Hsp90α and Hsp90β Co-Operate a Stress-Response Mechanism to Cope With Hypoxia and Nutrient Paucity during Wound Healing

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Running title: A novel tissue repair mechanism

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Abstract
When tissues are injured and blood vessels clotted, the local environment becomes ischemic – lack of adequate supply of oxygen and glucose delivered to the surrounding cells. The heat shock protein-90 (Hsp90) family proteins protect tissues from various environmental insults and participate in the damaged tissue repair. Here we report discovery of a novel ischemia-responsive mechanism by which the two Hsp90 isoforms, Hsp90α and Hsp90β, work together to promote cell motility in wounded skin and accelerate wound closure. We demonstrate that Hsp90α and Hsp90β have distinct and non-exchangeable functions during wound healing. Under hypoxia and lack of serum factors, Hsp90β binds to the cytoplasmic tail of the LDL Receptor-Related Protein-1 (LRP-1) and stabilizes the receptor at the cell surface. Hsp90α, however, is secreted by the cell into extracellular space, where it binds and signals through the LRP-1 receptor to promote cell motility, leading to wound closure. In addition to skin injury, we suggest that this repair mechanism applies broadly to other non-cutaneous injured tissues.
Introduction
The microenvironment of wounded tissues is hypoxic and lack continued nutrient supply due to vascular disruption and high oxygen consumption by cells at the wound edge (Pai et al., 1992). Acute hypoxia in injured tissues is also a critical environmental cue that triggers initiation of the wound healing processes (Tandara et al., 2004). For instance, it has been shown that hypoxia promotes migration of human keratinocytes, the cell responsible for wound closure via re-epithelialization (O’Tool et al., 1997), and migration of human dermal fibroblasts, which deposit new extracellular matrices to the wound and support subsequent wound remodeling (Mogford et al., 2002). On the other hand, impaired responses to hypoxia are associated with impaired wound healing, such as the environment in chronic diabetic wounds (Botusan et al., 2008). In diabetic foot ulcers, in particular, the stability of the hypoxia-inducible factor-1 alpha (HIF-1α) protein is compromised due to the hyperglycemic environment, albeit the mechanism remains unclear (Catrina et al., 2004; Fadini et al., 2006; Gao et al., 2005). These in vitro and in vivo studies suggest that acute hypoxia is a natural shock signal to the injured tissues and, more importantly, a call for an immediate jump-start of wound healing. However, how the cells in the injured tissue cope with the new environment of hypoxia and then respond to it remained little beyond speculations. In this study, we show that the two heat shock protein-90 family members, Hsp90α and Hsp90β, work together in a unique fashion to repair skin wounds. Hsp90β works inside the cell to stabilize the LRP-1 receptor at the cell surface. Hsp90α, however, is being secreted to the extracellular space to bind and transmit pro-motility signal via the stabilized LRP-1 receptor. Together, these two Hsp90 isoforms promote the early phase of wound healing, i.e. wound closure, under hypoxia and nutrient paucity.

Results and Discussion
Hsp90α, but not Hsp90β, acts extracellularly to mediate hypoxia-triggered dermal fibroblast migration.
To establish the individual role of Hsp90α and Hsp90β in hypoxia-driven human dermal fibroblast (HDF) migration, a critical event during wound healing, we wanted to selectively down-regulate Hsp90α or Hsp90β in the cells. In order to prove isoform-specific down-regulation, we first verified the specificity of anti-Hsp90α and anti-Hsp90β antibodies. As shown in Figure 1A, indicated amounts of human recombinant Hsp90α and Hsp90β proteins were
resolved in an SDS gel and visualized by Coomassie blue staining (panel a). Duplicate membranes were immunoblotted with either anti-Hsp90α or anti-Hsp90β antibody, respectively. The results clearly show that anti-Hsp90α (panel b, lanes 1-3) and anti-Hsp90β (panel c, lanes 4-6) antibodies are specific for each of the Hsp90 isoforms with little cross reactivity. Using these antibodies, we were able to verify specific down-regulation of Hsp90α or Hsp90β protein. As shown in Figure 1B, sh-Hsp90α selectively downregulated Hsp90α (panel a, lane 2), but not Hsp90β (panel b, lane 2). Similarly, sh-Hsp90β only downregulated Hsp90β (panel b, lane 3), but not Hsp90α (panel a, lane 3). In these cells, however, simultaneously knocking down both Hsp90α and Hsp90β compromised the cells’ viability.

We next compared motility of these Hsp90α- or Hsp90β- knockdown cells in response either to PDGF-BB (the major growth factor for HDFs) stimulation (a physiological condition) or to an acute hypoxia (stress) using the colloidal gold migration assay (Li et al., 2004). In the absence of any stimuli and serum-free conditions, all cells showed a basal motility (Figure 1C, panels a, b, c). Both PDGF-BB and hypoxia stimulation increased migration of the control sh-LacZ-infected HDFs (panel d and g), as expected. Interestingly, neither Hsp90α nor Hsp90β downregulation affected the PDGF-BB-stimulated HDF motility (panels e and f vs. panel d). Instead we even detected a modest increase in PDGF-BB-stimulated HDF motility in Hsp90α downregulated cells (panels e vs. panel d). However, either Hsp90α or Hsp90β downregulation alone was sufficient to impair the hypoxia-induced HDF migration (panels h and i vs. panel g). We have previously reported that hypoxia triggers HDFs to secrete Hsp90 proteins during wound healing (Li et al., 2007). Thus, we tested if extracellular supplementation with Hsp90α or Hsp90β protein rescues the motility defect in Hsp90α- or Hsp90β- downregulated HDFs in response to hypoxia. We found that only the addition of recombinant Hsp90α, but not Hsp90β, protein was able to rescue the motility of Hsp90α-downregulated HDFs (panel j vs. panel l). However, neither Hsp90α nor Hsp90β was able to rescue the motility defect of Hsp90β-downregulated HDFs (panels k and m). Computer-assisted quantitation of the migration data was shown in Figure 1D. The above results indicated that 1) Hsp90α, but not Hsp90β, acts outside the cells to mediate hypoxia-induced HDF motility; 2) the extracellular role for Hsp90α could not be replaced by Hsp90β, and 3) the extracellular Hsp90α action requires the presence of intracellular Hsp90β.
To verify the finding that secreted Hsp90α mediates hypoxia-stimulated cell motility, we used antibody neutralization approach. As shown in Figure 2A, both PDGF-BB and hypoxia caused increased migration of serum-starved HDFs (bars 2 and 3 vs. bar 1). The addition of a control IgG did not affect hypoxia-induced migration (bar 4). However, anti-Hsp90α antibody blocked hypoxia-induced cell migration (bars 5-6 vs. bar 3), whereas anti-Hsp90β antibody showed little effect (bars 7 and 8). Neither anti-Hsp90α nor anti-Hsp90β antibody showed any detectable effect on the PDGF-BB-stimulated HDF migration (bars 9 and 10), indicating that the physiological and stress signals use distinct pathways to promote HDF migration. Moreover, extracellular Hsp90α showed significant chemotactic effect on HDFs using PDGF-BB as a positive control (Figure 2B, panel b vs. panel c), providing additional support to our previous finding that topical application of Hsp90α protein promotes both acute and diabetic skin wound healing (Cheng et al., 2011).

To confirm the differential mechanisms of action by Hsp90α and Hsp90β in vivo, we tested topical application of the proteins on wound healing using our newly established model in pigs (O’Brien et al. 2014). As shown in Figure 2C, with the control CMC (Carboxymethylcellulose) vehicle treatment, 1.5 x 1.5 cm full thickness wounds closed slightly more than 50% on day 7 (panel d vs. panel a). Treatment with recombinant Hsp90α protein accelerated wound closure to approximately 75% (panel e vs. panel b). Wounds treated with recombinant Hsp90β protein also showed a less degree of acceleration of wound closure (panel f vs. panel c). However, when we carried out histological analysis of wounds from three treatment groups, we noticed a dramatic difference in quality between Hsp90α- and Hsp90β- repaired wounds. As shown in Figure 2D, the Hsp90α-treated wounds achieved the similar epidermal thickness as that of unwounded skin (panel c’ vs. panel a’). CMC treatment showed significant reduction in the epidermal thickness (panel b’). However, the worst outcome in thickness of the re-epithelialized epidermis came from the Hsp90β-treated wounds, which closed on the same day to the naked eye as other treatments but had extremely thin epidermis (panel d’). We concluded that extracellular Hsp90α is superior to Hsp90β in promoting wound healing quantitatively and, moreover, qualitatively. Nonetheless, the reason for Hsp90β to promote wound closure remains unclear and the possibilities include its effect on wound contraction or its lower pro-motility activity on skin cells as we previously reported (Cheng et al., 2008) or both.
The role for Hsp90β is to bind and stabilize LRP-1 receptor from inside the cell.

What is the role for Hsp90β in promoting HDF motility in response to hypoxia? We focused on the stability of LDL receptor-related protein-1 (LRP-1), the cell surface receptor that mediates hypoxia-induced cell migration (Cheng et al., 2008; Woodley et al., 2009). We first examined which Hsp90 isoform, Hsp90α or Hsp90β, binds to the short (100 amino acids) cytoplasmic tail of LRP-1 as a chaperone. To focus on the cytoplasmic tail only, we pre-incubated the cells with RAP (receptor-associated protein) prior to lysis of the cells. Since RAP is known to bind to and block the entire extracellular ligand binding domains of LRP-1, this “pre-occupying” approach prevented endogenous Hsp90 from binding to the extracellular part of LRP-1 after cell lysis. Anti-LRP-1 immunoprecipitates of the cell lysates were subjected to Western blot analysis with anti-Hsp90α or anti-Hsp90β antibodies to identify LRP-1-associated Hsp90 isoform protein(s).

As shown in Figure 3A, increasing concentrations of RAP protein in pre-incubation did not affect the amount of LRP-1-associated Hsp90β (panel a, lanes 1-3). The slight increase in Hsp90β binding to LRP-1 was likely due to less internalization of LRP-1 following RAP binding from outside the cells (Bu et al., 2000). Surprisingly, however, we were unable to detect any Hsp90α from anti-LRP-1 immunoprecipitates (panel b). Equal amounts of LRP-1 pull-down from the cells were verified by blotting the immunoprecipitates (10%) with an anti-LRP-1 antibody (panel c) (Note: the 150-kDa, instead of 55-kDa, IgG in the SDS-PAGE gel was due to the required non-reducing working condition for the anti-LRP-1 antibody). These data indicate that Hsp90β, but not Hsp90α, binds to the cytoplasmic tail of LRP-1 receptor. Therefore, Hsp90β acted as an intracellular chaperone to stabilize LRP-1 at the cell surface and allowed secreted Hsp90α to bind LRP-1 and initiate cell motility signaling. Indeed, as shown in Figure 3B, we found that Hsp90β downregulation (panel a, lane 3), but not Hsp90α downregulation (lane 2), caused almost complete disappearance of LRP-1 in the cells. Under the same conditions, the PDGF receptor-β and EGFR levels either remained unchanged (panel b) or slightly reduced (panel c). The Hsp90β downregulation-caused LRP-1 downregulation was at the post-translational level, since the LRP-1’s mRNA levels remained unchanged (Figure 3C, panel a, lane 3 vs. lane 1).

To confirm the specific chaperone function for Hsp90β, not Hsp90α, in stabilizing the LRP-1
receptor and to rule out possible off-target effect of the shRNA-Hsp90β used, we carried out gene rescue experiments. First, we overexpressed Hsp90α in Hsp90β-downregulated cells to test if simply a physical replacement of the downregulated Hsp90β with Hsp90α would restore LRP-1 receptor. As shown in Figure 3D, Hsp90β protein downregulation was evident (panel a, lane 2 vs. lane 1) and overexpression of Hsp90α (panel b, lane 3) did not change the Hsp90β protein level (panel a, lane 3). A slight increase in the Hsp90α protein level in Hsp90β-downregulated cells was always detected (panel b, lane 2) in our experiments as well as in previous reports by others (Chatterjee et al., 2007). However, the exogenous Hsp90α overexpression was unable to recover the downregulated LRP-1 (panel c, lane 3 vs. lane 2), indicating that the chaperone function of Hsp90β could not be substituted by Hsp90α inside the cells. In contrast, when we reintroduced Hsp90β into the Hsp90β-downregulated cells back to its endogenous level (Figure 3E, panel a, lane 3 vs. lane 1), the physiological level of LRP-1 was completely recovered (panel c, lane 3 vs. lane 2). The Hsp90α protein levels remained unchanged in these cells (panel b, lane 3 vs. lane 1). Therefore, Hsp90β is the chaperone that specifically stabilizes the LRP-1 receptor.

**Exogenous expression of Hsp90β or LRP-1 rescues Hsp90β-downregulated HDF motility in response to hypoxia and extracellular Hsp90α stimulation.**

We expected that the shRNA-Hsp90β + exogenous Hsp90β-overexpressed HDFs should behave like their wild type counterparts and regain enhanced motility in response to hypoxia and extracellular Hsp90α stimulation. As shown in Figure 4A, Hsp90β downregulation inhibited hypoxia-stimulated (bar 6 vs. bar 3) and Hsp90α-stimulated (bar 5 vs. bar 2) cell migration. Exogenous re-expression of Hsp90β, which restored the downregulated LRP-1 receptor, corrected the defects (bars 8 and 9). In contrast, overexpression of Hsp90α was unable to do the same (bars 11 and 12). More convincingly, directly re-introducing a LRP-1 gene into Hsp90β-downregulated HDFs also restored the cell motility. As shown in Figure 4B, a mini-LRP-1 receptor, LRP-1-II, which mediates extracellular Hsp90α signaling (Tsen et al., 2013), was successfully introduced to the cells (panel a, lane 3 vs. lane 2) and restored the ability of the cells to migrate in response to extracellular Hsp90α stimulation (Figure 4C, bar 9 vs. bar 6). Taken together, the above findings revealed for the first time the working relationship between extracellular Hsp90α and intracellular Hsp90β in response to a major tissue injury signal,
hypoxia and nutrient paucity. A schematic representation of this novel repair mechanism is shown in Figure 4D. We believe that this mechanism applies not only to skin wound healing but broadly to other tissue repairs, as well as tumor progression where secreted Hsp90α plays a critical role.

Hsp90β gene knockout is embryonic lethal in mice (Voss et al., 2000). In contrast, mice developed normally without functional Hsp90α (Grad et al., 2010; Imai et al., 2011). These studies support the notion that Hsp90β, but not Hsp90α, represents the long recognized “chaperone” protein that maintains the intracellular signaling networks essential for life. In contrast, Hsp90α plays less chaperone role inside the cells than Hsp90β. We argue that Hsp90α is mainly an extracellular tissue repair molecule. Two recent studies showed that conditional deletion of Hsp90α in adult mice causes defects in piRNA biogenesis and spermatogenesis (Ichiyanagi et al., 2014; Kajiwara et al. 2012). There are two possible explanations of the findings. First, Hsp90α acts as a chaperone in these cellular events. The other is that those defects may also be due to lack of the extracellular functions of Hsp90α, in particular for spermatogenesis where the local environment is hypoxic with lower temperature than rest of the body. A key future experiment is to test if extracellular supplementation of the cells isolated from the mice with purified Hsp90α protein rescues the defects.

**Methods**

Primary human dermal fibroblasts were purchased from Clonetics (San Diego, CA) and were cultured in DMEM supplemented with 10% fetal bovine serum. The 3rd or 4th passages were used in cell migration assays. rhPDGF-BB was purchased from R&D System. Antibodies against PDGFR-β (3169) and EGFR (4267) were from Cell Signaling Technologies (Dancers, MA). Anti-LRP1/CD91 antibody (37-7600) was purchased from Life Technologies (Grand Island, NY). Mouse monoclonal antibodies against Hsp90α (CA1023) and Hsp90β (SMC 107) were from Calbiochem (Billerica, MA) and Stressmarq Biosciences (Victoria, BC, Canada), respectively. Anti-GAPDH antibody (GTX28245) antibody was from Genetex (Irvine, CA). Recombinant RAP protein was as previously described (Cheng et al., 2008).

**Recombinant Hsp90α and Hsp90β proteins production and purification**
See previously described (Cheng et al., 2011).

**Cell migration assay**

The colloidal gold migration assay was modified and described previously in details by us (Li et al., 2004). Data from independent experiments (n ≥ 3) were averaged and calculated as Migration Index (MI, %).

**Chemotaxis assay**

The transwell motility assay was carried out according to the manufacturer’s instruction (Cat no. 3422, Corning Life Sciences, Tewksbury, MA).

**The lentiviral systems**

Utilization of lentiviral systems for down-regulation and overexpression of genes are as previously described (Li et al., 2007; Cheng et al., 2008; Sahu et al., 2012). The shRNA sequences for human Hsp90α: GGAAAGAGCTGCATATTAA (sense) and for human Hsp90β: GCATCTATCGCATGATCAA (sense).

**Preparation of carboxymethylcellulose gel with Hsp90, wound healing, H&E and IHC staining and Statistics**

See details as previously described (O’Brien et al., 2014).

**Reverse Transcriptase PCR (RT-PCR)**

The primers for RT-PCR of LRP-1 are: 5’ CTCCCACCGCTATGTGATCC 3’ (forward) and 5’ ACTCATCTTGTGCTCGGCAA 3’ (reverse) and for GAPDH 5’-CCATCACCATCTTCCAGGAG-3’ (forward) and 5’ -CCTGCTTCACCACCTTCTTG-3’ (reverse) (Primer-Blast software). The products were visualized on an 1.5% agarose gel using Ethidium Bromide staining.

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References


**Figure Legends**

**Figure 1. Distinct requirements for Hsp90α and Hsp90β for hypoxia-triggered cell migration**

(A) The specificity of anti-Hsp90α and anti-Hsp90β antibodies was confirmed using purified recombinant Hsp90α and Hsp90β proteins in Western blot analysis.

(B) Lentiviral infection-mediated downregulation of endogenous Hsp90α and Hsp90β in HDFs, shown by Western blots.

(C) The above cells were serum-starved for 16 hours and subjected to colloidal gold migration assay. Motility was visualized as “migration tracks” indicated by dotted circles. Human recombinant (hr) Hsp90α and Hsp90β proteins were used to rescue Hsp90 downregulation-caused cell migration defects in response to hypoxia.

(D) Quantitation of the cell migration (in C) as Migration Index (MI, %). *n* = 4, *p* < 0.05

**Figure 2. Secreted Hsp90α, not Hsp90β, mediates hypoxia-triggered HDF migration and promotes wound healing.**

(A) HDF migration under indicated conditions. Anti-Hsp90α antibody inhibited hypoxia-triggered HDF migration (bars 5 and 6).

(B) $1 \times 10^5$ serum-starved HDFs without or with PDGF-BB (15 ng/ml) or 10 µg/ml recombinant Hsp90α stimulation were tested for induced chemotaxis using the transwell assay and quantitated as average percentage (%) of cells over the total number of seeded cells that penetrated toward the lower chamber.

(C) hrHsp90α and hrHsp90β proteins were compared for their effects on promoting pig wound healing. Topical application of Hsp90 proteins (100µg/ml) or control vehicle (CMC) was carried out once on day 0. *n* = 3, *p* < 0.05

(D) H&E staining of fully closed wounds on day 21. *n* = 20-24 (sections) per treatment.

**Figure 3. Only Hsp90β stabilizes the LRP-1 receptor.**
(A) HDFs in 150 mm dishes were serum-starved for 16 hours and incubated with increasing amount of RAP blocker. Cell lysates were immunoprecipitated with anti-LRP-1 antibody. Anti-LRP-1 immunoprecipitates were divided into three portions: 45% for blotting with anti-Hsp90α, 45% for blotting with anti-Hsp90β and 10% for blotting with anti-LRP-1 antibodies.

(B) Hsp90α- or Hsp90β-downregulated HDFs were examined for their effects on expression of LRP-1 (panel a), PDGFRβ (panel b) and EGFR (panel c), in comparison to parental HDFs (lanes 1) by Western blot analyses.

(C) RT-PCR analysis of LRP-1 mRNA in the cells.

(D) Hsp90β-downregulated HDFs were re-infected with a lentiviral vector carrying a wild type Hsp90α gene. The cellular levels of Hsp90β (panel a), Hsp90α (panel b) and LRP-1 (panel c) were examined by Western blot analysis with corresponding antibodies.

(E) HDFs with Hsp90β downregulation were re-infected with a lentiviral vector carrying a wild type Hsp90β gene. The cellular levels of Hsp90β (panel a), Hsp90α (panel b) and LRP-1 (panel c) were examined by Western blot analysis with corresponding antibodies.

Figure 4. Exogenously expressed Hsp90β rescues endogenous Hsp90β-downregulated HDF motility.

(A) HDFs with control sh-LacZ (bars 1-3), Hsp90β-downregulation (bars 4-6), Hsp90β-downregulation and re-expression (bars 7-9) or Hsp90β-downregulation and Hsp90α re-expression (bars 10-12) were subjected to the migration assay in response to the stimulations indicated. Quantitation of the data as migration index (MI, %) is shown. n = 3, p < 0.05

(B) Overexpression of LRP-1-II in Hsp90β-downregulated cells.

(C) LRP-1-II rescues the motility defect of Hsp90β-downregulated cells in response to extracellular Hsp90α stimulation.

(D) Schematically, when tissue is damaged, acute hypoxia triggers cells in the wound edge to secrete Hsp90α. Hsp90β stabilizes LRP-1 and the secreted Hsp90α binds and signal through the LRP-1 receptor to promote cell migration and wound healing.
Figure 1

A. Stain/Blot

hrHsp90α (ng)∗
30 50 150
hrHsp90β (ng)∗
30 50 150

Coomassie

Stain/Blot

a.

b.

c.

anti-Hsp90α

anti-Hsp90β

B. Blot

Sh-LacZ
Sh-90α
Sh-90β

a.

b.

c.

anti-Hsp90α

anti-Hsp90β

anti-GAPDH

D. Migration Index (MI, %)

PDGF
Hypoxia

a. sh-LacZ
b. sh-Hsp90α
c. sh-Hsp90β

j. +hrHsp90α∗
k. +hrHsp90β∗
l. +hrHsp90α∗
m. +hrHsp90β∗

sh-Hsp90α
sh-Hsp90β

Extracellular rescue

* hrHsp90: Human recombinant Hsp90
Figure 2
Figure 3