Chapter 12
Role of Small Heat Shock Protein HspB5 in Cancer

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Abstract HspB5, also called αB-crystallin, is a ubiquitous small heat shock protein (sHSP) that is strongly induced by a variety of stresses, but that also functions constitutively in multiple cell types. Extensive research has demonstrated that HspB5 acts as an ATP-independent molecular chaperone by binding unfolding proteins and protecting cells from damage due to irreversible protein aggregation. As a result of its importance in protein homeostasis HspB5 is of significant interest to many areas of cell biology, including the development of cancer. However, the molecular understanding of HspB5's role in cancer is only beginning to emerge. In this chapter an overview is given of data that provide insight into the oncogenic role of HspB5 in human cancer.

Keywords HspB5 • Crystallin • Cancer • Expression • Cell survival

12.1 Introduction

HspB5 belongs to the family of stress proteins, grouped together based on stretches of sequence homology, of which the most conserved part is the α-crystallin domain (Kappe et al. 2010). The human family of sHSPs contains ten members (HspB1–HspB10), of which HspB5 is stress inducible and therefore belongs to the family of heat shock proteins (Lanneau et al. 2010). In general, sHSPs form large oligomers with dynamic quaternary structures, which diversification likely reflects an adaptation to tolerate all kinds of stresses and to cope with different client proteins (Hochberg and Benesch 2014). The abundance of the different human sHSPs varies enormously, depending on growth conditions, developmental states, differentiation and oncogenic status of the cell (Arrigo 2012). HspB5 is remarkably highly expressed in the eye lens, where it forms mixed complexes with HspB4 (αA-crystallin), and in

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heart and skeletal muscle, where it forms mixed complexes with HspB1 (Hsp27), HspB6 (Hsp20) and HspB8. HspB5 displays ATP-independent chaperone activity by suppressing the aggregation of a large set of client proteins, thereby contributing to the balance between cell survival and cell death (Boelens 2014; Acunzo et al. 2012). Its chaperone function is regulated by phosphorylation at three different phosphorylation sites, Ser19, Ser45, and Ser59, all located within the N-terminal domain. Phosphorylation can be stimulated by various kinds of stresses, such as hyperthermia, oxidative stress or the exposure to cytotoxic drugs (Kato et al. 1998; Ito et al. 1997). Phosphorylation of HspB5 has been shown to correlate with a reduction in average oligomer size and enhanced interaction with client proteins (Peschek et al. 2013). By binding different client proteins and thereby modifying the stability and/or activity of these proteins, HspB5 is able to affect all kinds of cellular activities (Arrigo 2013). Besides HspB5 other sHSPs have been shown to play crucial roles in human cancer pathologies by interacting with pro-oncogenic client proteins, recently reviewed by Arrigo and Gilbert (Arrigo and Gibert 2014). Here, I will focus on the expression of HspB5 in cancer and how this expression may promote cancer formation.

12.2 HspB5 Expression in Cancers

One of the first reports describing the association of HspB5 expression and cancer was a study of human brain tumors (Aoyama et al. 1993). Elevated levels of HspB5 were demonstrated to occur in the more aggressive stages of gliomas. Especially the more infiltrative glioblastoma cells showed higher levels of HspB5 (Goplen et al. 2010). In breast cancer patients HspB5 expression was closely associated with advanced tumor grade progression, lymph vesicular invasion, and mortality, a phenomenon that was particularly observed in triple-negative breast tumors (Chelouche-Lev et al. 2004; Kim et al. 2011; Moyano et al. 2006). Furthermore, breast cancer patients with brain metastasis formation show higher levels of HspB5 than breast cancer patients without brain metastasis (Malin et al. 2014). Overexpression of HspB5 can transform immortalized human mammary epithelial cells that can develop invasive mammary carcinomas in nude mice with basal-like breast tumor features (Moyano et al. 2006). Consequently HspB5 can be considered as a biomarker in the diagnosis of breast cancers, especially in those with advanced grade (Ivanov et al. 2008; Sitterding et al. 2008; Tsang et al. 2012). Similarly, high levels of HspB5 are associated with low survival rate in hepatocellular carcinoma (Huang et al. 2013; Tang et al. 2009), ovarian carcinoma (Volkmann et al. 2013), clear cell renal cell carcinoma (Ho et al. 2013) and head and neck squamous cell carcinoma (van de Schootbrugge et al. 2013). In non-small-cell lung cancer only the nuclear and not the cytoplasmic localized HspB5 correlated with poor survival (Cherneva et al. 2010).

HspB5 does not correlate with poor prognosis in all cancer types analyzed and negative effects of HspB5 on cancer progression have been described as well.

In nasopharyngeal carcinomas down regulation of HspB5 has been associated with tumor progression (Lung et al. 2008). Overexpression of HspB5 in cell lines of this type of cancer suppressed progression-associated phenotypes such as loss of cell adhesion, invasion and interaction with the tumor microenvironment (Huang et al. 2012). Similarly, patients with cutaneous squamous cell carcinoma having perineural invasion, which is associated with a poor prognosis, showed lower HspB5 expression compared to patients without perineural invasion (Solares et al. 2010). Since HspB5 expression can have opposite effects in different cancer types, it is likely that the molecular mechanisms by which HspB5 influences cancer development will be diverse.

12.3 Transcription Factors Involved in the Expression of HspB5

The molecular mechanisms that regulate HspB5 transcription in normal, stress or pathological conditions are extremely complex and rely on the combinatory effects of many transcription factors (Fig. 12.1) (de Thonel et al. 2012). Multiple pathways coordinate the spatial and temporal expression of the *HspB5* gene during lens development through the action of master genes, like members of the Maf protein family and Pax6. The MyoD family proteins (muscle regulatory-binding site), regulate HspB5 expression in muscle cells by binding the E-box (Gopal-Srivastava and Piatigorsky 1994), but additional elements are required, since one E-box is not sufficient for full transcriptional activity (Gopal-Srivastava et al. 2000). The induction of HspB5 transcription by various stresses proceeds via heat shock transcription factor (HSF)-dependent mechanisms, by which also concomitant induction of other stress proteins is observed.

The mechanisms underlying deregulated HspB5 expression in cancers are poorly understood. The overexpression of HspB5 in some cancers has been reported to be, at least partly, independent of HSF regulation (de Thonel et al. 2012). In basal-like breast cancer, in which the expression of HspB5 is correlated with a poor prognosis

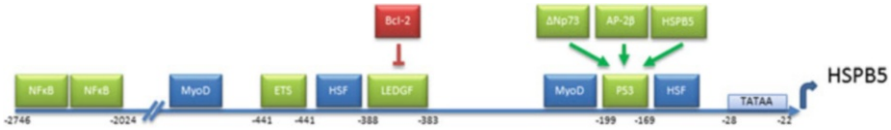

Fig. 12.1 Transcription factors able to interact with the promoter region of *HspB5*. The transcription factors known to influence the expression of HspB5 in tumors are indicated in *green*. Transcription factors that regulate the expression of HspB5 in normal tissues are indicated in *blue*. The factors above the transcription factors indirectly stimulate (*green*) or inhibit (*red*) HspB5 expression. The numbers indicate the location of transcription binding sites relative to the transcription start site. TATAA indicates the TATA box. The *HspB2* gene located upstream of *HspB5* is not shown

and may contribute to the aggressive phenotype, the proto-oncogene *Ets1*, a member of the ETS transcription factor family, appears to be involved in the upregulation of HspB5 expression. Silencing *Ets1* reduced both *HspB5* promoter activity and protein levels. Furthermore, Ets1 recognized a palindromic element corresponding to the ETS-DNA consensus motif in the human *HspB5* promoter, which is highly conserved among mammalian species (Bosman et al. 2010).

A substantial fraction of tumor cells may experience cycling hypoxia, characterized by transient episodes of hypoxia and reoxygenation (Koritzinsky and Wouters 2013). Such tumor cells are under a unique burden of stress, mediated by excessive production of reactive oxygen species (ROS). ROS has been shown to induce the expression of HspB5 (van de Schootbrugge et al. 2014). One of the transcription factors stimulated by ROS is the lens epithelial derived growth factor (LEDGF), which is able to bind to the stress response element of the *HspB5* gene, thereby stimulating its transcription (Shin et al. 2008). Remarkably, the anti-apoptotic family member Bcl-2, a pro-oncoprotein able to protect cells under various stress conditions, has been shown to reduce the activity of LEDGF in rabbit lens epithelial cells, thereby down-regulating the expression of HspB5 (Mao et al. 2001). The Bcl-2-induced down-regulation of HspB5 was shown to be responsible for a decreased protection against oxidative stress. However, the expression of another Bcl-2 family member, Bcl-2-Like 12 (Bcl2L12) showed a significant upregulation of HspB5 on mRNA and protein levels in cortical astrocytes (Stegh et al. 2008). This protein is only distantly related to the canonical Bcl-2 family members and part of its anti-apoptotic activity might be a consequence of its ability to increase the expression of HspB5. Whether Bcl2L12 enhances HspB5 expression by affecting the activity of LEDGF is not known.

The tumor suppressor protein p53 is a transcription factor that trans-activates various genes in response to DNA-damaging stress. By a search for new target genes using a cDNA microarray system, *HspB5* was identified as a gene that is trans-activated by p53 (Watanabe et al. 2009). Furthermore, a conserved response element for p53 was found in the *HspB5* promoter (Evans et al. 2010). Induction of *HspB5* by genotoxic stress could be inhibited by siRNAs targeting p53 and ectopic expression of p53-induced HspB5 mRNA and protein expression, showing that p53 is responsible for HspB5 induction in response to DNA-damaging stress. However, p53 does not seem to be sufficient for HspB5 induction. The N-terminal truncated form of p73, a p53-related transcription factor lacking a transactivation domain, the expression of which is also induced by p53, was needed as well (Evans et al. 2010). In addition, the transcription factor AP-2β was shown to upregulate the transcription of the *HspB5* gene by stimulating the activity of p53 (Hu et al. 2012). Notably, HspB5 is able to directly interact with the p53 protein and can affect the functioning of p53 (Watanabe et al. 2009; Jin et al. 2009; Liu et al. 2007). HspB5 has been shown to contribute to p53 stability, which is probably related to the chaperone activity of HspB5 (Peschek et al. 2013; Jin et al. 2009). Overexpression of HspB5 increased p53 protein and, in contrast, repression of HspB5 decreased p53 protein (Watanabe et al. 2009). These findings point to a link between HspB5 and genotoxic stress that is regulated by cooperative actions of p53.

Another transcription factor that plays a role in the oncogenic activity of HspB5 is NFκB, a key factor that regulates expression of a large number of genes involved in apoptosis, proliferation, tumorigenesis and metastasis. Loss-of-function mutations of the tumor suppressors tuberous sclerosis complex 1 or 2 (*TSC1/2*) have been shown to cause a rare multi-system genetic disease that causes benign tumors to grow in the brain and on other vital organs such as the kidneys, heart, eyes, lungs, and skin. Patients with this disease showed an increased expression of HspB5 in the afflicted organs (Wang et al. 2013). One of the transcription factors that was found to be activated in cells with inactivated *TSC1* or *TSC2* genes is NFκB. By computational analysis, two NFκB-binding sites were found within the promoter region on *HspB5* gene. Mutations in these putative NFκB-binding sites markedly attenuated the *HspB5* promoter activity in *TSC2*$^{-/-}$ MEFs, indicating that these sites were responsible for the upregulation of HspB5.

The studies described above have brought many insights on the action of control elements and transcription factors that operate on *HspB5* gene transcription, however further elucidation of the mechanisms of *HspB5* gene transcription is still needed to help to decipher the role of HspB5 in cancer development.

12.4 Involvement of HspB5 in Cell Survival

Several chemotherapeutic agents act through inducing cell death of neoplastic cells, which means that protective effects are disadvantageous for the outcome of the treatment. Accumulating evidence suggests that HspB5 can prevent cell death triggered by various stimuli, including hyperthermia, oxidative stress or the exposure to cytotoxic drugs (Acunzo et al. 2012). The cellular protection might be due to the prevention of intracellular damage induced by the stressors. HspB5 may prevent the aggregation or facilitate renaturation of proteins and protect cytoskeletal elements against ischemic injury or depolymerizing agents (Xi et al. 2006; Singh et al. 2007). Furthermore, HspB5 may prevent cell death by interacting with key pro-apoptotic proteins, thereby blocking apoptosis at different levels (Fig. 12.2). In human retinal pigment epithelial cell, HspB5 inhibits apoptosis induced by staurosporine by interacting with the pro-apoptotic proteins Bax and Bcl-Xs and inhibiting their translocation from the cytosol to the mitochondria (Mao et al. 2004). Also cell death induced by hydrogen peroxide and staurosporin might be inhibited this way (Mao et al. 2001; Hamann et al. 2013). Remarkably, phosphorylation at Ser59 may specifically stimulate the interaction with the anti-apoptotic protein Bcl-2 in MCF cells, thereby reducing the anti-apoptotic activity of this protein and making the cells more sensitive for the chemotherapeutic agent vinblastine (Launay et al. 2010). This effect is opposed to the phosphorylation-induced anti-apoptotic activity observed in cardiac myocytes and astrocyte cell lines (Morrison et al. 2003; Li and Reiser 2011). The pro- and anti-apoptotic activities of HspB5 indicate that the regulation of the protective mechanisms of phosphorylated HspB5 is complex and may depend on cellular background and stress conditions.

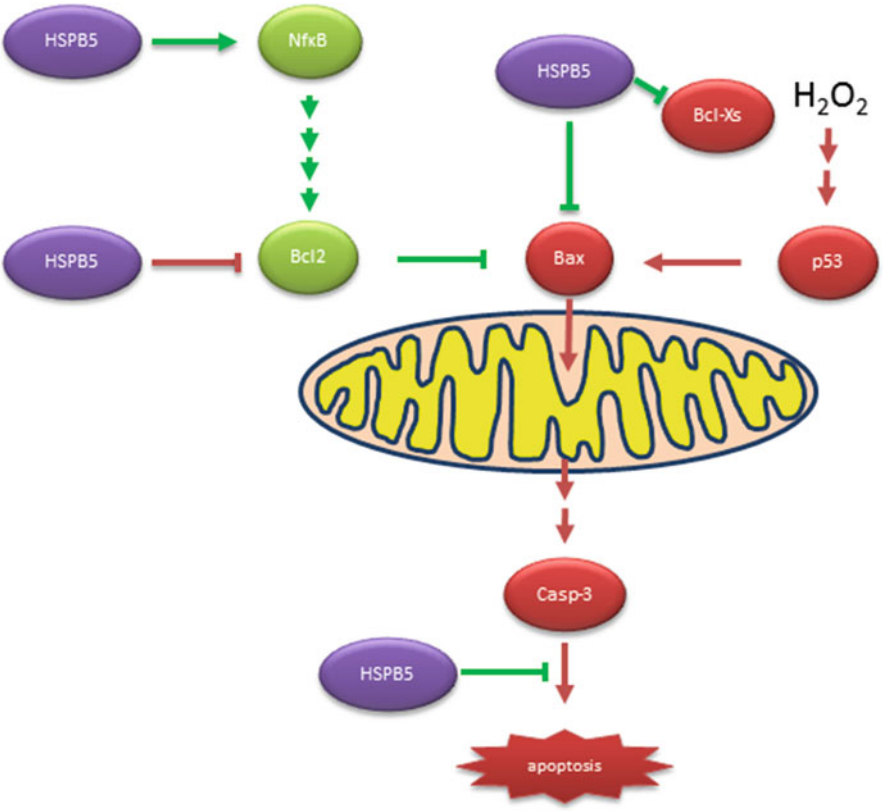

Fig. 12.2 The involvement of HspB5 in regulation of apoptosis. Schematic illustration showing the apoptotic and survival pathways controlled by HspB5. The pro-apoptotic signals are indicated in *red* and the anti-apoptotic signals in *green*

Downstream in the apoptotic pathway, HspB5 directly binds to partially processed caspase-3, thereby inhibiting the pro-apoptotic function of this protein (Kamradt et al. 2001). This interaction desensitizes cancer cells to chemotherapeutic treatments. Moreover, HspB5 may inhibit caspase-3 activation in cells that are primed for apoptosis by inactivation of the retinoblastoma tumor suppressor protein Rb. Rb plays an integral role in G1-S checkpoint control and consequently is a frequent target for inactivation in cancer. Prevention of Rb-induced apoptosis in cells may promote oncogenic transformation and thus in this context HspB5 may actually have an oncogenic activity (Petrovic et al. 2013).

In the mouse skeletal myoblast C2C12 cells HspB5 was observed to prevent apoptosis induced by hydrogen peroxide treatment (Liu et al. 2007). As described above, HspB5 is able to interact with p53 (Liu et al. 2007; Jin et al. 2009; Watanabe et al. 2009). Activated p53 can induce apoptosis by transactivating the expression of multiple pro-apoptotic genes, but also participates in apoptosis by acting directly at

the mitochondrial membrane. At this location, p53 physically interacts with anti-apoptotic Bcl-2 family proteins, thereby stimulating the release of cytochrome c from the mitochondria, subsequently leading to apoptosis (Galluzzi et al. 2011). By interacting with p53, HspB5 may inhibit the oxidative-stress induced translocation of p53 from cytoplasm to mitochondria and in this way prevent induction of apoptosis (Liu et al. 2007).

In another study using C2C12 cells, treatment with TNF-α induced apoptosis, but also led to association of HspB5 with IKKβ. This interaction facilitated the degradation of phosphorylated IκBα, a prime step in NFκB activation. The ability to activate NFκB was dependent on the phosphorylation status of HspB5. This process may be stimulated by a feed-forward loop, because activated NFκB can enhance the expression of HspB5 (Wang et al. 2013). The HspB5-dependent NFκB activation protected myoblasts from TNF-α induced cytotoxicity by enhancing the expression of the anti-apoptotic protein Bcl-2.

Taken together, these studies show that HspB5 has several mechanisms to exert its anti-apoptotic activity, allowing protecting cancer cells in different ways, likely depending on cell type and stress conditions.

12.5 Involvement of HspB5 in Cell Cycle Progression

Cell cycle progression is determined by the balance of positive regulators, cyclin-dependent-protein kinases, relative to negative regulators, cyclin-dependent kinase inhibitors. The family of D-type cyclins (D1, D2, and D3) are the regulatory subunits that control the G1/S-phase transition. Of the three D-type cyclins, cyclin D1 is most frequently overexpressed in human cancer (Pestell 2013). Accumulation of cyclin D1 is tightly regulated through various mechanisms including transcription, protein localization and ubiquitin-dependent proteolysis. The inhibition of ubiquitin-dependent proteolysis of cyclin D1 is thought to be a primary mechanism of cyclin D1 overexpression in human tumors. For the proteolysis, cyclin D1 phosphorylation at Thr-286 by GSK3β is required. One of the ubiquitin ligases involved in the degradation of cyclin D1 is the Skp1-Cul1-F box (SCF) E3 ubiquitin ligase, which contains the F-box protein Fbx4 and HspB5 (Lin et al. 2006). HspB5 has been shown to directly interact with Fbx4, which was stimulated by mimicking the phosphorylation of HspB5 (den Engelsman et al. 2003). Both Fbx4 and HspB5 are responsible for SCF substrate specificity. Knockdown of either Fbx4 or HspB5 in cells reduced cyclin D1 ubiquitination and reduced cyclin D1 proteolytic turnover resulting in accelerated cell cycle progression (Lin et al. 2006). To assess the potential contribution of HspB5 and Fbx4 to cyclin D1 overexpression in human cancer, the expression of HspB5 and Fbx4 have been assessed in primary esophageal cancers where cyclin D1 is known to be overexpressed in nearly 40 % of the cases (Lin et al. 2006). Strikingly, approximately 20 % of esophageal carcinomas exhibited loss of either HspB5 or Fbx4 as determined by immunohistochemistry. Furthermore, in 15 % of the primary esophageal tumors inactivating mutations in the *Fbx4* gene

were observed (Barbash and Diehl 2008). In melanoma cells the serine/threonine kinase B-RAF-MEK signaling is often hyperactivated to stimulate cell proliferation. This aberrant signaling has been shown to reduce the expression of HspB5 leading to a partial stabilization of cyclin D1 (Hu and Aplin 2010). These results show that inactivation of $SCF^{Fbx4/HspB5}$E3 ligase activity is not only restricted to esophageal cancers, a tumor where cyclin D1 is thought to be a driving force, but may also affect cell growth in other types of cancers.

12.6 Involvement of HspB5 in Modulating Tumor Neovascularization

HspB5 interacts with several important human growth factors, including vascular endothelial growth factor (VEGF) (Ghosh et al. 2007). The interaction of HspB5 with VEGF is dependent on the same region HspB5 that is involved in the binding of misfolded proteins, indicating that HspB5 may chaperone and stabilize misfolded VEGF. VEGF is an endothelial cell-specific mitogen that promotes vascular angiogenesis and is induced by hypoxic stress, a known stimulator of angiogenesis. As solid tumors grow, the inner mass become hypoxic, which will stimulate the production of VEGF to enhance neovascularization. In HspB5-deficient mice the tumor vasculature has been found to display high levels of apoptotic cells and decreased vessel formation, indicating that HspB5 is an angiogenic modulator (Dimberg et al. 2008). Furthermore, in HspB5 knock-out mice the expression of VEGF was found to remain low during retinal angiogenesis (Kase et al. 2010). A possible explanation for these observations is that increased VEGF expression results in both properly folded and misfolded VEGF in the endoplasmic reticulum (ER). The misfolded VEGF cannot be transported to the Golgi apparatus for secretion and will be exported to the cytoplasm for degradation by the ubiquitin-proteasome system. However, when HspB5 is present, it may bind and stimulate the refolding of misfolded VEGF, thereby enhancing the production of properly folded VEGF. Part of the rescued VEGF may not to be secreted, but used for the intracrine VEGF signaling, a process crucial for the vascular homeostasis (Ruan et al. 2011).

12.7 Involvement of HspB5 in Metastasis Formation

Invasion of cancer cells in other tissues is one of the main causes of death of cancer patients, but the molecular and cellular mechanisms underlying tumor metastasis are still not well understood. The spreading of cancer cells through the blood and lymph systems is a multistep process (Geiger and Peeper 2009). An important step in cell spreading is Epithelial-to-Mesenchymal Transition (EMT), a process that is characterized by the loss of polarity, the loss of epithelial markers, reorganization of

the cytoskeleton and the acquisition of mesenchymal markers (Lamouille et al. 2014). EMT can be seen as the acquisition of extreme plasticity by epithelial cells. In cancer, EMT enables malignant cells to acquire a migratory phenotype and is thus associated with tumor invasiveness. In hepatocellular carcinoma (HCC) HspB5 expression has been shown to induce EMT through activation of the extracellular-regulated protein kinase (ERK) cascade (Huang et al. 2013). The up-regulation of ERK1/2 activity in this cell type may occur via the interaction of HspB5 with 14-3-3ζ protein. Both overexpression of HspB5 and 14-3-3ζ correlated with poor survival outcome of HCC, indicating that the HspB5-14-3-3ζ complex promotes HCC progression.

Another important step in cell spreading is that cells have to detach from their environment. Normally, detached cells undergo anoikis, a type of cell death induced by inappropriate loss of cell adhesion. Hence, anoikis suppression is required for tumor cells to be able to metastasize to distant sites (Tan et al. 2013). HspB5 may confer protection to cells by its protective activity (see above) and this way may help to avoid anoikis.

A next step in cell spreading concerns the invasion of neighboring tissue. To this end tumor cells must acquire the ability to migrate through the basal lamina that separates tumor mass from stroma, which often occurs at sites where the lamina is incomplete. Many proteins are involved in the process of cell migration and in metastasizing tumors mutations in genes coding for proteins that mediate the attachment between cells and their environment have been found (Guo and Giancotti 2004). Overexpression of HspB5 in human mammary epithelial cells caused loss of polarity and increased migration and invasion (Moyano et al. 2006). Furthermore, these cells formed invasive carcinomas in nude mice. The oncogenic changes were dependent on HspB5 overexpression, since knockdown of HspB5 expression suppressed the abnormal phenotype. The transformation appeared to be dependent on the phosphorylation state of HspB5, as a pseudophosphorylation mutant of HspB5, which mimics an irreversible form of stress-induced phosphorylation, did not confer neoplastic changes.

Cell motility is tightly coupled to the biochemical and mechanical properties of the actin cytoskeleton. Actin filaments are semi-flexible polymers, which in conjunction with the molecular motor myosin are able to exert or resist against force in a cellular environment. To modulate the mechanical properties, actin filaments can organize into a variety of architectures generating a diversity of cellular organizations including branched or crosslinked networks in lamellipodia, parallel bundles in filopodia, and antiparallel structures in contractile fibers. In migrating lens epithelial cells, HspB5 was found to localize to the lamellipodia (Maddala and Rao 2005). HspB5 exhibited a clear co-localization with the actin meshwork and regulatory proteins involved in actin dynamics and cell adhesion, suggesting a role for HspB5 in actin dynamics during cell migration. Localization of HspB5 to the lamellipodia appeared to depend on Ser59 phosphorylation, since inhibition of the p38 MAP kinase diminished the accumulation in lamellipodia.

Another important step in cell spreading entails intravasation into pre-existing and newly formed blood and lymph vessels. Normally, angiogenesis is rare and occurs mainly during wound healing and the female reproductive cycle. The growth

of new vessels is controlled by a balance of angiogenic activators and angiogenic inhibitors (Bergers and Benjamin 2003). During the "angiogenic switch", upon which a tumor activates vascularisation, this balance is disturbed. A major player in angiogenesis is vascular endothelial growth factor (VEGF) (Roskoski 2007). Increased VEGF secretion is often correlated with metastasis formation (Hu et al. 2009) and worse outcome for the patient (Bremnes et al. 2006). HspB5 is able to enhance the production of VEGF (see above) and this may affect the intravasation of tumor cells, thereby promoting metastasis formation (van de Schootbrugge et al. 2013).

12.8 Conclusion and Future Perspective

The combined literature data described here indicates that HspB5 is involved in several vital cellular processes, finely regulating the balance between life and death of the cells by protecting cells under unfavorable conditions. Until recently, it was believed that HspB5, like most other sHsps, is a specialized chaperone whose activity was to attenuate the damages to cellular proteins by mediating their storage until they could be refolded. The recent findings show that HspB5 is involved in an impressive number of cancer-related processes. The multiple cellular functions of HspB5 might be a result of its fundamental property to interact with client proteins to control their folding. If the folding of client proteins is not adapted to cellular conditions, HspB5 can participate in their refolding, degradation or modulation of their enzyme activity. Hence, the proteins that are controlled by HspB5 could have crucial functions in the development of cancer. However, a major drawback to understand the functions of HspB5 in cancer is the fact that the cellular conditions that allow HspB5 to interact with specific client proteins are still not well characterized. Future work should be directed toward analysis and definition of HspB5 interactions with client proteins involved in crucial cellular functions, such as proteins of signaling pathways. These studies might indicate certain client proteins as useful targets for therapeutic manipulation, for example to induce cancer cell death or to sensitize them for current therapeutic approaches.

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