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MINI REVIEW

Extracellular small heat shock proteins: exosomal biogenesis and function

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Abstract Small heat shock proteins (sHsps) belong to the family of heat shock proteins (Hsps): some are induced in response to multiple stressful events to protect the cells while others are constitutively expressed. Until now, it was believed that Hsps, including sHsps, are present inside the cells and perform intracellular functions. Interestingly, several groups recently reported the extracellular presence of Hsps, and sHsps have also been detected in sera/cerebrospinal fluids in various pathological conditions. Secretion into the extracellular milieu during many pathological conditions suggests additional or novel functions of sHsps in addition to their intracellular properties. Extracellular sHsps are implicated in cell-cell communication, activation of immune cells, and promoting anti-inflammatory and anti-platelet responses. Interestingly, exogenous administration of sHsps showed therapeutic effects in multiple disease models implying that extracellular sHsps are beneficial in pathological conditions. sHsps do not possess signal sequence and, hence, are not exported through the classical Endoplasmic reticulum-Golgi complex (ER-Golgi) secretory pathway. Further, export of sHsps is not inhibited by ER-Golgi secretory pathway inhibitors implying the involvement of a nonclassical secretory pathway in sHsp export. In lieu, lysoendosomal and exosomal pathways have been proposed for the export of sHsps. Heat shock protein 27 (Hsp27), αB-crystallin (αBC), and Hsp20 are shown to be exported by exosomes. Exosomes packaged with sHsps have beneficial effects in in vivo disease models. However, secretion mechanisms and therapeutic use of sHsps have not been elucidated in detail. Therefore, this review aimed at highlighting the current understanding of sHsps (Hsp27, αBC, and Hsp20) in the extracellular medium.

Keywords Small heat shock proteins · Exosomes · Plasma · Export

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Introduction

Small heat shock proteins (sHsps) constitute a structurally divergent group of heat shock proteins (Hsps) characterized by a conserved sequence of 80–100 amino acid residues termed the α-crystallin domain (Basha et al. 2012; Garrido et al. 2012; Gusev et al. 2002; Sun and MacRae 2005a). The α-crystallin domain is located towards a highly flexible and variable C-terminal extension and is usually preceded by a poorly conserved N-terminal region. The molecular mass of sHsp subunits ranges from 13 to 43 kDa. The sHsps occur as homo- or heteromeric complexes, comprising 2–40 subunits. The subunits assemble into large globular complexes up to 1 mDa and are often polydispersed and dynamic with readily exchangeable subunits. The functional significance of sHsps is attributed to their ability to prevent in vitro aggregation of unfolded proteins through hydrophobic interactions (Horwitz 1992; Horwitz et al. 1998; Reddy et al. 2006). Subsequently, sHsps transfer the bound proteins to ATP-dependent chaperones such as Hsp70. Failure in appropriate protein folding will eventually result in proteasomal degradation of the unfolded

proteins (Haslbeck et al. 2005; Vos et al. 2008). Thus, molecular chaperone function of sHsps may be limited to storing (holding of aggregation-prone proteins as folding-competent intermediates and conferring enhanced stress resistance to cells by preventing aggregation of proteins). There are ten members in the sHsp family: Hsp27/Hsp25 of rodents (HSPB1), MKBP (myotonic dystrophy protein kinase; HSPB2), HSPB3, αA-crystallin (αAC; HSPB4), αB-crystallin (αBC; HSPB5), Hsp20 (HSPB6), cvHsp (HSPB7), Hsp22/H11/H2IG1 (HSPB8), HSPB9, and ODF (sperm outer dense fiber protein; HSPB10). While some sHsps are tissue-specific (MKBP, HSPB3, αAC, cvHsp, HSPB9, and ODF), the rest are ubiquitous (Hsp27, αBC, Hsp20, and Hsp22) (Garrido et al. 2012) Small Hsps can also modulate intracellular redox status suggesting that sHsps are not only involved in the cellular defense mechanisms against protein aggregation but also participate in essential physiological processes including cytoskeletal reorganization and apoptosis. Traditionally, Hsps including sHsps are considered to possess intracellular chaperone activity and confer protection from stress-induced cell death (Arrigo 2005; Sun and MacRae 2005b). However, the emerging evidence envisioned that there is the existence of extracellular Hsps that might contribute toward the alleviation of pathological conditions. Extracellular Hsps are first reported by Tytell et al. (1986) in giant squid axon functioning as a glia-axon transfer protein. Similarly, Hsp70 has been reported to be present in the extracellular milieu independent of ER-Golgi-mediated protein trafficking (Hightower and Guidon 1989). At the molecular level, Hsps lack classical N-terminus signal sequence required for conventional protein secretory pathway (Mambula et al. 2007) similar to that of immune-modulators such as IL-1α, IL-1β, IL-16, and IL-18 (Prudovsky et al. 2003). Extracellular release of Hsps has been reported during necrotic cell death (Basu et al. 2000). However, Hightower and Guidon (1989) noticed that the presence of lysine amino acid analogues/aminoethyl cysteine prevented the release of Hsp70 into extracellular milieu, suggesting that only a structurally intact protein is secreted out from the cells. In support of these results, subsequent studies demonstrated the extracellular presence of large (higher molecular weight) Hsps including Hsp60 and Hsp70 (Luo et al. 2008; Mambula and Calderwood 2006; Merendino et al. 2010; Vega et al. 2008).

Existing literature shed light on the unconventional release of Hsps and their possible extracellular functions in disease conditions including cancer (Calderwood et al. 2007; De Maio 2011; De Maio and Vazquez 2013) (Santos et al. 2017). Adding to this, the presence of sHsps (Hsp27, αBC, and Hsp20) is reported to be present in an extracellular milieu with undefined functions. Intriguingly, these studies also opened up new exploratory research avenues with the possibility of identifying novel functions performed by these secreted sHsps. Therefore, the current review tries to summarize the possible export mechanisms, functions, and therapeutic effects of extracellular sHsps with special emphasis on Hsp27, αBC, and Hsp20, which are known to be well characterized to date.

Hsp27

Hsp27, a member of the sHsp family, responds to stress and acts as a chaperone to facilitate appropriate folding of proteins (Rogalla et al. 1999). Hsp27 acts as an antioxidant and reduces reactive oxygen species (Mehlen et al. 1997) and also protects cells from programmed cell death by interacting with mediators of mitochondria (Havasi et al. 2008). Contrasting observations on the functional role of Hsp27 suggests that Hsp27 can positively and negatively regulate actin polymerization during cellular stress conditions (Benndorf et al. 1994; Huot et al. 1996; Miron et al. 1991).

Extracellular release of Hsp27 from tumor cells with unknown functions has been reported in plasma (4-fold increase) and interstitial fluid (2500-fold increase) in breast cancer patients (Banerjee et al. 2011; Ciocca et al. 1984; Fanelli et al. 1998; Feng et al. 2005; Thuringer et al. 2015). It is reported that secretion of Hsp27 is involved in differentiation of monocytes to macrophages. The secreted protein might be involved in immunological signaling by interacting with plasma membrane proteins thereby promoting tumor growth. Therefore, knock-down or knock-out approach or inhibition of secretion of Hsp27 in tumor cell lines or animal models might be an effective strategy in reducing the tumor growth. The secretion of Hsp27 in the extracellular medium is increased under stressful/pathological insults with different fold changes (Table 1**)**. Elevated levels of Hsp27 are found in the plasma of type 1 diabetic patients in the European population. Interestingly, high levels of Hsp27 are correlated with distal symmetrical polyneuropathy in diabetes and have been reported to be a marker for diabetic neuropathy (Gruden et al. 2008). Further, studies from our laboratory reported altered expression and phosphorylation of intracellular sHsps in target tissues of diabetic rat model (Reddy et al. 2015; Reddy et al. 2014; Reddy et al. 2013; Reddy and Reddy 2015; Reddy and Reddy 2016). In addition, we recently reported elevated levels of Hsp27 in the plasma of nephropathy patients in type 2 diabetes, and its levels were positively correlated with serum creatinine thereby suggesting that Hsp27 might serve as a marker for diabetic nephropathy (Jakhotia et al. 2017). Also, Hsp27 protein and its antibodies are elevated in insulin resistance in glucose-intolerant patients with a history of cardiovascular diseases (Burut et al. 2010). Further, the release of Hsp27 from platelets is accompanied by phosphorylation in type 2 diabetic patients and is correlated with accelerated aggregation of platelets (Tokuda et al. 2015). Taken together, these studies strongly suggest that Hsp27 is released into the serum in diabetes as perhaps a response to stressful events in

Table 1 Secretion of sHsps into the extracellular medium in pathological conditions

sHsp	Pathology	Fold change	Effect	References
Hsp27	Breast cancer	4-fold in serum and 2500-fold increase in tumor interstitial fluid.	Induced differentiation of monocytes to macrophages, anergy in T-cell, and neovascularization	Banerjee et al. (2011)
	Hepatocellular carcinoma (HCC)	Increased levels of Hsp27 found in HCC patients analyzed by 2-DE based serum proteome	Developed as a biomarker for HCC	Feng et al. (2005)
	Colon cancer	4-fold increase (secreted into culture medium from primary tumor derived SW480 cells)	Increased the endothelial gap junction coupling by promoting the phosphorylation of connexin 43	Thuringer et al. (2015)
	Diabetic neuropathy	2-fold increase	Novel biomarker of diabetic neuropathy	Gruden et al. (2008)
	Insulin resistance	Increased antigen and antibodies	Related to glucose intolerance	Burut et al. (2010)
	Chronic obstructive pulmonary disease (COPD)	1.8-fold increase (COPD with severe disease)	Diagnostic marker for immune activation and tissue destruction in COPD	Hacker et al. (2009)
	Multiple sclerosis (MS)	4.8-fold increase in the attack phase	Guiding the accurate detection of an attack in MS	Ce et al. (2011)
	Gastric adenocarcinoma (GA)	1.7-fold increase	Marker for detection of GA	Huang et al. (2010)
	Thymic epithelial tumors (TET)	1.5-fold increase	Can be developed as diagnostic marker for TET	Janik et al. (2016)
	Myasthenia gravis	2.3-fold increase	Can be developed as diagnostic marker for TET	Janik et al. (2016)
	Acute coronary syndromes	2.3-fold increase	Represent the systemic inflammation and oxidative stress	Park et al. (2006)
	Atherosclerosis	20-fold decrease	Could be developed as potential index of atherosclerosis	Martin-Ventura et al. (2004)
	Ovarian cancer (OC)	The secretion of Hsp27 increased after overexpression (2.38-fold) and heat shock	Non-invasive biomarker for OC	Stope et al. (2017)
αBC	Obesity	Increased in Conditioned media of cultured primary human adipocytes derived from obese patients	Could be developed as a novel adipokine	Lehr et al. (2012)
	Renal cell carcinoma (RCC)	Increased	Biomarker of RCC	Holcakova et al. (2008)
	Multiple sclerosis	Increased auto-antibodies	Inflammatory response	Celet et al. (2000); van Noort et al. (2006)
	Multiple sclerosis	5-fold increase	Anti-inflammatory response	Rothbard et al. (2012)
	Experimental auto-immune myelitis	3-fold increase	Anti-inflammatory response	Rothbard et al. (2012)
	Neuro-Behçet's disease	Increased auto-antibodies	Inflammatory response	Celet et al. (2000)
	Guillain–Barré syndrome	Increased auto-antibodies	Inflammatory response	Celet et al. (2000); Hegen et al. (2010)
	Chronic inflammatory demyelinating polyneuropathy			Hegen et al. (2010)
	Brain stroke in mice	2.7-fold at 12 h, 1.7-fold at 2 days, 0.5-fold compared to naïve mice	Anti-inflammatory response	Arac et al. (2011)
	Ischemic brain stroke in humans	1.7-fold at < 4 h, 1.2-fold at 24 h, 1.4 at 48 h in younger patients compared to 0.3-fold in healthy ones	Anti-inflammatory response	Arac et al. (2011)
Hsp20	Cardiomyopathy	126-fold increase	Regulator of platelet function	Kozawa et al. (2002)
	Acute dissecting aneurysm	11-fold increase	Acts as anti-platelet regulator	Niwa et al. (2000)
	Myocardial ischemia/reperfusion (I/R)	3.4-fold increase	Acts as a cardiokine which mediates angiogenesis by directly interacting with VEGFR2	Zhang et al. (2012)

hyperglycemia. Hsp27 and Hsp70 are also elevated in the serum of patients who smoke and have chronic obstructive pulmonary disease (COPD), and these Hsps were found to be diagnostic markers for COPD (Hacker et al. 2009). Extensive tissue damage in COPD patients with chronic disease symptoms correlates with the extracellular release of Hsp27 into circulation (1.8-fold vs healthy). Hsp27 is released not only into plasma but also into cerebrospinal fluid in the central nervous system during ischemia (Hecker and McGarvey 2011). Moreover, elevated serum levels of Hsp27 have been detected in multiple sclerosis (Ce et al. 2011), gastric adenocarcinoma (Huang et al. 2010), pancreatitis (Liao et al. 2009), thymic epithelial tumors, and myasthenia gravis (Janik et al. 2016). Very recently, secretion of Hsp27 into circulation in ovarian cancer (OC) patients has been noticed and considered to be a potential biomarker for prognosis and diagnosis for OC (Stope et al. 2017). The functions of extracellular Hsp27 are unknown, although a few studies have reported that it is involved in signaling by binding to the receptors of immune and endothelial cells (Schmitt et al. 2007; Binder et al. 2004). Additionally, a few studies reported its role in cardiovascular diseases as it was found in the serum of patients with acute coronary syndromes (Park et al. 2006), atherosclerosis (Martin-Ventura et al. 2004), and reperfusion after ischemic clamping during heart bypass surgery (Jin et al. 2014). Additionally, Hsp27 has been shown to be an atheroprotective agent by decreasing the uptake of atherogenic lipids and thereby attenuating inflammation (Rayner et al. 2008). The addition of exogenous Hsp27 to in vitro macrophage culture reduced the acetylated LDL uptake by 41% with a concomitant increase in the expression of anti-inflammatory (IL-10 and GM-CSF) and pro-inflammatory mediators (IL-1β and TNF-α) (Rayner et al. 2008; Salari et al. 2013). Extracellular Hsp27 reduced the LDL uptake by binding to the scavenger receptor-A (SR-A). It reduced the ability of SR-A to engulf acetylated LDL on the surface of macrophages and acquired the foam cell phenotype (Rayner et al. 2008). Hsp27 also activated the NF-kB in mouse coronary endothelial cells by interacting with TLR2 and TLR4 and resulted in upregulation of monocyte chemoattractant protein-1 (MCP-1) and intercellular adhesion molecule-1 (ICAM-1) (Jin et al. 2014). Similarly, Hsp27 engagement on TLR3 was implicated in the activation of NF-κB and secretion of IL-8 and vascular endothelial growth factor (VEGF) in human microvascular endothelial cells (HMECs) (Thuringer et al. 2013). Recombinant Hsp27 rapidly internalized into the endosomal compartment (within 15–30 min) with the pool of TLR3, which is required for NF-κB activation in HMECs. Treatment of bone marrow-derived dendritic cells with recombinant Hsp27 also activated IL-6, TNF-α, IL-1β, IL-12p35, and IL-12p40 by interacting with TLR4 (Yusuf et al. 2009). Estrogen receptor-β is also known to interact with Hsp27 (Al-Madhoun et al. 2007; Miller et al. 2005). In addition to inducing inflammation, extracellular Hsp27 shows chaperone and therapeutic activity in in vitro and experimental disease models. Recombinant Hsp27 inhibited the DTT-induced aggregation of insulin in vitro, indicating that exogenous Hsp27 has effective chaperone activity (Kurnellas et al. 2012). Likewise, intraperitoneal injection of recombinant Hsp27 reduced the paralytic symptoms by modulating inflammatory cytokines IL-2, IL-6, and IFN-λ in the experimental auto-immune encephalomyelitis (EAE) mouse model at the peak of the disease (Kurnellas et al. 2012). However, ceasing of Hsp27 supplementation resulted in complete return of the paralytic symptoms, indicating that Hsp27 acts as a biological inhibitor, and the therapeutic effects seem to depend on consistent circulatory levels of Hsp27, rather than inducing a long-acting immunological state of reduced inflammation or tolerance (Kurnellas et al. 2012). A short sequence of Hsp27 (73 to 92 amino acids; 1 μg/animal) ameliorated the paralytic symptoms in EAE mouse model, suggesting that peptides of Hsp27 also have effective therapeutic activity similar to that of the full-length protein (Kurnellas et al. 2012). In another study, the sequence of 93 to 113 amino acids of Hsp27 (10 μg/ml) protected the HeLa cells from STS- or H_2O_2-induced apoptosis by inhibiting the release of cytochrome c from mitochondria and activating caspase-3 (Nahomi et al. 2015). Intraperitoneal (i.p.) administration of the same peptide (93 to 113 amino acids; 50 μg/animal) for 6 days prevented selenite-induced cataract development in mice by inhibiting the oxidative stress, protein insolubilization, and apoptosis indicating that peptides of Hsp27 are effective in preventing disease phenotype (Nahomi et al. 2015). Detection of injected peptide (i.p.) in the lens confirmed that the peptide is able to traffic through barriers to execute its function. Finally, these observations culminate to suggest that Hsp27 is mainly involved in modulating the inflammatory response by interacting with certain receptors (secretion of cytokines), chaperone activity (prevention or suppression of aggregation of proteins), cell migration (upregulation of MCP-1 and ICAM-1), cell death (preventing apoptosis), and cell proliferation (interacting with ER-β) (Fig. 1).

Export of Hsp27

As discussed earlier, sHsps are not secreted through the classical ER-Golgi secretory pathway. Until now, lysoendosomal or exosomal/extracellular vesicular pathway, direct protein translocation, and release after cell death (necrosis) have been proposed toward the release of Hsps (De Maio 2011; De Maio and Vazquez 2013). Among these, it is unclear whether the release of Hsps after cell death is a valid mechanism for export of Hsps. Some recent studies have provided evidence for the release of sHsps via the lysoendosomal or exosomal pathway (Batulan et al. 2016; Rayner et al. 2009; Sreekumar et al. 2010; Zhang et al. 2012). Rayner et al. (2008) reported that

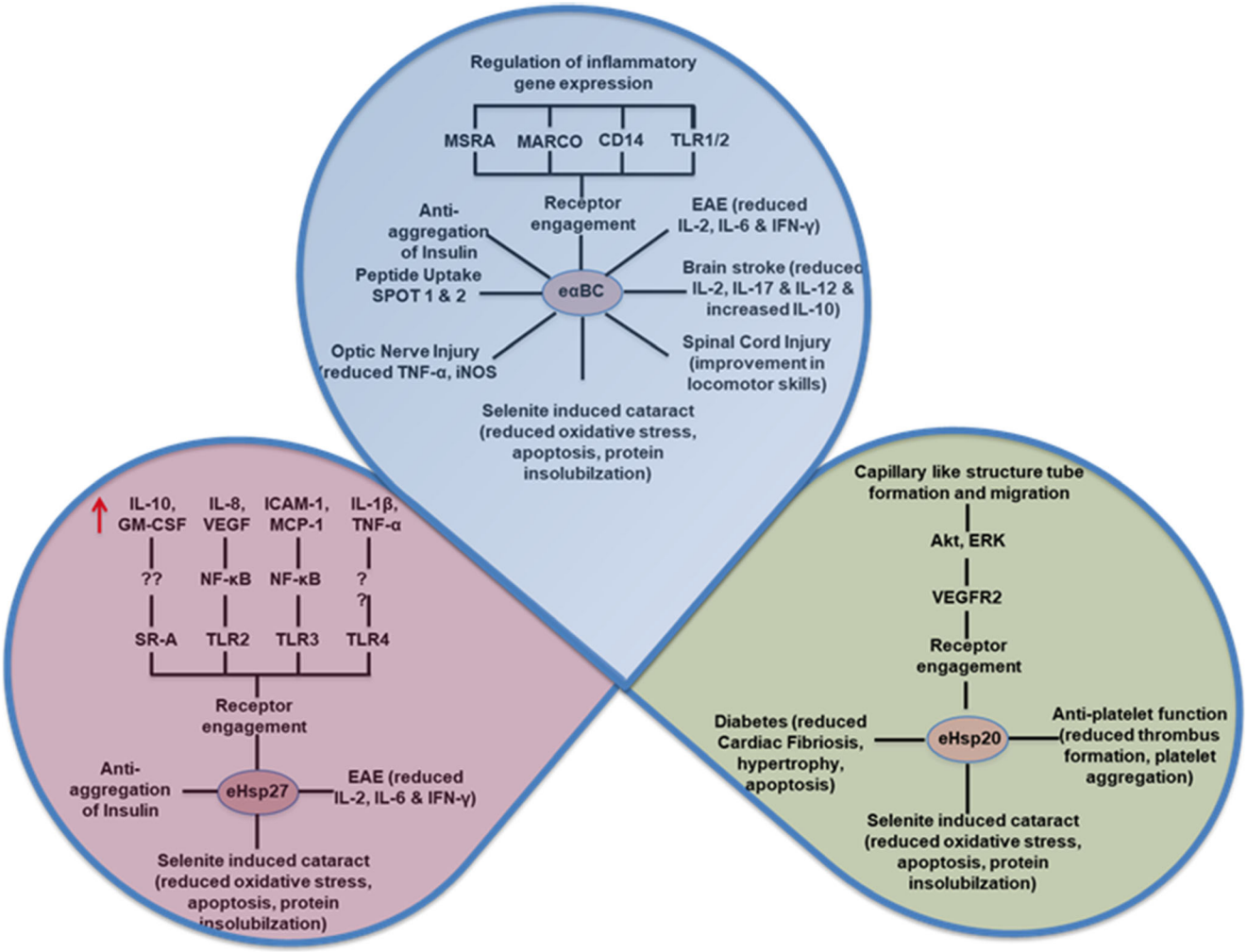

Fig. 1 Functions of extracellular heat shock protein 27 (eHsp27), αB-crystallin (eαBC), and Hsp20 (eHsp20). TLR: toll-like receptor; SR-A: scavenger receptor-A; VEGF: vascular endothelial growth factor; GM-CSF: granulocyte macrophage colony stimulating factor; ICAM-1: intercellular adhesion molecule1; MCP-1: monocyte chemoattractant protein-1; TNF-α: tumor necrosis factor; IL-6: interleukin-6; MSRA: macrophage scavenger receptor A; MARCO: macrophage scavenger receptor with collagenous structure; TNF-α: tumor necrosis factor; IL-2: interleukin-2; SOPT1: sodium oligopeptide transporter 1; VEGFR2: vascular endothelial growth factor receptor 2

treating macrophages with acetylated LDL or estradiol-induced secretion of Hsp27 and co-localization with lysotracker or lysosomal marker LAMP. Another group also reported the co-localization of non-phosphorylatable mutants (S15A/S82A) of Hsp27 fused to GFP with lysosomal marker, LAMP-1, indicating that Hsp27 also exits into the extracellular space through secretory lysosomal-like vesicles (Lee et al., 2012). Utilization of phosphomimetic forms of Hsp27 (S15D/S28D) further confirmed that phosphorylation of Hsp27 at specific serine residues is crucial for its secretion exclusively through the endosomal pathway. Another exit of Hsp27 is through the exosomal pathway, and numerous studies are supporting the release of Hsp27 through the exosomes. Rayner et al. (2009) showed that extracellular levels of Hsp27 are reduced upon treatment of human macrophages with exosomal inhibitor dimethyl amiloride. Nafar et al. (2016) detected Hsp27 in membrane fractions of exosomes originating from rat astrocytes upon treatment with amyloid-β. Under physiological conditions, exosomes are positive for Hsp27, and exposure of lymphoblastoid cells to heat stress (42 °C for 3 h) enhanced the quantity of exosomes with a ratio of 1.2:1 compared to that of exosomes from the control cells. Heat stress not only increased the quantity of exosomes but also selectively enriched Hsp27 along with Hsp70 and Hsp90 (Clayton et al. 2005). Exosomes are small-membrane microvesicles ranging from 4 to 100 nm in diameter and are found in all biological fluids. Lipids (lipid rafts) and proteins are major components of exosome membranes. Exosomes are released into the extracellular space after fusion with the plasma membrane and contain membranous and lumen portions. The inward budding of the plasma membrane forms early endosomes, and the membrane of early endosomes buds inwardly into various late endosomes. Late endosomes contain intraluminal bodies that are also called

multivesicular bodies (MVBs). These MVBs fuse with the plasma membrane to release intraluminal bodies, i.e., exosomes, while the microvesicles, ranging from 100 to 1000 nm directly bud off from the plasma membrane. In the recent past, the exosomes have become crucial in understanding the cell-to-cell communication and as delivery agents of miRNA. Large Hsps including Hsp70 are localized to the membrane (surface) of exosomes (Gastpar et al. 2005) as well as to the luminal compartment (Clayton et al. 2005; Jia et al. 2017). It is also subject to debate whether sHsps are localized in the luminal or membrane portion in exosomes. While the presence of Hsp27 in lumen or membrane of exosomes is still under debate, studies proposed that Hsps are localized in the lumen and as well as on the surface. Localization of Hsps in the lumen but not on the exosome surface suggests that Hsps in exosomes may not interact with target cells through cell surface Hsp receptors. However, the presence of Hsps on the surface can also be accepted, as it is important for secretion of Hsps into circulation and interaction with other cell surface receptors. Furthermore, the presence of sHsps on the cell surface is corroborated by the fact that Hsp27 interacts with cell surface receptors such as SR-A and TLRs (Yusuf et al. 2009). Hsp27 was seen to be released into the extracellular medium through exosomes when OVCAR-3 and SK-OV-3 cells were cultured in vitro (Stope et al. 2017). Blocking of the ER-Golgi pathway with brefeldin-A has not affected the secretion of Hsp27 suggesting secretion of Hsp27 may depend on intracellular levels rather than the phosphorylation status of Hsp27. In support to this, Stope et al. (2017) demonstrated that phosphorylation did not affect the secretion of sHsps. This study further provided significant insights into the understanding of the factors that influence the secretion of Hsp27. Interestingly, heat shock treatment not only increased the expression of intracellular Hsp27 but also enhanced the incorporation of Hsp27 by 10-fold into extracellular exosomes imparting a crucial role of the exosomal pathway in Hsp27 secretion.

αBC

Recently, many studies have demonstrated the extracellular presence and therapeutic functions of exogenous αBC in multiple in vitro cell culture as well as in in vivo disease models. The increased secretion of αBC into the culture medium has been shown during primary human adipocyte culture from obese patients and corroborates with its increased level in plasma and adipose tissue from the patients (Lehr et al. 2012). Therefore, it is concluded that αBC also acts as an adipokine. The secretion of αBC into plasma under several disease conditions with altered fold change is listed in Table 1. One study identified that αBC can act as a biomarker for renal cell carcinoma with its increased levels in serum samples of patients (Holcakova et al. 2008). An increasing number of studies also have been reported that the export of αBC into the extracellular milieu is through the exosomes. Sreekumar et al. (2010) reported the presence of αBC in secreted exosomes when retinal pigment epithelial cells (RPE) were cultured in exosome-free medium (to avoid contamination from endogenous exosomes present in the serum). The presence of αBC in exosomes has been further confirmed by performing immunogold labeling and immunoblotting for CD63, as a marker for exosomes (Sreekumar et al. 2010). Multiple factors including stress stimuli, cell polarity, and inflammation can induce the release of αBC. For example, αBC secreted within exosomes by highly polarized human RPE cells toward the apical photoreceptor facing neural retina confers neuroprotection. Extracellular αBC can also act as an auto-antigen by eliciting auto-antibodies in multiple sclerosis (van Noort et al. 2006). Subsequently, many studies reported the presence of auto-antibodies in CSF and serum of Neuro-Behçet's disease, Guillain–Barré syndrome, and inflammatory neurologic disorders (Celet et al. 2000; Hegen et al. 2010). However, the perception of αBC as an auto-antigen in these disorders is equivocal as recent studies reported that αBC through its chaperone activity can bind immunoglobulin with a high affinity, which contradicts the αBC as an auto-antigen (Rothbard et al. 2011). Of note, αBC promotes anti-inflammatory rather than proinflammatory response. Exogenous administration of 50 μg/100 g of body weight of α-crystallin (combination of αAC and αBC) reduced the expression of cytokines including IL-1α, TNF-α, and NO in the silver nitrate-induced rat inflammation model. Also, α-crystallin treatment in the same experimental model reduced the levels of neurotransmitters including dopamine, 5-hydroxytryptamine, and norepinephrine and increased their metabolites such as 3,4-dihydroxyphenylacetic acid, homovanillic acid, and 5-hydroxyindoleacetic acid in the neocortex and hippocampus regions of the brain (Masilamoni et al. 2005). Additionally, the same group reported alleviation of inflammation-induced expression of NF-κB and GFAP and decreased activation of acetylcholine esterase activity in neurons upon α-crystallin treatment (Masilamoni et al. 2006). However, the mechanism responsible for extracellular αBC in promoting an anti-inflammatory response upon administration remains unclear. The receptors including CD14, TLR1, and TLR2 are a prerequisite for αBC-mediated activation of HEK-293 cells secreting alkaline phosphatase under the control of transfected NF-kB (van Noort et al. 2013). Exogenous αBC activated the cells expressing CD14 and TLR1/2 through initial binding with macrophage scavenger receptor A (MSRA) and macrophage scavenger receptor with collagenous structure (MARCO) (van Noort et al. 2013). Further, it also inhibited the lymphocyte and neutrophil infiltration in the cigarette smoke-induced lung inflammation mouse model (van Noort et al. 2013). It has been observed that there is an increased level of αBC in plasma of individuals with the

disease in advanced stages than that of individuals with later stages of the disease in stroke patients (Arac et al. 2011). Intriguingly, increased levels of αBC have been noticed in patients with stroke at the time of presentation in the hospital (< 4 h from the point of stroke) and subsided within the next 48 h similar to that of mice suffering from a stroke. Supplementation of αBC i.p. to the αBC knock-out mice suffering from brain stroke reduced the lesion size. Administration of αBC 1 h before and 12 h after the onset of stroke in mice did not reduce the lesion size compared to the PBS-treated group. However, administration of αBC 1 h before and 12 and 24 h after followed by daily administration for 7 days after the stroke onset in mice led to a reduction in the lesion size compared to that of PBS-treated group, implicating the neuroprotective function to αBC. Furthermore, systemic augmentation of αBC reduced both the stroke volume and inflammatory cytokines (decreased proinflammatory cytokines and increased anti-inflammatory cytokines) in the stroke-induced mouse model (Arac et al. 2011). Intraperitoneal administration of αBC rapidly and efficiently reduced the paralytic symptoms in EAE mouse model. It is also noted that extracellular αBC does not directly affect T- or B-lymphocyte proliferation; rather, it limits the inflammation induced by TLRs and innate immunity. In support to this, Rothbard et al. (2012) reported that systemic administration of αBC in the EAE (induced by adoptive transfer of myelin-specific Th17 lymphocytes) mice did not ameliorate the clinical symptoms. Another study reported that intravenous administration of human recombinant αBC attenuated the spinal cord injury (SCI) in mice by ameliorating secondary tissue damage, improving locomotor skills, and reducing the recruitment of inflammatory macrophages (Klopstein et al. 2012). The extracellularly administered αBC is able to enter into some of the tissues including the spinal cord. The intravenous administration of αBC is able to enter injured spinal cord when injected into a SCI mouse model and showed 2-fold increase in protein levels at the site of injury 12 h post-injection as compared to a saline-treated group. Altogether, these results indicate that αBC can enter into inflammatory site in neuronal tissues and can show an anti-inflammatory response. However, endogenous αBC protein levels were reduced by 20% in recombinant human αBC-treated SCI mice compared with that of the physiological levels in normal spinal cord (Klopstein et al. 2012). The injected αBC protein has been shown to enter into the white matter at the injured epicenter and into white and gray matter in the adjacent areas (45% taken up by neurons, 38.8% by astrocytes, and 30.7% by oligodendrocytes) and not found in microglial cells, as well as granulocytes. αBC enhanced the survival of retinal ganglion cells, inhibited the retinal microglia cells, and reduced the expression of TNFα and iNOS in the rat model of optic nerve crush (Wu et al. 2014). Wu et al. (2014) administered αBC in a dose-dependent manner (0.05, 0.5, and 5 g/kg of body weight) to optic nerve crush rat model, but 5 g/kg of b.w. showed agitated behavior indicating that higher concentration of αBC is toxic to rats. Therefore, one should be cautious about using higher concentrations of αBC for retinal optic neuropathy, and one has to study the range of concentrations that are deleterious or beneficial to other diseases. However, the mechanism by which extracellular αBC exhibits beneficial functions remains largely unknown. One study revealed that αBC was able to bind to > 70 proteins in plasma of multiple sclerosis, amyloidosis, and rheumatoid arthritis patients, mouse model of EAE, and interestingly, these proteins have been identified to be part of proinflammatory signature including acute phase proteins, members of complement, and coagulation cascade (Rothbard et al. 2012). A study reported that αBC or its peptide blocks the function of platelets by preventing their aggregation in in vitro, ex vivo, and in vivo mouse models induced by thrombin or botrocetin (Kozawa et al. 2001). Inhibition of platelet aggregation is mediated by suppression of Ca^{2+} mobilization and activation of protein kinase C (Kozawa et al. 2001). Furthermore, αBC attenuates the ADP-induced granule secretion via p38 MAPK and p42 MAPK (Enomoto et al. 2009) upon incubation with platelets. Not only full-length αBC but also the intravenous administration of αBC peptides also inhibited thrombus formation in vivo indicating that both extracellular full-length and peptide αBC are important in the regulation of platelet function with an application in anti-platelet therapy (Kanno and Matsuno 2006). αBC is also found to be instrumental in performing extracellular chaperone activity in addition to anti-inflammatory and anti-platelet functions. In support to this, Mannini et al. (2012) showed that extracellular addition of αBC reduced the misfolded oligomer-induced toxicity even at a low level rather than overexpressing in the cytosol implicating the potential role for extracellular αBC to that of cytosolic counterpart. Exogenous recombinant αBC (12.5 μg/ml) protected the human lens' epithelial cells from heat- and oxidative stress-induced cell death indicating that exogenous αBC displays anti-apoptotic activity (Christopher et al. 2014). It is also shown that uptake of αBC is enhanced by fusion with cell penetration peptides (CPP) including TAT protein from human immunodeficiency virus and glycoprotein-C from hepatitis C virus. Cell penetration peptides fused to αBC including TAT-αBC and gC-αBC tagged with fluorophore showed enhanced uptake in HLE-B3 cells within 60 min of exposure to cell. The exogenously added gC-αBC was able to co-localize with endogenous αBC in cytoplasm as revealed by confocal microscopy indicating the efficiency of CPP in increasing the cellular uptake. It is also established that administration of αBC (i.p.) peptides inhibits protein insolubilization, epithelial cell apoptosis, and finally opacification in selenite-induced cataract in mice (Nahomi et al. 2013). This group administered the increasing dose of 2.5, 5, and 10 μg/animal of both αBC peptide and acetyl-αBC

peptide at 6 h prior to selenite injection in rat pups as a single injection separately. For multiple doses, the first dose was given 6 h prior to sodium selenite injection, followed by subsequent injections each day for 4 days. Both the peptides significantly inhibited cataract progression at 10 μg (single dose), as well as multiple doses. Treatment with 5 μg of both peptides also significantly inhibited cataract progression with multiple injections, while acetyl-αBC alone inhibited the cataract with single injection, indicating a dose-dependent therapeutic effect of native as well as modified αBC peptides. This study suggests that peptides of αBC are capable of crossing the blood/aqueous barrier to inhibit the opacification of the lens and shows the efficiency of exogenous αBC peptides in ameliorating the disease phenotype. The above findings suggest that the beneficial effects of exogenous administration of αBC are not only limited to a single disease or mechanism but also to multiple diseases or mechanisms where inflammation, cell death, and protein aggregation are involved in disease initiation or progression. The functions of extracellular αBC were depicted in Fig. 1.

Export of αBC

There is also compelling evidence suggesting the presence of αBC in exosomes. Like Hsp27, αBC secretion occurs independently of the classical secretory pathway wherein inhibiting the classical secretory pathway by using brefeldin and tunicamycin did not alter the secretion of αBC. It has been shown that αBC is secreted from the apical side of polarized human RPE cells (Sreekumar et al. 2010). In exosomes, αBC resides in detergent-resistant membrane microdomains, which are essential sites for exosome biogenesis. Treatment of human RPE cells with β-methyl cyclodextrin (lipid raft and cholesterol depletor) as well as dimethyl amiloride (exosome inhibitor and inhibitor of H^+/Na^+ and Na^+/Ca^{2+} exchanger) significantly inhibited αBC secretion, indicating the need of lipid rafts and exosomes for αBC secretion. Further, the secretory levels of αBC from human RPE cells are only a small fraction to that of the steady-state levels found in mitochondria and cytosolic compartments (Sreekumar et al. 2010). The polarization of cells is also equally important for secretion of αBC, which is further corroborated by the fact that a 5-fold increase in αBC secretion has been found in polarized RPE cells, compared to that of the non-polarized cells. Stress also affects the polarization and secretion of αBC. In support to this, oxidative stress induced by H_2O_2 in RPE cells induced the increased secretion of exosomes on the basolateral side compared to the apical side, and the secretion of αBC is found to be more from the exosomal fraction of the basolateral side when compared to non-stressed cells. Moreover, αBC has been shown to co-localize with tetraspanin CD63 in some of the MVBs/exosomes and perinuclear Golgi in ARPE cells. Interestingly, αBC did not co-localize to some of the CD63 positive exosomes/MVBs in the peripheral regions of the cell (Bhat and Gangulam 2011). Sreekumar et al. (2013) first reported the uptake of the αBC-derived peptide by two novel sodium-dependent oligopeptide transporters SOPT1 and SOPT2 in fetal RPE (fRPE) cells. These transporters are involved in transporting of endogenous and other synthetic opioid peptides and the iron regulatory peptide hormone hepcidin. αBC-derived peptides are transported into fRPE cells by competing with DADLE and deltorphin II that are substrates for SOPT1 and SOPT2. αBC-derived peptides have a high affinity for SOPT1 and SOPT2. On the contrary to Hsp27, exosomal secretion of αBC is influenced by post-translational modification including phosphorylation and O-GlcNAcylation (Kore and Abraham 2016). Phosphorylation and prevention of O-GlcNAcylation deterred the incorporation and exosomal secretion of αBC. αBC plays an crucial role in exosome biogenesis. Using shRNA, inhibition of αBC expression resulted in aggregated staining patterns of CD63 (LAMP3) and inhibited exosome secretion from ARPE19 cells (Gangalum et al. 2016). Further, inhibition of αBC expression increased the number of vacuoles and enlarged (fused) vesicles with increased expression of CD63 (LAMP3) and LAMP1 (a marker of lysosomes) indicating the shift toward the endolysosomal pathway which is further supported by increased levels of Rab7 (a marker for late endosome). This study demonstrates that αBC is crucial in the biogenesis of exosomes and that their secretion and inhibition of αBC cause shift into the endolysosome pathway. Further studies are required to address other mechanistic details of sHsps in the biogenesis of exosomes. It is possible that αBC might be involved in passaging the MVBs to the plasma membrane for the release of exosomes because in the absence of αBC, markers including Rab7 and LAMP1 increased, indicating that MVBs progress toward lysosomes instead of progressing toward the plasma membrane. However, it is also noted that exosome biogenesis normally occurs in many cell types without the involvement of αBC. Therefore, one should address whether αBC acts in a specific manner according to cell type.

Hsp20

Hsp20 is an important member of the sHsp family and is also referred to as HSPB6. It is expressed in multiple tissues with abundant expression in skeletal, cardiac, and smooth muscle cells (Fan et al. 2005; Salinthone et al. 2008). A few studies have reported the secretion of Hsp20 into the extracellular medium in pathological conditions. Whether secretion of Hsp20 in pathological conditions is advantageous or deleterious remains to be elucidated. Kozawa et al. (2002) reported that there is an elevated level of Hsp20 in plasma of cardiomyopathic hamsters. These researchers detected both

dissociated and aggregated forms of Hsp20 with elevated levels of the dissociated form in plasma of cardiomyopathic hamsters. Furthermore, circulating levels of Hsp20 increased 3.4-fold in cardiac-specific Hsp20-overexpressed transgenic mice cardiomyocytes in vitro (Zhang et al. 2012). These researchers also reported the secretion of Hsp20 through exosomes rather than through the classical ER-Golgi secretory pathway. Extracellular Hsp20 promoted the capillary-like structure tube formation and migration in HUVEC cells by interacting with VEGFR2, which is associated with activation of Akt and ERK (Zhang et al. 2012). Hsp20 levels were also elevated up to 11-fold in plasma of acute dissecting aneurysm patients. Extracellular Hsp20 shows anti-platelet activity by inhibiting thrombin-induced platelet aggregation in a dose-dependent manner by inhibiting receptor-mediated calcium influx in vitro and ex vivo (Matsuno et al. 1998; Niwa et al. 2000). Nevertheless, Hsp20 levels were markedly lower in a carotid artery endothelial injury model indicating that Hsp20 is secreted into circulation in response to endothelial injury to act as a regulator of platelets extracellularly (Kozawa et al. 2002). The intravenous administration of Hsp20 into hamsters prevented thrombus formation after endothelial injury, and its peptide also reduced the platelet aggregation implicating the anti-platelet function of exogenous Hsp20 (Kanno and Matsuno 2006). Intraperitoneal administration of recombinant Hsp20 (10 μg/day for 20 days) has shown similar therapeutic activity along with other sHsps by reducing the paralytic symptoms in the EAE mouse model, suggesting that exogenous Hsp20 acts as a therapeutic protein by decreasing the inflammation (Rothbard et al. 2012). In vitro treatment of HeLa cells with Hsp20-peptide (G71HFSVLLDVKHFSPEEIAVK91) caused it to translocate to the nucleus from the cytosol during thermal stress. Hsp20 peptide also prevented apoptosis by blocking the release of cytochrome c from mitochondria and caspase-3 activation in HeLa cells (Nahomi et al. 2015). Intraperitoneal administration of 50 μg of Hsp20 peptide/animal/day for six consecutive days inhibited the protein aggregation and oxidative stress thereby preventing the cataract formation in selenite-treated rats suggesting that exogenous Hsp20 peptide shows therapeutic activity by anti-aggregation and anti-oxidant activities (Nahomi et al. 2015). Previous studies have reported the secretion of Hsp20 through exosomes rather than a classical ER-Golgi secretory pathway. Accumulating evidence suggests that exosomes secreted from cardiomyocytes contain Hsp20 and play crucial role in cardiovascular pathology. Exosomes secreted from diabetic rat cardiomyocytes contain lower levels of Hsp20 compared to that of healthy controls. Exosomes secreted from diabetic cardiomyocytes from cardiac-specific overexpressing-Hsp20 mice (conditional transgenic mice overexpressing Hsp20 in the heart) showed increased levels of Hsp20 compared to those in control rats (Wang et al. 2016). Cardiac-specific overexpression of Hsp20 increased the biogenesis of exosomes upon interaction with tumor susceptibility gene 101 (Tsg101). Tsg101 is the component of Endosomal Sorting Complex Required for Transport-1 (ESCRT-1) which recognizes the ubiquitin tagged proteins and required for the sorting of endocytic ubiquitinated cargos into multivesicular bodies (MVBs). Further, cardiac-specific overexpression of Hsp20 increased the exosomes packed with Hsp20 and protected the cells from hyperglycemia-induced cell death in in vitro and in streptozotocin-induced diabetic mouse model in vivo, altogether suggesting that Hsp20 is instrumental in cardioprotection by increasing the biogenesis of exosomes (Wang et al. 2016). The functions of extracellular Hsp20 are depicted in Fig. 1.

Export of Hsp20

Zhang et al. (2012), for the first time, reported the secretion of Hsp20 from rat cardiomyocytes through exosomes. Hsp20 is released into the extracellular medium in physiological and myocardial infarction conditions, but its secretion is increased in myocardial infarction. It implies that secretion of Hsp20 via exosomes is increased under stress conditions such as ischemia/reperfusion. Interestingly, increased secretion of Hsp20 from cardiomyocytes of cardiac-specific overexpressing Hsp20 mice correlated with increased expression of Rab11a, Rab11b, Rab35, and Tsg101 that are involved in exosome generation/biogenesis. It has also been proven that recombinant Hsp20 is known to interact with Tsg101 but not with Rab11 a/b and Rab35 indicating that Hsp20 increases the generation of exosomes by interacting with Tsg101 (Wang et al. 2016). These studies strongly support that Hsp20 is strongly associated with the biogenesis of exosomes. It is also shown that extracellular Hsp20 promotes angiogenesis by acting as a VEGF-receptor agonist. Furthermore, the same research group has demonstrated that exosomes derived from cardiomyocytes of conditional transgenic-Hsp20 mice are enriched with SOD1, survivin, p-Akt, and Hsp20 and are capable of protecting the mouse cardiac endothelial cells from high glucose-induced stress (Wang et al. 2016). Moreover, exosomes derived from cardiomyocytes of transgenic-Hsp20 mice attenuated the cardiac fibrosis, hypertrophy, and apoptosis when injected into diabetic mice. All these studies indicate that Hsp20 promotes exosome biogenesis and exosomes encased Hsp20 protect the cells from multiple stressful conditions.

Hsp22

Hsp22 is also called as HSPB8 or H11 kinase or E2IG1. Abundant expression of Hsp22 has been noticed in skeletal muscle, heart, retina, brain, and spinal cord. Hsp22 plays an important role in neuronal cell survival by regulating the mitochondrial pathway. However, the extracellular presence

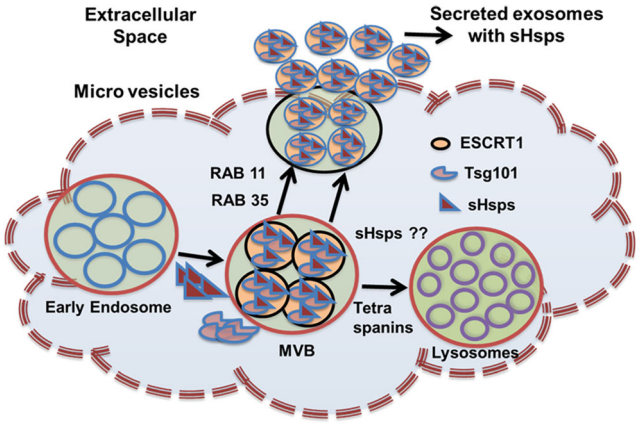

Fig. 2 Hypothetical model for export of small heat shock proteins (sHsps) into the extracellular medium. sHsps may be part of the ESCRT-I complex and involved in the biogenesis of exosomes by directly interacting with Tsg101, an important component of the ESCRT-I machinery, and they might also act as a branch point to decide the fusion of MVBs with plasma membrane or lysosome

of Hsp22 is least studied. Hsp22 is also known to be involved in inflammation of several pathologies. Immunohistochemistry and Western blot analysis in synovial tissues of rheumatoid arthritis patients showed increased expression of Hsp22 (Roelofs et al. 2006). It is also shown that Hsp22 activates dendritic cells upon its engagement with TLR4. Wilhelmus et al. (2009) reported the association of Hsp22 with cerebral amyloid angiopathy, hereditary cerebral hemorrhage, amyloidosis (Dutch type), and senile plaques in Alzheimer's disease. Hsp22 also induced the secretion of IL-6 in human brain pericytes, astrocytes, and microglia. However, the secretory pathway associated with Hsp22 extracellular levels is yet to be elucidated.

Conclusion and future prospects

Heat shock proteins are chaperones that prevent the aggregation of proteins and safeguard the cells from vicious insults. Previous studies reported the intracellular presence of Hsps. However, recently, this perception has changed owing to the extracellular presence of Hsps. Hsp27, αBC, and Hsp20 are secreted into the extracellular medium by a non-classical pathway. Secreted sHsps play an instrumental role in cell-to-cell communication, signaling, immunity, and inflammation. These proteins may act as part of the larger protein complex, for example by interacting with auto-antibodies in plasma or acting by binding to the cell surface receptors on immune/endothelial cells. sHsps also play a pivotal role in the biogenesis of exosomes. These proteins have also been shown to have therapeutic activity when administered extracellularly in in vitro and in vivo disease models. They attenuated the disease phenotype by chaperone, anti-apoptotic, anti-inflammatory, and anti-platelet activities. Many theories have been proposed to explain the secretion of sHsps into the extracellular medium including endolysosomal and exosomal pathways, direct translocation, and release after cell death. Among these, endolysosomal or exosomal pathways are shown to be predominant pathways utilized for the export of sHsps. Hsp27 shown to be secreted via exosomes and endolysosomes while αBC and Hsp20 via exosomes. The crucial role of sHsps in exosome biogenesis/generation should be studied in greater detail as αBC and Hsp20 are involved in exosome biogenesis and their interacting partners like Tsg 101 in the case of Hsp20. The contributory role of all the available sHsps in exosome biogenesis remains to be unanswered till date. Additionally, switching of secretory pathways from exosomes to endolysosomes and vice versa by sHsps and cell type-specific pathways is yet to be elucidated in greater detail. It is also possible that sHsps are involved in exosome biogenesis by participating in the ESCRT machinery in the generation of exosomes. The ESCRT multiprotein complex contains a set of cytosolic proteins that are incorporated into the endosomes by limiting membrane proteins. Proteins recruited to endosomes are usually tagged with ubiquitin on their cytosolic domains. Various types of ESCRT machinery are used in the biogenesis of exosomes including ESCRT-0, -I, -II, and -III. ESCRT-0 is involved in the trafficking of the ubiquitin-tagged proteins to ESCRT-I that are recognized by Tsg-101. It has been established that Hsp20 mediates biogenesis of exosomes by directly interacting with Tsg101. Hsp20 has a high affinity for Tsg101 and less affinity for Rab11 a/b and Rab35 indicating that Hsp20 might be part of the ESCRT-I complex helping Tsg101 to recognize ubiquitin proteins. These studies also support the idea that Hsp20 is localized in the lumen of exosomes as Tsg101 is used as an internal marker of exosomes. Another hypothesis is that, owing to the anti-aggregation effect of sHsps, they might participate in ESCRT complexes to sort out the proteins into vesicles. sHsps might also decide the fate of MVBs in fusion with lysosomes or plasma membrane as suggested in the case of αBC. The role of sHsps in exosome biogenesis was depicted in Fig. 2.

After all these observations, a lot to be resolved regarding the mechanisms by which sHsps find their way to the extracellular milieu with varied functions: in-depth molecular mechanistic details and interactions involved in secretion, role of sHsp auto-antibodies in blood, elucidating the presence of sHsps on the membrane/luminal portion of extracellular vesicles, determining how they bind to cell surface receptors and communicate with distant cells, determining the half-life of extracellular sHsps, and explicating several activities of extracellularly administered sHsp and how they cross the various barriers to exert their effect. Furthermore, studies should be conducted to exploit the benefits of exosomes packed with sHsps in various disease models. The therapeutic effect of exosomes encased with Hsp27, αBC, and Hsp20 should be studied by injecting them in disease models including

neurodegeneration, cancer, diabetes, and ischemia/reperfusion. All these studies are likely to help develop extracellular sHsps as therapeutic molecules and as native proteins/peptides/exosomes encased with sHsps, encapsulation of sHsps with nano- and microparticles or cell penetration peptides.

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