Chapter 1
Regulatory Roles of HSP90-Rich Extracellular Vesicles

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Abstract HSP90 is an essential protein in protein folding, cancer progression and wound healing. Originally, most studies were focused on the intracellular molecular chaperone role of HSP90. However, more recent studies, including ours, have reported the secretion of HSP90 and novel functions for this protein in the extracellular space (ex-HSP90). Additionally, HSP90 has been found to be a major cargo contained in extracellular vesicles (EV) such as exosomes. HSP90 can directly bind to and promote functions of CD91/LRP1 and receptor tyrosine kinases such as EGF receptor. HSP90 also regulates the recycling of Rab proteins that control the secretion of exosomes. This chapter reviews current knowledge and the future potential of ex-HSP90 and EV-HSP90.

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Abbreviations

ECM	Extracellular matrix
EGFR	Epidermal growth factor receptor
EMT	Epithelial-mesenchymal transition
EV	Extracellular vesicle
ex-HSP90	Extracellular HSP90
FN	Fibronectin
HIF-1	Hypoxia-inducible factor-1
HNC	Head and neck cancer
HSP	Heat shock protein
ic-HSP90	Intracellular HSP90
LRP1	Lipoprotein receptor-related protein 1
MV	Microvesicle
MVB	Multivesicular body
OSCC	Oral squamous cell carcinoma
RTK	Receptor tyrosine kinase
TM	Transmembrane
TSP1	Thrombospondin 1

1.1 Introduction

Heat Shock Protein (HSP) is a protein universally present in every cell, while the HSP family consists of two types; a cell stress-response type of HSP and a constitutively expressed housekeeping type of HSP. The stress-responsive HSP is expressed when cells are exposed to stress such as heat, cold, and hypoxia. Among members of the HSP family, HSP90 is one of the most abundant molecular chaperones playing key roles in proteostasis in the cells. The HSP90 family consists of four members; the proteotoxic stress-inducible HSP90α encoded by *HSP90AA1*, the constitutively expressed HSP90β encoded by *HSP90AB1*, the mitochondria-localized TRAP1, and an ER resident paralog GRP96/HSP90B1. Of note, HSP90α is often highly expressed in cancer cells and is secreted to extracellular space as a soluble protein so-called chaperokine (Eguchi et al. 2018) or as a cargo protein of extracellular vesicles (EVs) (Ono et al. 2018). Additionally, HSP90β, TRAP1, and some members of HSP70 were recently found to be secreted with EVs from cancer cells (Ono et al. 2018). However, the mechanism by which HSP is incorporated with EVs and their significance are unknown.

1.2 Extracellular HSP90 in Skin Wound Healing and Cancer

Intracellular HSP90 (ic-HSP90) impacts many components of the cellular proteostasis network, cytoplasmic protein quality control, and the stress response (Neckers et al. 2018). More recently the roles and clinical applications of extracellular HSP90 (ex-HSP90) have been established by Drs Wei Li and David Woodley since 2007. Dr. Li's studies have shown a novel homeostatic mechanism involving the pathway: hypoxia>HIF-1>ex-Hsp90α secretion>skin cell migration>wound healing, and identified ex-Hsp90α as a potential therapeutic agent for the healing of skin wounds (Li et al. 2007; Jayaprakash et al. 2015). This group subsequently showed that TGFα, a member of the EGF ligand family, also stimulates secretion of HSP90α (Cheng et al. 2008). In addition, the low-density lipoprotein receptor-related protein 1 (LRP1/CD91) was identified as a key receptor for ex-HSP90 to promote human skin cell migration (Cheng et al. 2008). ex-HSP90 was demonstrated to bind to the subdomain II of LRP1 and it was shown that the intracellular NPVY motif of LRP1 was essential for activation of Akt1/2 signaling (Tsen et al. 2013). These studies regarding wound healing were also relevant to cancer studies. Secreted ex-HSP90α and ex-HSP90β were found in the conditioned media (CM) of breast cancer cell lines such as MDA-MB-231, MDA-MB-468, MDA-MB-361, BT474, T47D, and Skbr3, in which HIF-1α is also constitutively active, but not in HBL100 or HS-578-T cells (Dong et al. 2016). In breast cancer MDA-MB-231 cells, the secreted ex-HSP90 increased cancer cell survival in a hostile hypoxic environment via LRP1-mediated activation of Akt, a kinase that is known to mediate cell survival (Tsen et al. 2013). The interiors of large tumors are hypoxic due outgrowing the local capillary system (LePage 1948; Najafi et al. 2019). It is noteworthy that hypoxia induces LRP1 (Koong et al. 2000; Kawata et al. 2012) and HSP90 expression (Eguchi et al. 2018). LRP1-HSP90 interaction on the surface of cells and of exosomes might therefore promote tumor growth.

1.3 Extracellular Vesicles (EVs)

Recent studies have demonstrated the significance of extracellular vesicles (EVs) in many biological and medical phenomena, including: cancer (Peinado et al. 2011; Fujita et al. 2016; Kalluri 2016; Fujiwara et al. 2018a, b), the immune system (Carstens et al. 2017), tissue development and repair (Barile and Vassalli 2017; Mathiyalagan et al. 2017), bone metabolism (Taverna et al. 2017), microbiology (Beveridge 1999), and amyloidogenesis (van Niel 2016) as well as EVs as drug delivery system (DDS) (Fais et al. 2016; Ha et al. 2016) and carriers of biomarkers (Kalluri and LeBleu 2016; Minciacchi et al. 2017b; Mendt et al. 2018; Ono et al. 2018). In a brief classification, EVs include exosomes (50–200 nm), ectosomes (100–1000 nm, a.k.a. shed microvesicles, MVs), large EVs such as large oncosomes (1–10 um) (Minciacchi et al. 2017a), exophers (~4 um) generated upon neurotoxic

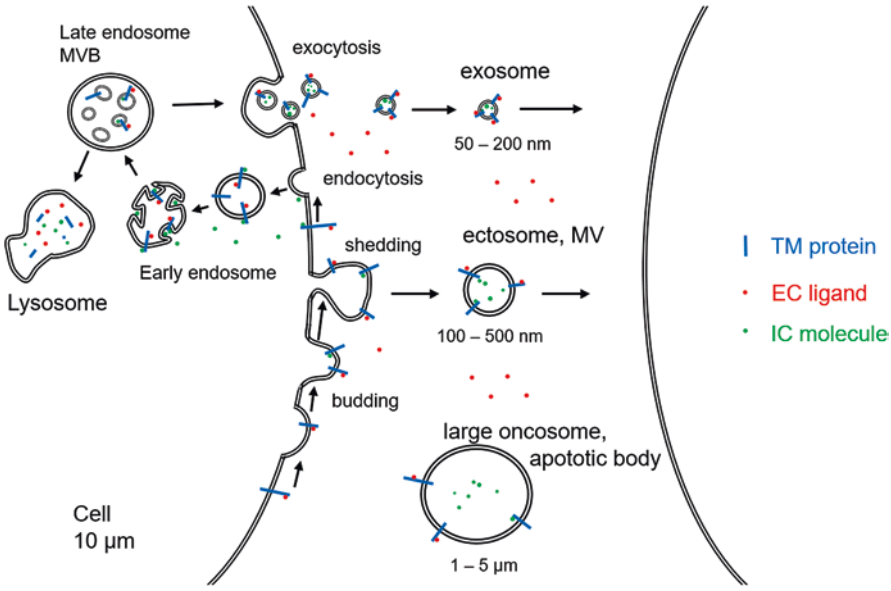

Fig. 1.1 Roles of HSP90 and LRP1 within EVs. EVs are a heterogeneous mixture of structures classified respectively as exosomes, ectosomes (a.k.a. shed MVs), large oncosomes and apoptotic bodies as shown at the center in this figure. Exosomes are secreted via exocytosis of late endosomes a.k.a. multi-vesicular bodies (MVBs) (top). Distinctively, budding and shedding of plasma membrane generate ectosomes (center). Transmembrane (TM) proteins such as LRP1 (blue) can localize on the surface of EVs. Extracellular (EC) ligands such as ex-HSP90 (red) bind to the extracellular domain of LRP1 on the surface of EVs. LRP1-positive EVs can gather ex-HSP90. Intracellular HSP90 (green) can be kept bound to the intracellular domains of the TM proteins such as receptor tyrosine kinases (RTKs) including EGFR, which is incorporated into EVs with HSP90. In this case, HSP90 can enter into EVs

stress (Melentijevic et al. 2017) and apoptotic bodies (Fig. 1.1, at the center), migrasomes associated with cilia (Ma et al. 2015), and exomeres (~35 nm) (Zhang et al. 2018; Zijlstra and Di Vizio 2018). Differences in the generation mechanisms involved in their production define these EVs rather than their size. Exosomes are secreted via exocytosis of late endosomes a.k.a. multivesicular bodies (MVBs) (Fig. 1.1, upper left). By contrast, the budding and shedding of plasma membrane lead to the generation of ectosomes (Fig. 1.1, center).

1.4 EV-Associated HSP90

We recently reported that the secretion of ex-HSP90α was boosted along with the formation of tumor organoids (tumoroids/cell aggregates/spheroids) of prostate cancer PC-3 cells, in which intra-tumoral hypoxic milieu was reconstituted (Eguchi et al. 2018). ex-HSP90α receptor LRP1 was robustly expressed in the PC-3 cells

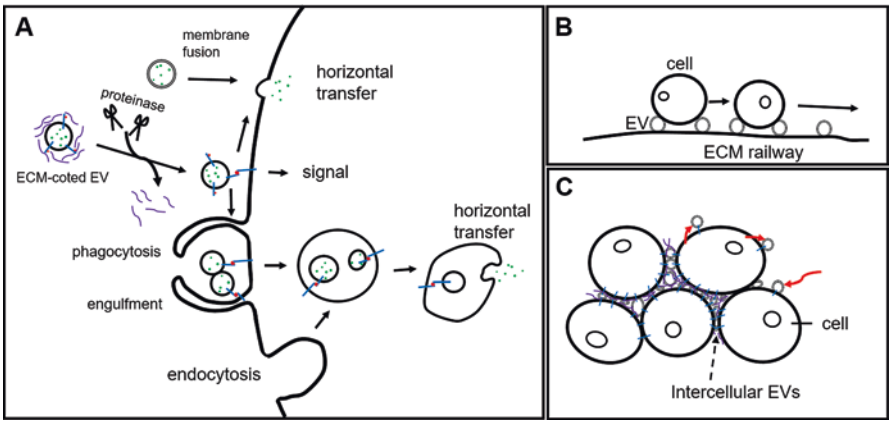

Fig. 1.2 The multiple actions of EVs on/to the cells. The action of EVs on cells can be classified as: (i) horizontal transfer of EV cargos (**a**, top), which can largely change the recipient cells, (ii) signal transduction to the recipient cells using the EV-surface molecules such as ex-HSP90 and cell surface receptor such as LRP1 (**a**, center), which can trigger subsequent membrane fusion, phagocytosis, macropinocytosis (Nakase et al. 2015) or endocytosis (**a**, bottom) (Horibe et al. 2018). After the phagocytosis and endocytosis, EV cargos can be processed in lysosomes, horizontally transferred into the cytoplasm (**a**, right) or recycled in recycling endosomes. EVs can stay on the surface of cells via interaction between cell surface molecules and extracellular matrix (ECM). EVs can also be coated by ECM (purple), which may physically interfere with membrane fusion and horizontal transfer from the EVs to the cells (**a**, left). EVs can be used as wheel-like adaptors that enable cells to drive (migrate) on the cell migration highway made by ECM in tissues, most notably in cancer metastasis (**b**). EVs can also be kept on the surface of cells and at intercellular space, where EVs could mediate cell aggregation (**c**). ECM molecules such as fibronectin (FN) and TM proteins such as LRP1, EpCAM (Eguchi et al. 2018) and integrins that associate with both cells and EVs could mediate such new roles of EVs in cell aggregation

(unpublished data) and thus a mechanism of tumoral hypoxia>HIF-1α>secretion of ex-HSP90>LRP-1>hypoxia-resistant survival signal (e.g. Akt) can be generalized in many types of tumors. However, we showed that HSP90 and LRP1 were carried by EVs secreted by the prostate cancer PC-3 cells (Eguchi et al. 2018), indicating that LRP1-HSP90 on the surface of EVs can act on recipient cells (Figs. 1.1 and 1.2). ex-HSP90 species including EV-HSP90 and EV-free ex-HSP90, are also secreted by oral squamous cell carcinoma (OSCC) cells (Ono et al. 2018). These cells are classified among head and neck cancers (HNC). HSP family members such as EV-HSP90α and EV-HSP90β were more significantly detected in the lymph node (LN)-metastatic OSCC-derived EV compared with low-metastatic OSCC-EVs (Ono et al. 2018). We thereafter demonstrated that high-expression of HSP90s, including HSP90α, HSP90β, TRAP1 (mitochondrial HSP90) and HSPH1/HSP105 were correlated with increased metastatic tendencies and poor prognosis of patients suffering from HNCs (Ono et al. 2018), indicating that high HSP90 in tumors and EVs are potential prognostic biomarkers of HNCs. Double targeting of HSP90α and HSP90β using siRNA reduced the survival of OSCC cells (Ono et al. 2018), suggesting potential usefulness of small RNA medications that target HSP90 mRNA in cells and EVs.

Although many papers have reported that HSP90 is included in exosome or EV fractions, it is still unclear whether HSP90 is located inside the vesicles or outside (on the surface of) vesicles. Such sub-vesicular localization of HSP90 is important for their functional properties. Cytoplasmic HSP90 and its cochaperone CDC37 play key roles in functions of receptor tyrosine kinases (RTK). Many RTKs including EGFR have been found in exosomes (Fujiwara et al. 2018a, b; Ono et al. 2018). Therefore, RTK-HSP90 complex is thought to be incorporated into vesicles. By this mechanism, HSP90 can be incorporated inside EVs, whose membrane fusion with recipient cells enable HSP90/RTKs transfer into recipient cytoplasm. Intra-exosomal HSP90 could be horizontally transferred into recipient cells where it could exert chaperone functions (Fig. 1.2). Horizontal transfer of HSP90 from the EVs to the recipient cells could thus increase the cellular proteostasis networks, including autophagy, and the UPR in the ER, as well as cytoplasmic protein quality control and stress response in tumoral immune cells such as tumor-associated macrophages (TAMs), T cells, cancer-associated fibroblasts (CAFs), tumor endothelial cells (TECs), normal subtumoral epithelial cells, and other cells in the local and distant milieu.

1.5 Roles of LRP1 in Vesicle Traffic

LRP1/CD91 is a macromolecule that is composed of the extracellular α-subunit (approx. 500kD) and the transmembrane β-subunit (approx. 85kD). Although LRP1 is one of the receptors for ex-HSP90, this macromolecule receptor binds with many ligands that control signal transduction (Misra et al. 1995, 1999; Zilberberg et al. 2004; Yang and Williams 2017), endocytosis (Marynen et al. 1982; Actis Dato and Chiabrando 2018), transcytosis (Burgess and Stanley 1997; Fillebeen et al. 1999; Kawata et al. 2012; Jarosz-Griffiths et al. 2016), and exocytosis (Meng et al. 2011; Roy et al. 2015; Leca et al. 2016). Interestingly, a recent study has shown that LRP1 expression is significantly elevated on exosomes, especially in lung adenocarcinoma patients' sera (Ueda et al. 2014). Strong staining patterns of LRP1 were observed in stromal cells surrounding cancer cells in tissue sections from lung adenocarcinoma patients with poor clinical outcomes, while minimal expression of LRP1 in lung cancer cells was observed in the normal tissues (Meng et al. 2011). These reports suggested that the high level of serum LRP1-expressing exosomes might be secreted from stromal cells surrounding lung adenocarcinoma cells (Ueda et al. 2014). LRP1-rich EVs were also found in studies that model tumor milieu as follows. CAFs under physiopathologic conditions such as coculture with macrophages, under hypoxia and lipid deprivation, were enriched in EVs that contain annexin A6 (ANXA6)/LRP1/thrombospondin 1 (TSP1) complex (Leca et al. 2016). In this study, the ANXA6/LRP1/TSP1 complex was necessary for the uptake of these EVs by cancer cells and for the increased tumor cell aggressiveness (Leca et al. 2016). It is noteworthy that hypoxia induces LRP1 expression (Kawata et al. 2012) as well as HSP90α (Eguchi et al. 2018). Elevated expression of LRP1-HSP90α in tumor milieu might promote further tumor growth via LRP1-mediated control of vesicle molecular traffic.

In addition to roles for LRP1 in vesicle traffic, this receptor plays key roles in intracellular signaling including the Akt1/2 pathways (Tsen et al. 2013), STAT3 (Signal transducers and activator of transcription), and β-catenin signaling. It was shown that glioblastoma-derived plasminogen activator inhibitor 1 (PAI1) binding to LRP1 increased STAT3 phosphorylation and subsequent exocytosis in mast cells (Roy et al. 2015). It was also shown that LRP1 is a receptor for lipoproteins that alter canonical Wnt/β-catenin signals and sterol signals (Willnow et al. 2007). We recently showed lipoprotein transport to be a key for tumorigenesis (Namba et al. 2018).

1.6 ex-HSP90 Promotes Epithelial-Mesenchymal Transition (EMT)

TGFα/EGFR signaling is another inducer of secretion of ex-HSP90 (Cheng et al. 2011). We recently reported that EGF-EGFR signaling is essential for secretion of EGFR/HSP90-contained exosomes from OSCC cells (Fujiwara et al. 2018a, b). Interestingly, OSCC-derived EGFR/HSP90-containing exosomes had an ability to induce carcinogenic epithelial-mesenchymal transition (EMT) in the oral epithelial cell line RT7 (Fujiwara et al. 2018a, b). HSP90 is often found in cancer EVs and EV-free CMs (Eguchi et al. 2018; Ono et al. 2018). We hypothesize that ex-HSP90 may be enclosed in EVs and displayed on the outer surface of the EV membrane via its receptor EV-LRP1. The HSP90 inside EVs could also be horizontally transferred into recipient cells, which subsequently acquire multiple functions of cytoplasmic HSP90 as mentioned above. The HSP90 outside EVs could bind to cell surface receptors such as LRP1 on the surface of recipient cells leading to signal transduction and ligand-dependent endocytosis. As ex-HSP90 has been shown to promote EMT, EV-associated HSP90 can also promote EMT potentially via recipient cell surface receptor LRP1.

ex-HSP90-driven promotion of EMT was first reported by a group of Dr. Jennifer Isaacs in 2012 (Hance et al. 2012). This report showed that ex-HSP90 binding of LRP1 promoted EMT in prostate cancer cells via activation of ERK signaling. This group subsequently added a mechanism underlying ex-HSP90>LRP1>p-ERK>repression of E-cadherin gene by showing that polycomb group repressor EZH2 is induced under ex-HSP90>LRP1>pERK and the induced EZH2 repressed E-cadherin gene by histone H3 K27 methylation (Nolan et al. 2015). Alternatively, intracellular HSP90 was also shown to promote EMT, motility, and invasion of colorectal cancer cells via activation of HIF-1α and NF-κB (Nagaraju et al. 2015). Therefore, a positive feedback loop of hypoxia>HIF-1α>HSP90 (ic-HSP90 and ex-HSP90)>multiple signaling>EMT may play a key role in the promotion of cancer. Secreted ex-HSP90 promotes not only EMT but also heterogeneity of cancer stem cells or cancer-initiating cells (CSC/CIC) (Nolan et al. 2017). We recently reported that tumor organoids with CSC/CIC/EMT traits of prostate cancer cells profoundly secreted ex-HSP90α that may play autocrine and paracrine roles in tumor progression (Eguchi et al. 2018).

A new role of HSP90β was recently shown that promotes aggressive vasculogenic mimicry (VM) via EMT in hepatocellular carcinoma (HCC), a typical hypervascular solid tumor (Meng et al. 2019). In this study, HSP90β interacted with Twist1 and promoted its deubiquitination, stabilization, and nuclear translocation and enhanced the vascular endothelial (VE)-cadherin promoter activity. An HSP90 inhibitor NVP-BEP800 suppressed VM formation by releasing the HSP90β and Twist1 interaction. Such a new role of HSP90 in VM could also be mediated by HCC cell-derived HSP90-contained EVs.

1.7 LRP1- HSP90 Complexes Can Gather Extracellular Matrix (ECM) on the Surface of Cells and EVs

We have also identified many ECM proteins in the fraction of EVs (Ono et al. 2018). It was shown that fibronectin (FN) on the surface of myeloma cell-derived exosomes mediates exosome-cell interaction (Purushothaman et al. 2016). Dr. Adrienne Edkins group has reported that FN is a stress-responsive gene regulated by HSF1 (Dhanani et al. 2017) and is anchored to the plasma membrane by LRP1-HSP90 complex (Hunter et al. 2014; Boel et al. 2018). We reported that cancer EVs often carry abundant levels of FN, LRP1, and HSP90 (Ono et al. 2018). FN often coats EVs (Purushothaman et al. 2016). Therefore, EV-coating FNs can be anchored to the EV membrane via HSP90-LRP1. Not only FNs but also many species of ECM are found in the EV fractions (Ono et al. 2018). EV-coating ECM can physically interfere with membrane fusion and horizontally transfer from the EVs to recipient cells (Fig. 1.2a, left). EVs can be used as wheel-like adaptors that enable cells to drive (migrate) on the "metastasis highways" made by ECM in tissues, most notably cancer cells (Sung et al. 2015) (Fig. 1.2b). EVs can also be kept on the surface of cells and at intercellular space, where EVs mediate cell aggregation (Fig. 1.2c) (Eguchi et al. 2018). ECM proteins and transmembrane proteins such as LRP1, EpCAM (Eguchi et al. 2018), and integrins were found to be associated with both cells and EVs and might thus mediate such new roles of EVs in cell aggregation.

However, we showed that HSP-rich metastatic OSCC-EVs lost ECM whereas low-metastatic HSP-poor OSCC-EVs were ECM-rich. Therefore, HSP90 might not connect EVs with ECM. We also identified members of matricellular CCN protein family in cancer exosome fractions (unpublished data). CCNs have been shown to bind with ECM, transmembrane (TM) proteins such as LRP1 and integrins, and growth factors. Therefore, we prospect not HSP90 but CCN proteins are essential adaptors between EVs, TM proteins, and ECM and thus contribute for generation of ECM-coating of EVs. Importantly, CCN2/CTGF plays a key role in bone metastasis (Shimo et al. 2006), indicating that CCN-positive, ECM-positive EVs may play a key role in pre-metastatic niche formation in bone metastatic breast cancer and prostate cancer.

1.8 Roles of Rab Proteins in Exosome Secretion

MVBs are formed by endocytic budding from an endosomal membrane into the lumen side of the compartment such as late endosome (Fig. 1.1). After vesicular accumulation, the MVBs are either sorted for cargo degradation in the lysosome or released from the cells into the extracellular space as exosomes by fusion with the plasma membrane. Recently, a large number of Rab proteins have been associated with the exocytic pathway, including Rab3, Rab11, Rab26, Rab27, Rab37 and Rab38 (Masuda et al. 2000; Nashida et al. 2006; Wasmeier et al. 2006; Rupnik et al. 2007; Tolmachova et al. 2007; Takahashi et al. 2012). Rab proteins belonging to Ras GTPase superfamily are small GTPases (20–25 kDa) comprising more than 60 proteins in *homo sapiens*. They play pivotal roles in regulating intercellular membrane trafficking including endocytosis and exocytosis such as exosome secretion and vesicles delivery between organelles (Chavrier and Goud 1999; Pereira-Leal and Seabra 2000; Stenmark 2009). The Hsp90 chaperone complex regulates GDI-dependent Rab (Rab1 and Rab3A) recycling (Chen and Balch 2006). Among Rab members, Rab11, Rab27, and Rab35 have been shown to play crucial roles in exosome secretion. Rab11 exists as two isoforms, Rab11A and Rab11B, and involved in recycling from an endosome to the plasma membrane, so-called slow recycling. Overexpression of the wild-type of Rab11 slightly stimulated exosome secretion (Savina et al. 2002). On the other hand, the inhibition of Rab11 function by overexpression of a dominant-negative mutant decreased exosome release (Savina et al. 2002). Similar to Rab11, Rab27 is widely conserved and are existed two isoforms, Rab27A and Rab27B. Their regulation of exosome secretion was confirmed by using breast cancer cell lines. Their inhibition of both Rab27 A and B was observed the fewer exosomes into the culture medium (Zheng et al. 2013). Rab35 regulates a fast endocytic recycling pathway for a lot of proteins to the plasma membrane (Kouranti et al. 2006). The inhibition of Rab35 activity in oligodendrocytes leads to intracellular accumulation of endosomal vesicles and reduces exosome secretion (Hsu et al. 2010). Such roles of Rab proteins in vesicle molecular trafficking might play roles in controlling cell differentiation. Indeed, Rab27A regulates the transport of cell surface receptors, modulating multinucleation and lysosome-related organelles in osteoclasts (Shimada-Sugawara et al. 2015). Additionally, Rab44, a novel large Rab GTPase, negatively regulates osteoclast differentiation by modulating intracellular calcium levels followed by NFATc1 activation (Yamaguchi et al. 2018). Although it is likely that more Rab family proteins are involved in secretion of exosomes and cell differentiation, the detailed mechanism remains to be clarified.

1.9 Conclusions

The significance and potential of EV-HSP90 and ex-HSP90 in the progression of cancer and wound healing is becoming apparent (Table 1.1). HSP90-LRP1 binding to the surface of EVs and cells appears to impact both cellular and tissue homeostasis and tumor progression at local regions and distant milieu. On-going hypotheses and theories of HSP90/LRP1-EVs were also explored in the review and suggested new areas of investigation particularly in the cancer field.

Table 1.1 Classification and functions of intracellular and extracellular HSP90

Alias	Localization of HSP90	Mechanism of generation	Function
HSP90, ic-HSP90	Cytoplasm, mitochondria, ER	1. Translation of cellular mRNA 2. Translation of EV-derived mRNA upon horizontal transfer	Impact on cellular proteostasis network, including; 1. Autophagy 2. The UPR in the ER 3. Cytoplasmic protein quality control 4. The cytoplasmic stress response
ex-HSP90 (EV-free)	Extracellular space	1. Hypoxia and HIF-1alpha 2. TGFα-EGFR signal 3. Stemness 4. Membrane damages of cells and EVs upon injuries, inflammation and cell stress	1. Chaperokine role via binding to its receptor LRP1, whose signaling promote wound healing, cancer cell survival, and EMT 2. Activity control of extracellular proteins, e.g. MMP (Eustace et al. 2004)
EV-HSP90	Inside EVs	1. Intracellular HSP90 is engulfed and incorporated into exosomes when MVB is generated 2. Intracellular HSP90 is engulfed and incorporated into ectosomes, apoptotic bodies or large oncosomes 3. Heat shock stress increased EV-HSP90 (unpublished data)	1. EV-HSP90 can be released into cytoplasm of recipient cells after membrane fusion between EVs and cells 2. New MVBs can be generated in recipient cells after endocytosis or phagocytosis of EVs
	On the outer surface of EVs	1. ex-HSP90 are bound to LRP1 on the surface of EVs after/upon secretion 2. HSP90 kept bound to LRP1-EVs before secretion	1. Chaperokine role via binding to its receptor LRP1, whose signaling promote wound healing 2. EV-cell interaction mediated by HSP90 and/or LRP1 triggers membrane fusion, endocytosis or phagocytosis 3. Activity control of extracellular proteins, e.g. MMP

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