Extracellular HSP90 in Cancer Invasion and Metastasis: From Translational Research to Clinical Prospects

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Abstract: During the last decade, the extracellular molecular chaperone HSP90 (eHSP90) has been identified as a critical effector in cancer cell invasion and metastasis by virtue of its interaction with a diverse cohort of molecules that serve as key nodal points in oncogenic pathways. Thus eHSP90 has most recently emerged as a novel target in cancer therapeutics, subsequently becoming the focus of several drug development efforts. This review highlights recent studies on the mechanisms through which eHSP90 exhibits its tumor cell invasion action. It also presents latest efforts to translate this cumulative knowledge into clinical practice to disable eHSP90-driven metastasis.

Keywords: Cancer therapeutics, wound healing, cell impermeable antibodies, signal transduction, pro-motility factors, extracellular.

INTRODUCTION

Heat shock protein 90 (HSP90), a ubiquitously expressed molecular chaperone essential for eukaryotic homeostasis, plays an important role in cell function under normal and stress conditions [1, 2]. There are two cytosolic HSP90 isoforms, referred to as HSP90α and HSP90β [3]. Intracellular forms of HSP90 are among the most abundant proteins in unstressed normal cells, where they perform housekeeping functions, such as regulation of the stability, activity, intracellular disposition and proteolytic turnover of numerous client proteins. Moreover, cytoplasmic HSP90 interacts with proteins involved in the development and/or survival of genetically unstable malignant cells, allowing mutant clients to retain or gain function. More specifically, HSP90 functions as a cellular buffer that enables tumor cells to tolerate genetic alterations, including the accumulation of potentially lethal mutant signaling molecules. As a result of this buffering capacity, phenotypic diversity within the tumor population increases, and the evolution of invasive metastatic and drug resistant phenotype accelerates [4].

While the regulatory functions and cellular importance of intracellular HSP90 have been extensively studied for over thirty years, it is only in the last decade that attention has been re-focused upon the comparatively less abundant population of extracellular HSP90 (eHSP90). This designation of eHSP90, which includes HSP90 detected in the extracellular space and on the cell membrane, has been linked to mechanisms related to cell migration during normal development as well as the invasion and metastasis of tumor cells [5-8]. This conserved feature of eHSP90 as a regulator of cell motility is further evident in studies demonstrating that inhibition of eHSP90 *in vivo* prohibits both wound healing [9] and tumor invasion [10]. Importantly, eHSP90 has also been detected in the blood of cancer patients, where its levels are positively associated with tumor malignancy [11]. Therefore, eHSP90 holds promise as a molecular target for the development of anti-cancer drugs to inhibit tumor metastasis. In this review, we will discuss recent developments in our understanding of the mechanisms by which eHSP90 promotes cell migration during development and wound healing and pay particular emphasis on the role of eHSP90 during cancer cell invasion and metastasis and the down-stream pathways and interacting molecules through which it exerts its pro-invasive action in tumors. We will finally discuss progress in efforts to inhibit the function of eHSP90 in supporting tumor invasion, an approach that bears great promise for the development of a new generation of anti-metastatic drugs.

1. EXTRACELLULAR ACTIVITY OF MOLECULAR CHAPERONES: A RECENTLY IDENTIFIED PHENOMENON

In recent years, findings from several groups support the premise that components of the intracellular chaperone machinery, including HSP90, HSP70, Hop, p23 and Cdc37, are present on the cell surface and have been associated with promotion of cell motility, which is a crucial event for development, wound healing and cancer invasion [5, 8, 12-16].

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Intracellularly molecular chaperones consist a central role in signaling networks, interacting with an array of client proteins exerting essential housekeeping functions, by controlling their folding, stability, activation, function and proteolytic turnover, but also through their interaction with client oncoproteins leading to tumor cell invasion. Although the components of the intracellular chaperone machinery do not act as classical chaperones extracellularly, in many cases they continue to act as pro-motility molecules during development and metastasis or repairing factors in wound healing processes. Due to their proximity in the extracellular space and their multi-tasking properties, primarily related to increased tumor cell invasion and metastasis as highlighted below, extracellular forms of HSP70, HSP90 and Cdc37 have been also arbitrarily characterized as extracellular chaperones.

1.1. eHSP70

Extracellular HSP70 (eHSP70) has recently been identified as a component of the extracellular HSP90 pro-motility complex. As described in detail below, this extracellular complex plays an essential role in matrix metalloproteinase 2 (MMP-2) activation and contributes to the invasion of breast cancer cells [17]. In addition, the co-chaperones Hop, HSP40 and p23 have been similarly detected on the surface of breast cancer cells, and biochemical studies confirm their co-immunoprecipitation with HSP90α *in vitro* in conditioned media.

1.2. eHSP90

A number of studies have correlated the high expression of eHSP90 both on the surface and in conditioned media derived from cancer cells with the tumor invasive properties of these cells [5], while cancer cell invasion and metastasis could be inhibited by an eHSP90 cell impermeable monoclonal antibody [7]. Additionally, the involvement of eHSP90 in the re-organization of the actin cytoskeleton and cancer cell invasion in prostate and bladder cancer has been reported by Tsutsumi *et al*., while interaction of eHSP90 with the extracellular domain of HER-2 during invasion process has been documented [16]. Moreover, it has been shown that eHSP90 expression is not elevated only during cancer invasion, but several hostile environmental conditions such as serum starvation, hypoxia and oxidative stress also trigger the extracellular localization and migration promoting properties of HSP90 [18]. Recently there has been accumulating evidence highlighting the existence of a small population of cells within tumors, cancer stem cells (CSCs), that are resistant to hypoxia and nutrient shortage [19] and are considered to be highly metastatic [9]. Given the capacity of CSC to survive under severe hypoxia conditions and nutrient shortage as well as to give rise to secondary tumors, preliminary data (Stivarou *et al*. unpublished observations) show over-expression of eHSP90 on CSC, indicating a further involvement of this protein in cancer cell invasion and metastasis. Overall, increasing evidence indicates that eHSP90, either secreted in the extracellular matrix or loosely attached on the cell surface, acts as a chaperone for the activation of proteins involved in the processes of wound healing and cancer cell invasion and metastasis thus demonstrating its critical importance for the occurrence of these events.

1.3. eCdc37

Cdc37 is considered as a key co-chaperone of HSP90, playing a specialized and indispensable role in the maturation and/or stabilization of a large subset of protein kinases, implicated in signal transduction, proliferation and survival [20]. Cdc37 was identified intracellularly as part of the protein complex involving HSP90 and the Rous sarcoma virus-encoded oncogene pp60v-src [21]. It acts as an adaptor, facilitating client kinase interaction with HSP90 [22] and stabilizing them in a folding-competent conformation [23]. In 2012, El Hamidieh *et al*. identified a cell surface pool of Cdc37 (eCdc37) that participated in breast cancer cell invasion. More specifically the authors showed that eCdc37 is localized on the surface of MDA-MB-453 and MDA-MB-231 breast cancer cells, is necessary for their invasive capacity and, similarly to its intracellular counterpart, specifically interacts with eHSP90. Moreover, it was shown that eCdc37 also interacts with receptor tyrosine kinases HER-2 and EGF-R on the surface of MDA-MB-453 and MDA-MB-231 cells respectively, and that this interaction is disrupted by the anti-HSP90 antibody mAb4C5. These results support an essential role for surface Cdc37 in concert with eHSP90 on the cell surface during cancer cell invasion processes[13].

2. eHSP90 FROM WOUND HEALING TO CANCER CELL INVASION

2.1. eHSP90 in Wound Healing

An increasing number of studies have highlighted the central role of eHSP90 in wound healing implying

that it functions as a guardian of extracellular homeostasis. Compatible with this notion, eHsp90 has been characterized as an essential mediator of tissue repair following injury. To this end, early reports have documented the participation of eHSP90 in regeneration processes following mechanical injury of the sciatic nerve. More precisely, it was shown that eHSP90 participates in Schwann cell migration during regeneration following sciatic nerve injury, through a mechanism that involves actin re-organization and lamellipodia formation [24, 25]. While the release of eHSP90 and additional chaperones following necrotic death was reported by Basu a co-workers [26], a variety of stimuli, related to inflammation are capable of inducing HSP90's extracellular localization, including DNA damage [27], oxidative stress [28], chemotherapeutic agents [29], growth factors and signaling mechanisms [30], heat-stress [31] and hypoxia [9, 32]. It is interesting to note that the majority of these stimuli are linked with cellular stress and likely to be present in a wounded environment, supporting the notion that eHSP90 functions in a protective capacity to buffer cellular stress. Cheng *et al*. have provided three unique properties of eHSP90α which are absent from conventional factors participating in wound healing, such as growth factors [30]. First, by contrast to the majority of growth factors that act on specific cell types eHSP90 is a common pro-motility factor for all three types of human skin cells involved in wound healing, keratinocytes, dermal fibroblasts and dermal microvascular endothelial cells, and is therefore more effective in the multi-cell-type participating process of wound healing. Second, eHSP90α can effectively promote migration of all three types of human skin cells, even in the presence of TGFβ3, which suppresses the action of the wound-healing factor PDGF-BB. Thirdly, there is evidence that eHSP90 promotes diabetic wound healing in particular, by way of bypassing the hyperglycemia-caused HIF-1α down-regulation and promoting migration of the cells that otherwise cannot respond to the environmental hypoxia. The involvement of eHSP90 in cell motility processes was further supported by Li *et al*. who reported that hypoxia caused HSP90 secretion by dermal fibroblasts, which in turn mediated the *in vitro* migration of these cells. Furthermore, the authors demonstrated that treatment of fibroblasts with antibodies against HSP90 inhibited cell motility, while application of recombinant HSP90 stimulated cell migration. The unique property of eHSP90α of promoting migration without being a mitogen also plays a physiological role in wound healing [9]. Following skin injury, keratinocyte migration occurs almost immediately preceding cell proliferation during wound healing. It has been proposed that while the cells at the wound edge are moving toward the wound bed, they leave "empty space" between them and the cells behind them. At a next step cells behind the migrating ones start to proliferate after losing contact inhibition with the front moving cells in order to re-fill the space generated by the front-migrating cells. The specific role of eHSP90α is to help to achieve the initial wound closure as quickly as possible so as to prevent infection, water loss, and severe environmental stress. [33]. Therefore, eHSP90 has a profound role functioning as a guardian of extracellular homeostasis.

2.2. eHSP90 in Cancer

Over the past years much attention has been given in a previously unrecognized extracellular role of HSP90, associated with cancer cell invasion and metastasis. As previously mentioned, the first direct evidence for eHSP90 involvement in tumor cell invasion *in vitro* was reported in 2004, when HSP90 was shown to be present on the cell surface of HT-1080 fibrosarcoma cells, while conditioned media derived from these cancer cells promoted their invasive capacity *in vitro* [5]. These results were confirmed in breast cancer cells, where cell surface HSP90 was shown to participate in cell invasion processes, including actin cytoskeletal re-arrangement and formation of membrane motile structures, triggered by the presence of Heregulin (HRG) in the culture medium [16]. In support of these findings, it has been also suggested that surface HSP90 participates in re-organization of the actin cytoskeleton and cancer cell invasion processes also in bladder cancer, prostate cancer and melanoma models [8]. The presence of HSP90 on the surface of melanoma cells was reported by Becker *et al*., who showed that its expression was dramatically up-regulated in malignant melanomas when compared to begnin melanocytic lesions [34]. Stellas *et al*. further confirmed surface localization of HSP90 in melanoma cells and additionally demonstrated that the cell impermeable mAb 4C5, inhibited both melanoma cell invasion and metastasis [7].

Additionally, using recombinant human HSP90 (rHSP90), Song *et al* have demonstrated that eHSP90 is a positive regulator of tumor angiogenesis, whereas as analyzed below, an anti-HSP90α antibody suppresses angiogenesis in an MMP-2-dependent manner [35], signifying the potential role of anti-HSP90

cell impermeable antibodies on the inhibition of tumor angiogenesis and growth. In addition to the above, quite recently, Defee *et al*. have reported that eHSP90 serves as a co-factor for mitogen-activated protein kinase (MAPK) activation during Kaposi's sarcoma-associated herpes virus (KSHV) infection, leading to tumorigenesis. [36]. They have also found that eHSP90 serves as an important co-factor for canonical activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and that eHSP90 inhibition suppresses KSHV-induced, NF-κB-dependent secretion of the pro-migratory factors, interleukin-8 and vascular endothelial growth factor as well as invasiveness of primary cells following *de novo* infection. As NF-kB is a major mediator of angiogenesis facilitating pathogenesis related to a wide variety of cancers, this report further links eHSP90 to angiogenesis and highlights the importance of focusing on new drugs that inhibit eHSP90 as an approach to battle cancer metastasis.

2.2.1. Clinical Relevance of eHSP90 Secretion by Tumor Cells

Taking into account the above, it has come to the clinically important question of which tumors do or do not secrete HSP90. So far, at least one critical regulator of HSP90 secretion has been established. Sahou *et al* showed that the HIF-1-αlpha transcription factor mediates hypoxia-induced HSP90α secretion in human dermal fibroblasts and keratnocytes [37]. This finding is relevant to cancer, since HIF-1-αlpha over-expression is associated with increased patient mortality in approximately 40% of human solid tumors, including colon, breast, gastric, lung, ovarian, pancreatic, prostate, and renal carcinomas,. independent of other specific mechanisms [38]. In support, Dales *et al* carried out anti-HIF-1-alpha immunohistochemical assays on frozen sections of 745 breast cancer samples and found that the levels of its expression correlated to poor prognosis, lower overall survival and high metastasis risk among both lymph node-negative and lymph node-positive patients [39]. Additionally, Sahu *et al*. have recently shown that down-regulation of the endogenous and constitutively expressed HIF-1-αlpha in breast cancer cell lines, MDA-MB-231 and MDA-MB-468, completely blocked HSP90α secretion [37].

Clinical relevance for tumor-induced HSP90 secretion is supported by demonstration of elevated HSP90 in patient serum, exemplified by the elevated eHSP90 levels in serum or plasma of patients with prostate, liver, breast, lung, pancreatic, and hepatocellular tumors as compared to cancer-free controls [11, 40]. At the same time, presence of HSP90 auto-antibodies in patients with late stage ovarian cancer [41], breast cancer [42] and osteosarcoma [43] lends further support to tumor-derived circulating HSP90.

Taken together, these studies strongly indicate that eHSP90 is involved in the molecular pathways leading to cancer cell invasion and metastasis. To this end, while more than 100 intracellular HSP90 interacting proteins have been reported, a relatively small number of surface molecules have been identified so far to interact with the extracellular pool of HSP90. However, the above mentioned studies demonstrate that the presence of HSP90 on the cell surface and/or the surrounding environment is a wide-ranging phenomenon suggesting that this extracellular pool of the molecule performs 'extra-ordinary' chaperoning activities to a number of extracellular molecules similarly to its intracellular counterpart [8].

3. eHSP90 MECHANISMS OF ACTION DURING CANCER CELL INVASION AND METASTASIS

eHSP90 promotes cell motility during cancer cell invasion by directly activating its various down-stream targets localized both in the extracellular matrix and on the cell membrane, such as matrix remodeling proteases and growth factor receptors. Thus eHSP90 seems to be a nodal molecule acting on multiple levels, which are summarized below.

3.1. Interaction of eHSP90 with Matrix Remodeling Metalloproteinases

Approximately 90% of cancer deaths are not from the primary tumor but due to metastasis to distant sites [44]. In order for tumor neo-vascularization and cell invasion processes to occur, degradation of the basement membrane as well as matrix remodeling are essential. Matrix metalloproteinases (MMPs) a family of zinc-binding endopeptidases that participate in the extracellular matrix (ECM) degradation molecular machinery during tumor invasion, have increased expression and activation in almost all human cancers [45]. The most common MMPs having a positive correlation with cancer cell invasion and metastasis are MMP-1, MMP-2, MMP-3, MMP-7, MMP-9, MMP-11 and MT-MMP [46]. Among these, MMP-2 and MMP-9 are of particular interest because, in addition to gelatin, they degrade type IV collagen, the basic component of the basement membrane, which is the main barrier separating in situ and invasive carcinoma from the

metastatic site [47, 48]. These MMPs are secreted in an inactive form and are activated extracellularly [49, 50]. Several lines of evidence support a role for extracellular HSP90α in MMP-2 activation. Eustace *et al*. in 2004 showed that HSP90α is secreted from fibrosarcoma cells and promotes their invasive capacity, through association with MMP-2,while inhibition of eHSP90 using the inhibitor geldanamycin, decreased MMP-2 activity. Additionally they showed that the decrease in MMP-2 activity caused by eHSP90 inhibition was directly responsible for the loss of invasiveness and could be rescued by adding back activated MMP-2 [5]. The association of eHSP90 with MMPs was further confirmed by Stellas *et al.,* who demonstrated that both HSP90α and HSP90β were secreted from breast cancer cells and that they participated in the activation of MMP-2 and MMP-9 necessary for cell invasion (Figure **1A**). Additionally, they showed that targeting eHSP90 with mAb 4C5, prevents maturation of the two metalloproteinases *in vitro* and inhibits metastatic deposition of breast cancer cells *in vivo* [51]. A step further, Sims *et al*., reported that eHSP90α-mediated activation of MMP-2 was assisted by a complex of co-chaperones outside of breast cancer cells. The authors demonstrated that the co-chaperones HSP70, Hop, HSP40, and p23 were present outside of breast cancer cells and co-immunoprecipitated with eHSP90a *in vitro* and in breast cancer conditioned media. This co-chaperone complex also increased the association of eHSP90α with MMP-2 and HSP90α-mediated MMP-2 activation *in vitro*, while inhibition of HSP70 in conditioned media reduced this activation and decreased cancer cell migration and invasion (Figure **1A**) [17]. Finally it has been shown that the extracellular interaction of MMP-3 with HSP90 occurs through the hemopexin domain of MMP-3 and that this interaction is critical for cell invasion [52]. These findings shift the focus from the well-studied proteolytic activity of MMP-3, that resides within its catalytic domain, to its non-catalytic hemopexin domain, as the central player in cancer invasion and add a new dimension to eHSP90 functions by revealing a hitherto un-described mechanism of MMP-3 regulation.

As an overall, targeting eHSP90-MMPs interaction in non-catalytic sites of MMPs with agents such as small inhibitors or antibodies may yield more effective and tissue-specific inhibitors.

3.2. Protease Plasmin Activation through HSP90 Exosomal Transfer

The secretion mechanism of eHSP90 is up to date not fully elucidated. However a major pathway leading to HSP90 extracellular localization is its secretion through exosomes. eHSP90 has been found in exosomes in immune and other physiologically normal cell types [30, 31, 53, 54] and was suggested to be in exosomes-derived from diabetic cells [55].

Recent studies demonstrate that eHSP90 is secreted via exosomes from mesotheloma cancer cells thus increasing their invasive capacity [56]. There is accumulating evidence that eHSP90α, once secreted through exosomes, activates the protease plasmin, consequently increasing tumor cell motility [57]. Additionally, it has been reported that interaction between eHSP90α, tissue plasminogen and annexin II, also found in exosomes, leads to plasmin activation and plasmin-dependent cell motility (Figure **1B**) [58]. One mode of action of exosome secreted HSP90α involves binding to plasminogen (tPA), whereas inhibiting eHSP90 decreased conversion of tPA to plasmin thus prohibiting cell migration. Therefore, it has been proposed that eHSP90 is part of an extracellular complex including annexin II, tPA and plasminogen that functions to increase cell motility [32]. To this end, Peinado *et al*., [59] identified an exosome-specific melanoma signature with prognostic and therapeutic potential comprised of TYRP2, VLA-4, HSP70, HSP90 and the receptor tyrosine kinase MET. The discovery of another protease, besides MMPs, activated by eHSP90 suggests the possibility that one role of eHSP90 in cancer cells is the activation of precursor proteins that contribute to cellular migration and invasion. Under the light of these events, it would be interesting to speculate that eHSP90 could activate a cassette of proteins that function collectively in cancer cell invasion. These proteins would act in concert to enhance breakdown and remodeling of the extracellular matrix and permit the tumor cell to invade its microenvironment. Thus, inhibition of eHSP90 could indirectly inhibit a growing number of proteins that are responsible for increased tumor cell movement making this molecule an attractive target for drug therapy to limit tumor invasion.

3.3. Interaction of eHSP90 with Fibronectin

ECM glycoprotein fibronectin (FN), a major cell-matrix and cell-cell adhesion mediator, is also involved in tumor invasion and metastasis [60-62] and is moderately or strongly over-expressed in specimens of various tumor types such as breast, lung, thyroid and esophageal cancer. Moreover expression of either epithelial-FN (E-FN) or stromal FN (S-FN) by cancer cells showed a significant correlation with

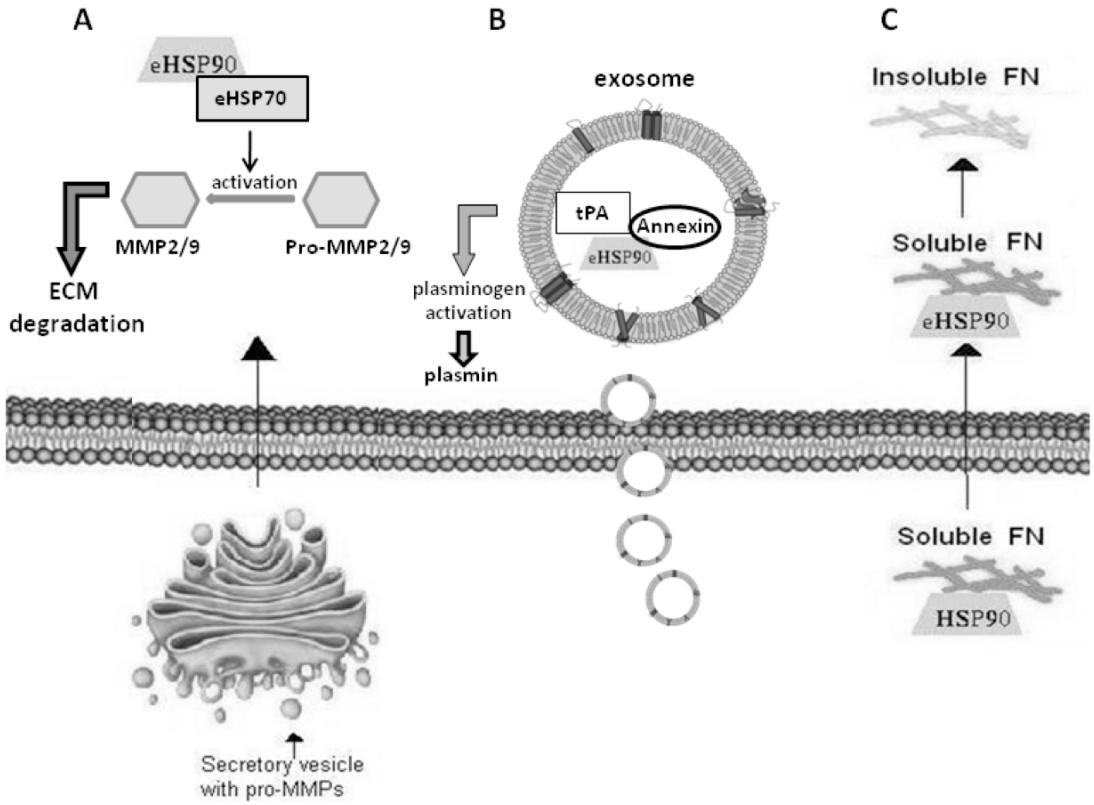

Figure 1: eHSP90 promotes cancer cell invasion by (**A**) inactivation of martrix remodeling matalloproteinases (MMPs) in concert with eHSP70, (**B**) exosomal transfer and plasminogen (tPA) activation and (**C**) conversion of extracellular fibronectin (FN) to its insoluble form.

clinicopathologic factors representing aggressive tumor biology [63]. Fibronectin interacts with HSP90 both intracellularly and extracellularly [61]. FN is secreted by cells as a soluble dimer that is then assembled into an insoluble network of fibers (Figure **1C**). Hunter *et al*. suggest that once soluble fibronectin is secreted from the cell, eHSP90 promotes its conversion to the insoluble form leading to increased cell invasion and metastasis through the degradation and remodeling of ECM [61].

3.4. Acetylation of eHSP90

In addition to co-chaperone association as well as ATP binding and hydrolysis, post-translational modifications such as hyper-phosphorylation [55, 64, 65], S-nitrosylation and reversible hyper-acetylation have also been shown to regulate the chaperone function of HSP90 [66, 67]. Among these post-translational modifications hyper-acetylation of HSP90 has been reported to promote its extracellular location and cause increased cancer cell invasion [68]. In particular treatment of cells with histone deacetylase inhibitor resulted in the extracellular location of hyperacetylated HSP90α that acted as a chaperone for MMP-2, promoting *in vitro* invasion of breast cancer cells. Additionally, Yang *et al*. [69], showed that K69Q, K100Q and K558Q substitutions promote the export and extracellular location of HSP90α. Thus, it seems that the acetylation status of HSP90α at specific sites may influence the extracellular localization of this molecule. Whether ATP binding and hydrolysis is also required for the extracellular chaperone function of HSP90α remains to be determined.

3.5. Interactions of eHSP90 with Growth Factor TGF-β1 and Growth Factor Receptors

Transforming growth factor-beta 1 (TGF-β1) is a multifunctional cytokine that regulates cell proliferation, migration and differentiation, as well as synthesis of the extracellular matrix [70-72]. Recent findings demonstrate that there is an abnormally high expression of TGF-β in osteosarcoma, which is the most common bone cancer in young adults [73, 74]. Additionally it has been reported that MG63 osteosarcoma cells secrete HSP90β into the extracellular space, thus inhibiting the activation of latent TGF-β1 resulting to a decrease in cell proliferation. On the other hand, TGF-β1-mediated

stimulation of MG63 cells resulted in the increased cell surface expression of HSP90β. Thus, it seems eHSP90β participates in a negative feedback loop inhibiting TGF-β1 signaling [75].

HER-2, a member of the ErbB family of receptor tyrosine kinases, is considered a ligandless receptor which shows preferred hetero-dimerization with HER-1, HER-3, and HER-4 [76-78]. HER-2 functions as a co-receptor to mediate signal transduction resulting in cell motility, mitogenesis, apoptosis, angiogenesis and/or cell differentiation. HER-2 over-expression is strongly associated with increased progression and metastasis in human breast and prostate cancer [76-80]. Whilst it had been shown already that intracellular HSP90 contributes to the stability of HER-2 via its cytoplasmic kinase domain [81-83], Sidera *et al*. [16] reported in 2008 that the molecular interaction of cell surface HSP90 with the extracellular domain (ECD) of HER-2 in MDA-MB-453 breast cancer cells is necessary for their invasion. More specifically, they showed that this interaction is essential for receptor activation and subsequent heterodimerization with HER-3 which in turn mediates signal transduction pathways via MAP kinase and PI3K/Akt, leading to actin re-arrangement necessary for cell motility (Figure **2A**). It has been reported previously that the ECD of HER-2 constitutively adopts an extended configuration with its dimerization arm exposed, suggesting that it is always poised to form heterodimers with ligand-activated forms of ErbB-receptors [76, 84, 85]. Taking this into consideration Sidera *et al*. speculated that surface HSP90 interacts with the HER-2 ectodomain in order for the receptor to maintain its active conformation. Finally, it should be noted that HER-2–HER-3 heterodimerization is essential for mediating the effects of growth factors such as HRG on cell motility. HRG binds to HER-3 to activate downstream kinase signaling pathways which lead to actin re-arrangement and cell invasion[16].

Epidermal growth factor receptor (EGF-R) another member of the ErbB family of receptors is expressed in various types of neoplasias including those in the lung, head and neck, colon, pancreas, breast, ovary, bladder and kidney as well as in gliomas [86]. The over-expression of EGF-R and TGFα by neoplasias confers a more aggressive phenotype by inducing cancer metastasis, resistance to chemotherapy and poor prognosis [87, 88]. Given its massive presence in several tumors and its key role in metastasis, EGF-R is defined as a principal target in anti-cancer therapies [89]. In 2012, El Hamidieh *et al*., identified EGF-R as another partner of eHSP90 acting together with eCdc37 in HSP90 extracellular chaperoning activities [13]. Additionally the authors showed that functional inhibition of eHSP90 using the cell impermeable anti-HSP90 mAb 4C5 antibody, led to significantly reduced formation of the eHSP90/eCdc37/ErbB receptor complexes in MDA-MB-453 and MDA-MB-231 breast cancer cells (Figure **2A** and **B**). Overall the above results support an essential role for the ErbB receptors in concert with eHSP90 on the cell surface during cancer cell invasion processes, with focus on the therapeutic potential of this interaction.

3.6. Role of eHSP90 as an Extracellular Signaling Peptide-Interaction with the LRP-1 Receptor

eHSP90 also acts as an extracellular signaling peptide, triggering across-the-membrane signaling. The best characterized receptor for eHSP90 is LRP-1 (low-density lipoprotein (LDL) receptor-related protein-1, also called α2-macroglobulin receptor, CD91 or TGFβR-V) [18]. LRP-1 has been shown to play an essential role in mediating eHSP90 signaling both *in vitro* and *in vivo*, resulting to activation of the Akt pathway, stimulation of cell migration, promotion of wound healing and tumor cell invasion. LRP-1 is widely expressed in various types of normal and cancer cells and has been reported to bind a wide variety of extracellular ligands, including lipoproteins, proteases and their inhibitors, ECMs and growth factors. The general function of LRP-1 is to protect tissue damage, a feature that all tumors can take advantage of [90]. LRP-1 expression is altered in certain cancer cells and this alteration influences the invasiveness of the cancer cells [90]. Cheng *et al*. provided direct evidence that the LRP-1 receptor mediates eHSP90 stimulated human skin cell migration *in vitro* and wound healing *in vivo*. Their study showed that neutralizing antibodies against LRP-1's ligand binding domain blocked recombinant HSP90-induced cell migration. In accordance, lentiviral-vector-mediated short hairpin RNA expression and down-regulation of LRP-1 abolished normal cell migration and cancer cell invasion in response to recombinant HSP90α, whereas re-introduction of LRP-1 (mini receptor) rescued the response [30]. Breast cancer cell invasion *in vitro* and tumor metastasis *in vivo* were greatly reduced by down-regulation of LRP-1 in these cells [37]. A step further, Gopal *et al*., showed that eHSP90-LRP1 interaction conferred invasive properties to highly aggressive glioblastoma cells and revealed a novel crosstalk mechanism where eHSP90-LRP1 signaling is an obligate step for the AKT-dependent activation of

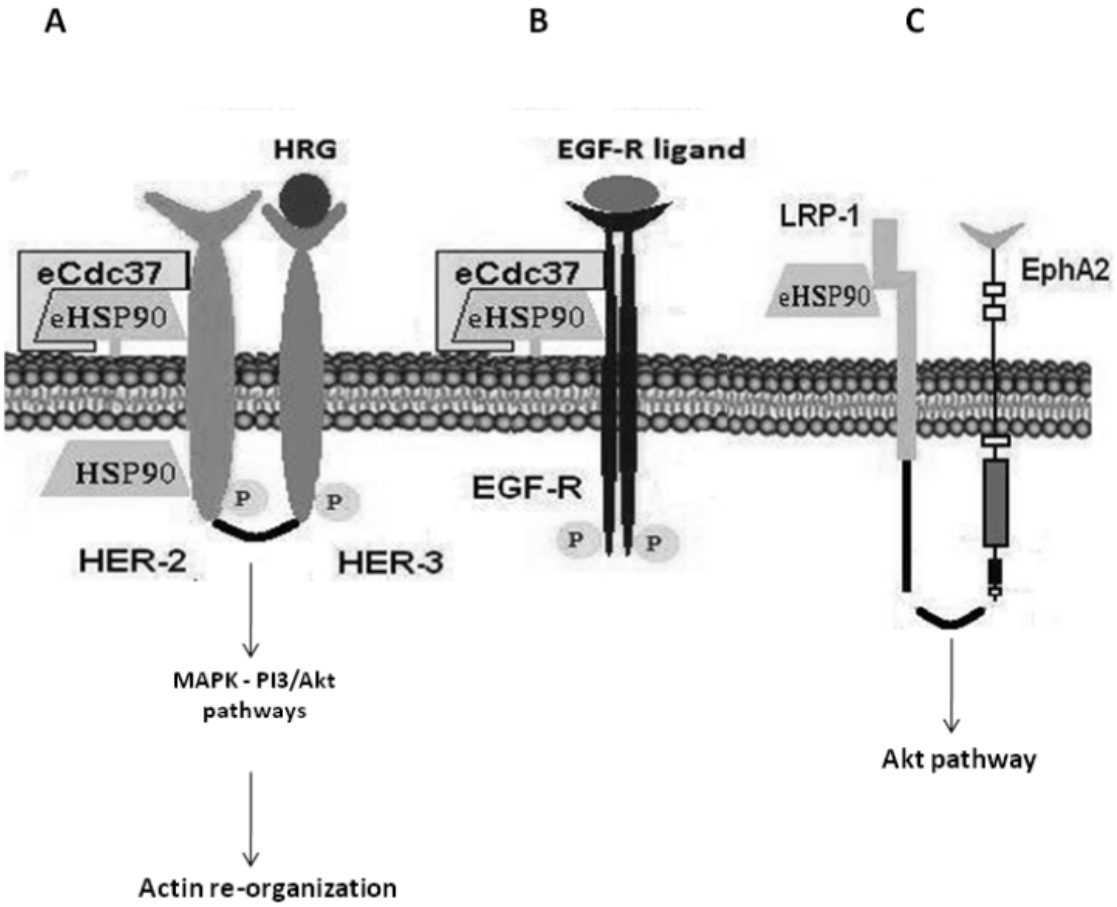

Figure 2: eHSP90 together with eCdc37 (**A**) induces HER-2 hetero- dimerization with HER-3 and subsequent cytoskeletal actin reorganization and (**B**) interacts with EGF-R, thus promoting cancer cell invasion (**C**) eHSP90 acts as a signaling peptide to LRP-1 receptor leading to the Akt-pathway activation and ultimately to cancer cell invasion.

the receptor tyrosine kinase EphA2 signaling leading to cancer cell motility and invasion [91] (Figure **2C**).

These reports, identifying another essential mechanism of eHSP90-dependent invasive function and the effector molecules participating in this mechanism, highlight new approaches to battle tumor aggressiveness.

3.7. Role of eHSP90 in Epithelial to Mesenchymal Transition Occurring in Cancer

Activation of the epithelial to mesenchymal transition (EMT) genetic program [92, 93] is a significant contributor to cancer progression. A universal hallmark of EMT is loss of epithelial cell polarity and acquisition of elongated mesenchymal morphology, concomitant with disruption of cell adhesion, increased cell motility, invasion, and metastasis [94]. The adherens junction protein E-cadherin acts as a gatekeeper in suppressing EMT events, and corresponding cell motility and dissemination, by maintaining the cuboidal phenotype and architecture that defines the morphology of normal epithelium [92, 93, 95]. As such, loss of E-cadherin function is a conserved and fundamental hallmark associated with early EMT events [93] and multiple preclinical models provide strong support for EMT in mediating cancer cell progression. There has been increasing evidence that eHSP90 modulates numerous events consistent with activation of EMT, such as promoting diminished cell adhesion and conversion to a more mesenchymal morphology (Figure **3**). Among these studies, eHSP90 has been identified as a pivotal regulator of E-cadherin function in prostate cancer, where it has been demonstrated that modest, physiologically relevant expression of eHSP90 reduced the expression and altered the localization of E-cadherin in epithelial cell lines [96] (Figure **3**). In addition to its role in initiating EMT events, eHSP90 has also been shown to be essential for sustaining the mesenchymal properties and behavior of more aggressive prostate cancer cell types and its expression levels are positively correlated with markedly elevated expression of a subset of pro-

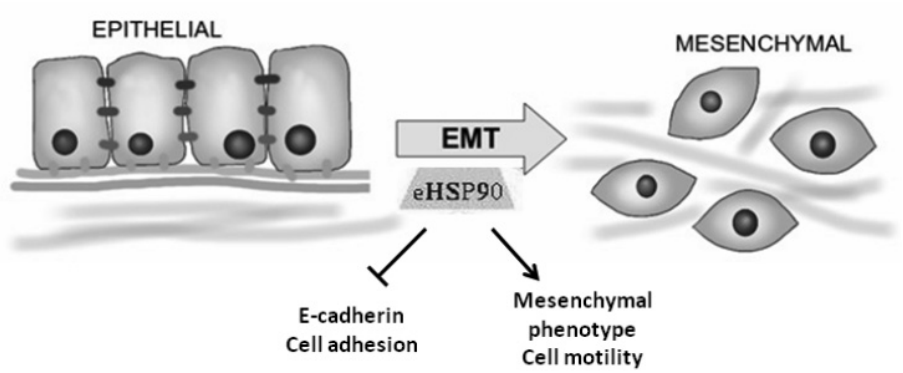

Figure 3: eHSP90 promotes epithelial to mesenchymal cell transition, necessary for cancer cell invasion, by reducing cell adhesion and E-cadherin expression (modified from Buddhini Samarasinghe Scientific American, October 2013).

tumorigenic eHSP90-regulated transcripts [96]. In the same study, it was further demonstrated that eHSP90 shifted cellular morphology towards a mesenchymal phenotype and promoted cell motility in an ERK and MMP-2/9-dependent manner. Conversely, inhibition of eHSP90 attenuated pro-motility signaling, blocked prostate cancer cell migration and shifted cell morphology toward an epithelial phenotype. The EMT initiating activity of eHSP90 was further supported by its ability to modulate a wide array of events consistent with this program, including up-regulation of the EMT effectors Snail, Twist, Zeb and Slug [96], which also serve as transcriptional repressors of E-cadherin [50, 97, 98]. Although more mechanistic details need to be elucidated, the above-mentioned data conclusively position eHSP90 as a novel and pivotal effector of tumor cell EMT plasticity.

4. FOCUS ON CLINICAL TRIALS USING INHIBITORS OF eHSP90

Over the last decade many efforts regarding HSP90 inhibitors for cancer therapy, have reached the clinical trial level. However the great majority of these inhibitors do not specifically target eHSP90. In this chapter we will present data concerning recent attempts to produce molecules that exclusively inhibit eHSP90, with the ultimate aim to develop agents for the treatment and cure of metastatic cancer.

4.1. Small Molecule Inhibitors of eHSP90

As a first attempt Eustace and co-authors covalently affixed the small molecule inhibitor geldanamycin to cell-impermeable beads and thus impaired cancer cell invasion by targeting exclusively eHSP90 [5]. Along the same lines, Tsutsumi *et al*., reported that a cell-impermeable and water-soluble form of 17-AAG, D-MAG-N-oxide, significantly reduced integrin/extracellular matrix-dependent cytoskeletal re-arrangement associated with cancer cell motility and invasion. Furthermore, this compound inhibited melanoma cell colonization *in vivo* [10]. The authors also showed that this inhibitor did not affect the stability of several known intracellular clients of HSP90, such as Akt and Raf-1, by contrast to its membrane-permeable counterpart 17-AAG that caused degradation of these signaling proteins. Under these conditions, the DMAG-N-oxide pre-treatment inhibited motility and invasion of bladder cancer, breast cancer, prostate cancer, and melanoma cells *in vitro* and reduced lung colonization by melanoma cells in mice [10]. Since these inhibitors target the ATPase activity of eHSP90, results of these two studies suggest that the N′-terminal ATP-binding region and ATPase activity of HSP90α are also required for eHSP90α function. More recently, McReady *et al*., introduced a novel cell impermeable eHSP90 inhibitor, STA-12-7191, derived from ganetespib and showed that it is markedly less toxic to cells and can inhibit cancer cell invasion in a dose dependent manner [99].

Based on the above-mentioned results of e-HSP90 inhibitors Bristol-Myers Squibb (BMS) acquired the California-based Kosan Biosciences in 2008 and launched a phase 3 clinical trial of tanespimycin on multiple myeloma in combination with Velcade (bortezomib). While results of the tests were described “very encouraging” by scientists involved in the trials, BMS permanently terminated the trial in 2010 without giving specific reasons. Additionally, Infinity Pharmaceutical, Inc. launched a phase 2 clinical trial of IPI-504 (retaspimycin hydrocloride) a water-soluble version of 17-AAG, in combination with Trastuzumab on breast cancers in 2009. This trial was terminated in 2011 because while it had modest clinical effect, the data fell short on the pre-specified efficacy criteria for continued trial expansion [100].

4.2. 'Bioconjugates' as Inhibitors of eHSP90

Sidera *et al*., [16] and Stellas *et al*., [7], by applying the cell-impermeable anti-HSP90 monoclonal antibody mAb 4C5, demonstrated that the *in vitro* MDA-MB-453 breast cancer cell invasion and the *in vivo* B16F10 melanoma cell metastasis was significantly reduced, respectively. Along the same lines, quite recently a number of patent applications regarding the inhibition of eHSP90 or its interaction with co-factors using 'bioconjugates' as a therapeutic strategy for metastatic cancer treatment have emerged. Jay *et al*., together with Xerion Pharmaceuticals claimed a series of 'bioconjugates' as eHSP90 inhibitors, including polypeptides, antibodies and antibody fragments, associated with coumarin and/or purine based HSP90 inhibitors and their analogs [101]. Udono *et al*., together with Riken disclosed the sequences of hybridomas 6H8 and 5H12 targeted against surface HSP90 [102], while Li *et al*., claimed a fragment of secreted HSP90 as a vaccine for monoclonal antibody drugs or target for small molecule drugs against a range of HIF-1-overexpressing tumors [33]. Finally Sidera *et al*., disclosed the sequence of a chimeric cell impermeable anti-HSP90 light chain immunoglobulin, named ch4C5, as a potential agent for the treatment of neoplasias [103].

The inhibition of the extracellular pool of HSP90 involved in cancer metastasis by cell-impermeable chemical or biological HSP90 inhibitors, is an interesting area for research, since these inhibitors could overcome the challenge of toxicity versus efficacy, that efforts with intracellular HSP90 anti-cancer drugs are confronted with. To this end, eHSP90 inhibitors are expected to selectively target eHSP90, present in high amounts on the cell surface of cancer cells, while minimizing potential damages in the physiological intracellular HSP90 chaperone functions in the surrounding normal cells.

In conclusion during the last years it has become evident that eHsp90 is a novel pro-motility factor, critical for wound healing and tumor progression. The mechanism of action and downstream targets of eHSP90 are being investigated and it is increasingly evident that eHSP90 provides a novel and promising molecular target for the development of effective anti-metastatic drugs. Although much progress is being made and compounds targeting eHSP90 already exist, it is noteworthy that no eHSP90 inhibitor has yet achieved an approval for clinical application in the treatment of cancer. The need for further development of next-generation cell impermeable eHSP90 inhibitors with improved pharmaceutical properties and tolerance remains a great challenge. A deeper understanding of the eHSP90 machinery structure and function as well as elucidation of the specific eHSP90-client interactions will most probably lead to novel molecules that prevent eHSP90 activity in metastatic cancers.

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