Stra13 and Sharp-1 can interact with various proteins and inhibit their activity in a DNA-binding independent manner. For instance, Sharp-1 interacts with the bHLH factor MyoD resulting in inhibition of its activity and myogenic differentiation (Azmi et al. 2004; Morosetti et al. 2007; Fujimoto et al. 2007).

The Helt/Heslike subfamily

The newest subfamily of bHLH-O factors contains only one member, Helt/Heslike/Megane (Miyoshi et al. 2004; Nakatani et al. 2004; Guimera et al. 2006a). The bHLH domain of Helt shares significant sequence homology with Hey (57%) and Hes (50%) proteins. There is also weak homology in the Orange domain, which is about 25% with Hey and Hes proteins. However, unlike Hes or Hey proteins, the basic domain of Helt lacks either a proline or glycine residue, and instead has a lysine residue in the corresponding position. In addition, Helt does not have a WRPW or YRPW motif. Helt can bind to class B E-box sequences, and function as a transcriptional repressor (Nakatani et al. 2004).

In Vivo Functions

Hes genes

Hes1 is widely expressed in many different tissues and cell types including neuronal stem cells (Sasai et al. 1992). Gene-disruption studies have demonstrated that Hes1 null mice die during gestation or shortly after birth with defects in neural tube closure (Ishibashi et al. 1995; Table 1). Neuronal differentiation markers are expressed earlier in Hes1 null embryos. This premature neuronal differentiation can be also seen in the retina (Tomita et al. 1996), olfactory placode (Cau et al. 2000), and inner ear (Zheng et al. 2000), indicating an essential role of Hes1 in maintenance of neuronal stem cells and suppression of neural differentiation. Consistent with this finding, constitutive expression of Hes1 in neural precursors inhibits neuronal and glial differentiation (Ishibashi et al. 1994). Hes1 is also required for expansion of precursor cells in the thymus (Tomita et al. 1999), pituitary gland (Kita et al. 2007), and pancreas (Jensen et al. 2000). Transfer of Hes1-/- fetal liver cells into Rag2 null mice results in defective TCR-dependent and -independent expansion of T precursor cells, leading to arrested T cell differentiation at the CD4-CD8-double negative stage (Tomita et al. 1999). In Hes1 null mutants, premature differentiation of endocrine cells results in depletion of pancreatic precursors and pancreatic hypoplasia (Jensen et al. 2000). In conditional knockout mice lacking both Hes1 and Hes5, the pituitary gland is severely hypoplastic and a loss of both intermediate and posterior lobes occurs due to premature differentiation (Kita et al. 2007). In addition to maintenance of stem cell or progenitor pools, Hes1 can also regulate binary cell fate decisions. In Hes1-deficient pancreas, premature differentiation occurs primarily in endocrine but not in exocrine cells, indicating that Hes1 specifically inhibits differentiation of endocrine cells (Jensen et al. 2000). Interesting, Hes1-/- biliary epithelium differentiates into both endocrine and exocrine cells which normally only exist in pancreas (Sumazaki et al. 2004), indicating that Hes1 prevents the biliary epithelium from a pancreatic differentiation program.

Hes 3 and Hes 5 are also expressed in neuronal stem cells. Mice lacking Hes 3 exhibit no obvious abnormality (Hirata et al. 2001). Hes5 null mice are viable, but exhibit premature neuronal differentiation similar to Hes1 mutants (Ohtsuka et al. 1999). Double mutants lacking both Hes1 and Hes5 die at E12.5, and have very few radial glia (Ohtsuka et al. 1999; Hatakeyama et al. 2004). Hes1 and Hes3 double null mice also exhibit premature neurogenesis, and lack midbrain and anterior hindbrain structures (Hirata et al. 2001).

Table 1. In vivo functions of bHLH-O factors as seen by gene disruption studies.

Gene (s)	Phenotype	References
Hes1	Null mice die during gestation or shortly after birth, and exhibit premature differentiation of precursor cells into neurons and ganglion cells. This premature differentiation can be also seen in the thymus, pituitary gland, pancreas and other neuronal tissues, such as retina, olfactory placode, and inner ear.	Ishibashi et al. 1995 Tomita et al. 1996 Tomita et al. 1999 Cau et al. 2000 Zheng et al. 2000 Jensen et al. 2000 Sumazaki et al. 2004 Takatsuka et al. 2004 Kita et al. 2007
Hes3	Null mice are viable, fertile, and have no apparent defects.	Hirata et al. 2001
Hes5	Apparently normal.	Ohtsuka et al.1999
Hes7	Hes7 null mice exhibit short trunk and tails, and most of them die within a few hours after birth due to respiratory failure.	Bessho et al. 2001
Hes1/Hes3	Hes1 and Hes3 double knockout mice die around E15.5 with neural tube defects, and defects in midbrain and hindbrain structures.	Hirata et al. 2001
Hes1/Hes5	Hes1 and Hes5 double null mice show similar but more severe phenotypes than Hes1 single null mice.	Ohtsuka et al. 1999
Hes1/Hes5 conditional	Hes1 and Hes5 conditional knockout mice show accelerated differentiation of progenitor cells, leading to severe pituitary hypoplasia, and lack the intermediate and posterior lobe of pituitary gland.	Kita et al. 2007
Hes1/Hes3/Hes5	All neural stem cells prematurely differentiate into neurons, and brain structures are severely affected	Hatakeyama et al. 2004
Hey1	No obvious abnormality Decrease in spontaneous locomotor activity and other behavioral defects	Fischer et al. 2004 Kokubo et al. 2005 Fuke et al. 2006
Hey2	Hey2 null mice display dysplastic AV valves, a ventricular septal defect (VSD) and an atrial septal defect (ASDII)	Donovan et al. 2002 Gessler et al. 2002 Kokubo et al. 2004 Sakata et al. 2002
HeyL	No obvious phenotype	Fischer et al. 2007
Hey1/Hey2	Hey1 and Hey2 double null mice die between E9.5 to E11.5 due to cardiovascular defects and massive hemorrhage	Fischer et al. 2004 Kokubo et al. 2005
Hey1/HeyL	Hey1 and HeyL double null mice die within a few days after birth due to congenital heart defects, and exhibit impaired epithelial to mesenchymal transition in developing heart.	Fischer et al. 2007
Stra13	Stra13 null mice have defective CD+T cell activation. DNA-damage induced apoptosis of thymocytes is reduced. Normal circadian phenotype but alteration in a subset of peripheral clocks. Aberrant skeletal muscle regeneration in response to injury.	Sun et al. 2001 Grechez-Cassiau et al. 2004 Thin et al. 2007 Sun et al. 2007

Interestingly, in Hes1/Hes3/Hes5 triple knockout mice, neuroepithelial cells are not maintained and radial glial cells prematurely differentiate (Hatakeyama et al. 2004).

Hes6 is expressed in nervous system, muscle, thymus, and the sensory lineage of the inner ear (Bae et al. 2000; Pissarra et al. 2000; Vasiliauskas and Stern, 2000; Qian et al. 2006). Misexpression of Hes6 in the developing retina and myoblasts promotes neuronal and muscle differentiation respectively (Bae et al. 2000; Gao et al. 2001). However, Hes6 null mice appear normal, and do not reveal any overt defects (Koyano-Nakagawa et al. 2000; Qian et al. 2006). Hes7 is specifically

expressed in the presomitic mesoderm in an oscillatory manner (Bessho et al. 2001a; Bessho et al. 2001b). In Hes7 null mutants, somites are not properly segmented and exhibit disrupted anterior-posterior polarity, demonstrating its essential role in somite segmentation (Bessho et al. 2001b).

Hey genes

During mouse embryonic development, all three Hey genes are expressed in many different tissues. Hey1, Hey2 and HeyL are expressed in the developing heart with distinctive but somewhat overlapping patterns (Nakagawa et al. 1999; Leimeister et al. 2000a). Hey1 is expressed in the atrial myocardium and epicardium, whereas Hey2 expression is seen mainly in the ventricle, the compact zone, and in the atrioventricular (AV) cushions. HeyL is located in endocardial cells in AV cushions. In blood vessels, Hey1 expression is restricted to endothelial cells, but Hey2 and HeyL are expressed in both endothelial and smooth muscle cells (Leimeister et al. 1999; Nakagawa et al. 1999; Leimeister et al. 2000b).

Several groups generated mice lacking Hey genes (Table 1). Hey1 (Fischer et al. 2004) and HeyL (Fischer et al. 2007) single knockout mice are viable and exhibit no overt abnormalities, although recently Hey1 null mice were reported to have decreased spontaneous locomotor activity and a reduction in exploration to novelty through the dopaminergic nervous system (Fuke et al. 2006). Hey2 null mutants die within 10 days after birth with cardiac defects (Donovan et al. 2002; Gessler et al. 2002; Sakata et al. 2002; Kokubo et al. 2004). Hey2 null mutant hearts display AV valve hypoplasia, ventricular septum defect (VSD), an atrial septal defect (ASD), as well as cardiomyopathy. Surprisingly, Hey1 and HeyL double null mutants also exhibit dysplastic AV valves, and VSD similar to Hey2 mutants (Fischer et al. 2007). In contrast to normal vascular development in all three Hey single mutants, Hey1 and Hey2 double null mutants exhibit severe vascular defects and die between E9.5 to E11.5 (Fischer et al. 2004). The defects in arterial-venous specification, septation and cushion formation in double null mice recapitulates the phenotypes in mutants with disrupted Notch signaling, suggesting that Hey genes are essential transducers of Notch signaling.

Helt gene

Helt/Heslike is expressed in the neural system during early embryonic development, specifically in a region that gives rise to GABAergic neurons (Miyoshi et al. 2004; Nakatani et al. 2004; Georgia et al. 2006). Co-expression of Helt with Mash1 in neural precursor cells promotes formation of GABAergic neurons (Miyoshi et al. 2004). Helt null mice die about 3 to 5 weeks after birth due to neurological defects, exhibiting a seizure-like phenotype prior to death (Guimera et al. 2006b; Table 1). GABAergic neurons are missing in the mesencephalon of Helt/Heslike null embryos, and are replaced by glutamatergic neurons (Guimera et al. 2006b; Nakatani et al. 2007), indicating a key role of Helt/Heslike in selection of GABAergic over glutamatergic neuronal fate.

Stra13/DEC genes

Both Stra13 and Sharp-1 are expressed in a number of tissues and cell types during embryonic development as well as in the adult (Boudjelal et al. 1997; Shen et al. 1997; Fujimoto et al. 2001; Sun H et al. 2001; Azmi and Taneja, 2002; Li et al. 2002; Azmi et al. 2004). Stra13 null mice are viable but exhibit defects in several cell types (Table 1). CD4+ T cell activation is defective in the mutants, resulting in insufficient cytokine production. Moreover, surface FasL levels are decreased resulting in reduced Fas-mediated apoptosis and accumulation of spontaneously activated T and B cells that leads to lymphoid organ hyperplasia and a systemic lupus-like immune disorder (Sun H et al. 2001). Similar to impaired Fas-mediated apoptosis of activated T and B cells, p53-dependent cell death is also defective in Stra13 null thymocytes supporting a common role for Stra13 in regulating apoptosis (Thin et al. 2007). Interestingly, skeletal muscle regeneration is impaired in Stra13 null mice along with increased Notch activity, indicating its role in antagonizing Notch signaling in myogenic cells (Sun et al. 2007). Thus, unlike Hes and Hey genes that are downstream effectors of Notch signaling, Stra13 regulates Notch activity. Stra13 is expressed rhythmically in peripheral tissues and Stra13 mutant mice exhibit a normal circadian phenotype, but an altered subset of peripheral clocks (Grechez-Cassiau et al. 2004). Stra13/Sharp-1 double mutants exhibit altered entrainment of the circadian system to external cues (M. Rossner, personal communication), improved learning in aversive

and non-aversive tasks and altered sleep patterns (M. Rossner, personal communication).

bHLH-O Proteins and Cancer

In contrast to the essential and established roles of bHLH-O factors in embryonic development, organogenesis, cellular differentiation and apoptosis, little is known whether these factors function in cancers. A growing body of evidence indicates deregulated expression of bHLH-O factors in several cancers (Table 2), suggesting that these factors may be important in neoplastic transformation.

bHLH-O proteins and p53

In a genome wide screen for potential p53 regulators, Hey1 and Hes1 were found to positively regulate p53 levels (Huang et al. 2004). Expression of Hey1 and Hes1 in human colon cancer HCT116 cells significantly induces endogenous p53 protein levels as well as promotes p53 transcriptional activity by inhibiting HDM2 transcription. Hey1 and Hes1 do not associate with the E- or N-box sequences in the HDM2 promoter, suggesting that HDM2 regulation by these proteins is independent of DNA-binding. Consistent with the ability of Hey1 and Hes1 to increase p53 levels, ectopic expression of Hey1 and Hes1 in zebrafish and chick embryos recapitulates p53-dependent apoptosis *in vivo*. Interestingly, Hey1 overexpression inhibits oncogene-induced transformation of wild-type

but not p53-null mouse embryonic fibroblast (MEF) cells, suggesting that Hey1 suppresses transformation in a p53-dependent manner. The ability of Hey1 and Hes1 to induce p53 levels and its activity in mammalian cells, as well as in zebrafish and chick embryos, suggest that in some contexts they may function as tumor suppressors.

Our recent studies also indicate that Stra13 is a regulator of p53 (Thin et al. 2007). Stra13-deficient thymocytes exhibit decreased γ-radiation induced apoptosis, along with reduced expression of p53 and its targets Puma and Noxa. Consistent with this, overexpression of Stra13 results in the accumulation of p53 in NIH3T3 cells but not in p53/Mdm2-/- MEF cells, suggesting that Stra13 modulates p53 levels in a Mdm-2-dependent manner. Stra13 can directly interact with p53, preventing its nuclear export and degradation via Mdm2-mediated ubiquitination.

bHLH-O proteins and hypoxia-inducible factor 1 (HIF-1)

As highly proliferating tumor cells outgrow their blood supply, a local hypoxic microenvironment is formed in the tumor, triggering expression of genes that allow cells to survive hypoxic stress, and stimulate formation of new blood vessels from the existing vasculature. These responses are largely initiated by HIF-1, which is a heterodimer of two bHLH-Pas proteins: HIF-1α and the aryl

Table 2. Changes in bHLH-O proteins in various cancers.

Factors	Cancer	Expression	References
Hes1	Breast cancer	Down regulated in estradiol treated MCF7 and T47D cells	Strom et al. 2000
	Lung cancer	High in NSCLC, low in SCLC	Chen et al. 1997
	Pancreatic cancer	High	Fukushima et al. 2004
	Ovarian cancer	High in adenocarcinoma, low in adenomas	Hopfer et al. 2005
Hey1	Lung cancer	High in NSCLC	Collins et al. 2004
	Prostate cancer	High	Belandia et al. 2005
HeyL	Lung cancer	High in NSCLC	Collins et al. 2004
		High in SCLC	Henderson et al. 2005
Dec1	Breast cancer	Elevated in both *in situ* and invasive carcinoma	Chakrabarti et al. 2004 Turley et al. 2004
	Lung cancer	Low in 62% NSCLC, high in 38% NSCLC	Giatromanolaki et al. 2003
	Pancreatic cancer	High	Yoon et al. 2001
	Colorectal cancer	High	Li et al. 2002
		Low in 68%, high in 32%	Koukourakis et al. 2006

hydrocarbon nuclear translocator (ARNT, also called HIF-1β) (Ke and Costa, 2006). While ARNT is constitutively expressed in many cell types, oxygen levels tightly regulate HIF-1α expression. Under normoxic conditions (21% oxygen tension), HIF-1α is constantly modified by prolyl hydroxylases at proline-402 and 564, leading to its degradation through von Hippel-Lindau protein (VHL)/E3 ligase-mediated ubiquitination. However, under hypoxic conditions, the activity of prolyl hydroxylases decreases due to insufficient oxygen levels, resulting in stabilization of HIF-1α. Once stabilized, HIF-1α translocates into the nucleus, forms a heterodimer with ARNT, binds to a specific DNA sequence (hypoxia response element, HRE) in the promoter of its target genes such as VEGF, glucose transporters, glycolytic enzymes, erythropoietin etc (Semenza, 2003; Keith and Simon, 2007).

Several lines of evidence indicate that both members of the Stra13/Dec subfamily are direct targets of HIF-1. First, Stra13 expression is down regulated by VHL under normoxic conditions (Wykoff et al. 2000), and can be induced by hypoxia in many cancer cell lines including lung carcinoma A549, bladder cancer EJ-28, breast cancer HBL-100, kidney carcinoma RCC4 (Wykoff et al. 2000; Ivanova et al. 2001), and pancreatic cancer Capan-2 cells (Yoon et al. 2001). Second, a consensus HRE sequence is present in the promoters of both Stra13/Dec1 and Sharp-1/Dec2 genes, and co-transfection of HIF-1α stimulates their promoter activity (Miyazaki et al. 2002). Third, Stra13/Dec1 levels in many different tumors are tightly correlated with HIF-1α levels (Giatromanolaki et al. 2003; Chakrabarti et al. 2004). As a direct target of HIF-1 heterodimers, the function of Stra13/Dec1 and Sharp-1/Dec2 in hypoxic signaling still needs to be clarified. It has been reported that Stra13 mediates hypoxia-induced inhibition of adipogenesis (Yun et al. 2002) and is also able to repress STAT1 expression under hypoxic conditions (Ivanov et al. 2007). These studies suggest that Stra13 acts as a mediator of hypoxia signaling. However, a recent report indicates that Stra13/Dec1 may be an upstream regulator of HIF-1α. In cultured human keratinocytes, UVB radiation strongly induces HIF-1α levels. This induction is almost completely abolished in Dec1-deficient HaCat cells (Li et al. 2006), suggesting Stra13/Dec1 is required for HIF-1α stabilization. In another study, it was reported that while hypoxia can induce HIF-1α levels in human carcinoma A549 cells, overexpression of Dec1 in A549 cells reduced both HIF-1α mRNA and protein levels (Zhang and Li, 2007), suggesting that Stra13/Dec1 can negatively regulate HIF-1α in these cells. Clearly hypoxia induces Stra13/Dec1, but depending on cellular context, Stra13/Dec1 may function in an opposing manner, resulting in either a positive or negative impact on hypoxic signaling.

Compared to the Stra13/Dec family, little is known about Hes and Hey genes in hypoxia signaling. Recent studies have indicated that HIF-1α can upregulate Hes1 and Hey2 in both P19 and C2C12 cells in a Notch-dependent manner (Gustafsson et al. 2005). Hypoxia also induces Notch signaling and activates Hey1 and Hey 2 in endothelial precursor cells, which then repress HIF-1α induced gene expression (Diez et al. 2007). In addition, Hey1 (CHF2) was identified as an interacting protein of ARNT (HIF-1β) (Chin et al. 2000), and suppressed HRE-dependent gene expression under both normoxic and hypoxic conditions (Takahashi et al. 2004), suggesting a role for Hey1 in negatively regulating hypoxia signaling.

bHLH-O proteins in human cancers

Breast cancer
Hes1 levels are down regulated in 17β-estradiol treated human breast cancer lines T47D and MCF-7, and this down regulation was found to be essential for estradiol induced cell proliferation (Strom et al. 2000). Interestingly, all-trans-retinoic acid can antagonize estradiol induced cell proliferation by up-regulating Hes1 expression in both cells (Muller et al. 2002; Hartman et al. 2004). Consistent with this anti-proliferative effect, the expression of Hes1 in T47D cells via a tetracycline-inducible system down-regulates E2F-1 expression and inhibits G1/S transition induced by estrogen (Hartman et al. 2004).

In normal human breast tissue, Dec1, the human homolog of Stra13, is weakly expressed predominantly in luminal epithelial cells, stromal cells, as well as in endothelial cells of capillaries (Chakrabarti et al. 2004) and is mostly nuclear, with occasional cytoplasmic staining. However, Dec1 expression is elevated in 10 out of 15 cases of in situ human breast carcinomas, and 59 out of 101 cases of invasive human breast carcinomas (Chakrabarti

et al. 2004). Consistent with this finding, elevated Dec1 was seen also seen in another study in 25 out of 26 cases of in situ human breast carcinomas (Turley et al. 2004). Within tumor cells, both nuclear and cytoplasmic Dec1 levels were increased. Elevated Dec1 level was also found in fibroblasts (86 out of 101 cases, 85%), macrophages (42%), and endothelial cells (78%) of invasive human breast carcinomas. Most interestingly, both studies reported a decrease or loss of Dec1 expression in cells immediately adjacent to necrotic areas suggesting that down regulation of Stra13 might confer a survival signal in cells adjacent to necrotic zones. In addition to whole tissue sections, 253 invasive human breast carcinomas from tissue microarrays were also screened for Dec1 expression. Similar to tissue sections, more than 74% carcinomas showed elevated Dec1 levels and a significant correlation was noted between Dec1 and tumor grade (p = 0.01), as well as HIF-1α (p = 0.04) (Chakrabarti et al. 2004).

Lung cancer

Based on the morphology of malignant cells, human lung cancers can be categorized into two major types: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). SCLC is less common accounting for 20% of human lung cancers, and is strongly associated with smoking. Almost 80% of lung cancers are NSCLC, including squamous cell carcinoma, adenocarcinoma, large cell carcinoma and bronchioloalveolar carcinoma.

Hes-1 expression is high in NSCLC cell lines compared to its low or undetectable levels in SCLC cell lines (Chen et al. 1997). High Hes1 expression is inversely correlated with expression of human achaete-scute homolog-1 (hASH1) (Chen et al. 1997), consistent with the negative regulation of Mash1 by Hes-1 (Ishibashi et al. 1995). However, it is not clear whether Hes1 plays any direct role in tumor cell growth, or is just the outcome of altered Notch signaling since NSCLC cells frequently express more Notch1 and Notch2, whereas SCLC cells rarely express Notch1 (Ball, 2004; Collins et al. 2004; Garnis et al. 2005). Constitutive activation of Notch signaling in SCLC cells induces a profound growth arrest (Sriuranpong et al. 2001) as well as rapid degradation of hASH1 (Sriuranpong et al. 2002). Overexpression of Hes1 in SCLC cells does not induce growth arrest,

suggesting other molecules may mediate the effects of Notch in SCLC cells (Sriuranpong et al. 2001). SAGA expression profiling data showed high levels of Hey1 and HeyL in NSCLC cells (Collins et al. 2004). Interestingly, high levels of HeyL were also found in SCLC cells (Henderson et al. 2005), which normally have low Notch activity.

Dec1 protein was also evaluated in 115 SCLC cases (Giatromanolaki et al. 2003). In normal lung tissue, Dec1 was expressed in the nuclei of bronchial epithelium, submucosal vessels, type II alveolar epithelial cells, and alveolar macrophages. In 71 out of 115 (62%) SCLC tumor cases, Dec1 levels are low in cancer cells compared to adjacent normal bronchial epithelium. But in the remaining cases (44%), Dec1 expression is high, and exhibits some degree of cytoplasmic expression, compared to its nuclear localization in normal cells. Interestingly, tumor samples expressing high levels of Dec1 exhibit poor histological differentiation, and also expressed elevated levels of HIF-1α and Carbonic anhydrase-9, a HIF-induced gene.

Pancreatic cancer

Pancreatic cancer, a malignant tumor within the pancreatic gland, is the fifth leading cause of cancer death in U.S. Most human pancreatic cancers are adenocarcinomas (95%), and the remaining includes other types of tumors, such as acinar cell cancer, pancreatic neuroendocrine tumor, and tumors from exocrine pancreas.

Using an oligonucleotide microarray to screen for genes that are overexpressed in human mucinous cystic neoplasm's (MSNs), the Notch ligand Jagged1 was found to be elevated in tumor cells compared to normal pancreatic tissue (Fukushima et al. 2004). Consistent with this finding, Hes1 levels are also increased in tumor cells (Fukushima et al. 2004).

Upon hypoxia treatment, Dec1 was increased about 7.5-fold in Capan-2 cells, and 2.6-fold in a poorly differentiated human pancreatic cancer MIAPaCa-2 cells (Yoon et al. 2001).

Other tumors

Prostate cancer

14 benign prostatic hyperplasia (BPH) and 10 prostate cancer samples were analyzed for Hey1 expression by immunohistochemistry. Hey1 was expressed in both the nucleus and cytoplasm of

epithelial cells. However, in 8 out of 10 prostate cancer samples, Hey1 was exclusively located in the cytoplasm (Belandia et al. 2005). Since Hey1 is localized in the nucleus, nuclear exclusion of Hey1 in prostate cancer cells suggests that inactivating Hey1 may be crucial for prostate cancer development. Interestingly, Hey1 can inhibit ligand-dependent activity of the androgen receptor (Belandia et al. 2005; Powell et al. 2006), indicating that its nuclear exclusion may also lead to abnormal androgen receptor activity in prostate cancer cells

Colorectal cancer

Four colorectal cancer tissues were analyzed for Dec1 expression, and both mRNA and protein levels were high in cancer cells compared to adjacent normal tissues (Li et al. 2002). In other analysis, 54 out of 79 cases (68%) of colorectal cancer expressed low levels of Dec1, and only 32% tumor samples express high nuclear Dec1 (Koukourakis et al. 2006). Interestingly, tumors with low levels of Dec1 were linked to a high proliferation index, and high Dec1 expression was linked to a low mitotic index (Koukourakis et al. 2006).

Ovarian cancer

Ovarian cancer is the fifth leading cause of cancer death in women. In a study with 32 ovarian tumor samples, Hes1 levels were significantly higher in 18 out of 19 ovarian adenocarcinomas and borderline tumors, but Hes was not expressed in ovarian adenomas (Hopfer et al. 2005).

Summary

The role of bHLH-O proteins in the development and differentiation of diverse cell types has expanded rapidly as a result of intensive genetic studies using mice ablated in individual or multiple bHLH-O factors. Increasing evidence demonstrating altered expression of bHLH-O genes in cancers suggests that these genes may play similarly important roles in tumorigenesis. Specifically, the involvement of Stra13, Hey1 and Hey2 in the hypoxic response, as well as the regulation of p53 and p53-dependent tumor suppressor functions by Hey1, Hes1 and Stra13 suggest an important role for these factors in tumorigenesis. In agreement with this contention, the expression of these genes is highly modulated in many cancer cell lines as well as in primary tumors. Among the bHLH-O

factors, Stra13/Dec1 expression has been recently analyzed to the greatest degree in various cancers. Intriguingly, Stra13 is upregulated in some tumors, and down regulated in others. For instance, studies in breast cancer have indicated that Stra13 is expressed endogenously at low levels in normal breast tissue, and exhibits a progressive increase in expression in in situ carcinomas and invasive tumors, which is associated with HIF-1α levels (Turley et al. 2004; Chakrabarti et al. 2004). In contrast to breast tissue, endogenous Stra13 is high in lung, and a majority of NSCLC specimens exhibit reduced Stra13 levels, although a smaller percentage of tumors show elevated Stra13 expression, which is associated with hypoxic markers (Giatromanolaki et al. 2003). Similarly, Stra13 expression is down regulated in most colorectal cancers (Koukourakis et al. 2006). The seemingly paradoxical findings that Stra13 expression is elevated in some tumors and down regulated in others, may be a reflection of its divergent cell-type and context-dependent functions, or alternatively a read out of the different stages of tumorigenesis. For instance, previous reports have suggested a role for Stra13 in cell survival by inhibition of caspases (Li et al. 2002) as well as in protection of podocytes from oxidative stress (Bek et al. 2003). In contrast, loss of Stra13 results in reduced apoptosis of lymphocytes (Sun et al. 2001; Thin et al. 2007), and its overexpression results in increased cell death in 293T cells (Ivanova et al. 2004). The down regulation of Stra13 in lung and colon cancer is consistent with many previous studies demonstrating that Stra13 causes growth arrest in a number of cell types (Sun and Taneja, 2000; Beadling et al. 2001; Li et al. 2002; Zawel et al. 2002; Seimiya et al. 2004), as well as the fact that it is expressed in most normal tissues that are differentiated (Turley et al. 2004; Ivanova et al. 2005; Sun et al. 2007). Moreover, Stra13 promotes differentiation of a number of cell types such as chondrocytes (Shen et al. 2002), osteoblasts (Iwata et al. 2006), trophoblasts (Hughes et al. 2004), and neurons (Boudjelal et al. 1997). Lastly Stra13 maps to a tumor suppressor locus and is localized on chromosome 3p26 in humans (Antonevich and Taneja, 1999; Teramoto et al. 2001), which is frequently lost in various tumors (Hung et al. 1995; Mertens et al. 1997; Johansson et al. 1997). It is therefore possible that loss of Stra13 expression in lung and colon tumors is an early event in tumorigenesis, and the subset of tumors that show increased

levels of Stra13 may reflect its reactivation at late stages, secondary to signals such as hypoxia or TGFβ. In this regard, it is interesting to note that in many late stage breast tumors, Stra13 is expressed in the cytoplasm (Chakrabarti et al. 2004; Ivanova et al. 2005), which may be another mechanism of inactivating its function as a transcriptional repressor. At this point it is unclear whether cytoplasmic Stra13 has any specific cellular function.

Clearly, our current understanding of the function of bHLH-O factors in the genesis of cancer is limited, and it is unclear whether their altered expression is a cause or consequence of deregulated signaling, or other genetic changes. Many bHLH-O factors are targets or regulators of the Notch signaling pathway, which plays a critical role in tumor formation. Many of them also are responsive to hypoxia, and may regulate cellular proliferation and differentiation under hypoxic conditions. The findings that Hes1, Hey1 and Stra13 modulate p53 activity suggest that their functions may converge in neoplastic transformation in some contexts. However, these findings need to be substantiated by further analyses that directly address the role of these genes in oncogenesis. Examination of bHLH-O null mutants for altered tumorigenic potential should help clarify these issues. Furthermore, analysis of the molecular mechanisms by which the expression of these genes is deregulated in various cancers is essential, as are investigations into the relevance of a switch between the nuclear and cytoplasmic compartments. Future studies in many of these areas will help elucidate the functions of bHLH-O factors in tumorigenesis which will likely lead to the identification of novel prognostic and therapeutic targets.

Acknowledgements

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