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Extracellular heat shock protein-90α: linking hypoxia to skin cell motility and wound healing

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Hypoxia is a microenvironmental stress in wounded skin, where it supports wound healing by promoting cell motility. The mechanism of the hypoxia action remained speculative. Here, we provide evidence that hypoxia promotes human dermal fibroblast (HDF) migration by inducing secretion of heat shock protein-90alpha (hsp90α) into the extracellular environment through hypoxia-inducible factor-1alpha (HIF-1α). The secreted hsp90α in turn executes hypoxia's pro-motility effect. Expression of an activated HIF-1α mimicked, whereas expression of an inactive HIF-1α or suppression of endogenous HIF-1α blocked, hypoxia-induced hsp90α secretion and HDF migration. Interestingly, the hypoxia–HIF-1 pathway-induced hsp90α secretion required neither changes in the steady-state mRNA level nor in the promoter activity of hsp90α. Recombinant hsp90α fully duplicated the hypoxia effect on HDFs. Inhibition of extracellular hsp90α function completely blocked the hypoxia–HIF-1 pathway-stimulated HDF migration. More intriguingly, topical application of hsp90α accelerated wound healing in mice. This study has demonstrated a novel mechanism of hypoxia > HIF-1 > hsp90α secretion > skin cell migration > wound healing, and identified extracellular hsp90α as a potential therapeutic agent for skin wounds.

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Introduction

Hypoxia plays a critical role in the pathophysiology of a variety of human disorders, such as ischemic cardiovascular disease, stroke, chronic lung disease, acute skin wounds and cancer (Semenza, 2000). After acute injury, the microenvironment of a skin wound is hypoxic, likely due to vascular disruption and high oxygen consumption by cells at the edge of the wound and in granulation tissue (Hunt *et al*, 1972; Niinikoski *et al*, 1972; Varghese *et al*, 1986). This hypoxic environment induces increased synthesis and secretion of growth factors, such as TGFβ1 (Falanga *et al*, 1991), PDGF (Kourembanas *et al*, 1990) and VEGF (Shweiki *et al*, 1992) by the resident skin cells. Induction of these growth factors could either be mediated by hypoxia-inducible factor (HIF)-dependent pathway or by a reactive oxygen species (ROS)-dependent pathway that does not involve HIF (Sen *et al*, 2002). Acute hypoxia enhances proliferation of human dermal fibroblasts (HDFs) (Falanga and Kirsner, 1993), whereas, under chronic hypoxia, HDFs decrease their proliferative capacity (Siddiqui *et al*, 1996). Hypoxia also promotes HDF migration (Mogford *et al*, 2002; Lerman *et al*, 2003) and human keratinocyte (HK) migration (O'Toole *et al*, 1997; Xia *et al*, 2001). Although the mechanisms were unclear, these observations suggest that acute hypoxia plays a positive role in early skin wound healing (Tandara and Mustoe, 2004). Subsequently, following the completion of wound healing and revascularization, oxygen tension gradually normalizes.

In mammalian cells, HIF-1 is a ubiquitously expressed heterodimeric transcription factor consisting of HIF-1α and HIF-1β subunits, and is widely known as a key regulator of cellular oxygen homeostasis (Semenza, 2000). In the HIF-1 complex, the HIF-1α subunit responds specifically to hypoxia, whereas the HIF-1β subunit is constitutively present within the cell. Under normoxic conditions, the HIF-1α subunit is rapidly degraded by an O_2-dependent prolyl hydroxylation that targets HIF-1α to the ubiquitin–proteasome pathway. As a result, HIF-1α is kept at a low steady-state level, preventing the formation of the functional HIF-1α–HIF-1β transcriptional complex. Under hypoxia, the degradation pathway is suppressed and HIF-1α levels increase rapidly. The increased HIF-1α dimerizes with HIF-1β and binds to hypoxia response elements of target genes, implicated in the control of cell proliferation, survival, motility, apoptosis, angiogenesis and cellular metabolism (Semenza, 2003). Elson *et al* showed that in skin HIF-1 and HIF-1 target genes are differentially expressed in multistage epithelial wound healing (Elson *et al*, 2000). Transgenic mice overexpressing a constitutively active HIF-1 show increased dermal capillaries and VEGF expression (Elson *et al*, 2001). HIF-1 also mediates hypoxia-induced TGF-beta3 expression, a potent anti-scarring cytokine, in fetal skin (Scheid *et al*, 2002). Thus, there has been little challenge for the well-established pathway of hypoxia > HIF-1 > gene expression > cell responses.

Cell migration is the result of repeated cycles of cytoskeletal-mediated protrusion and polarization, formation of adhesive contacts, cell contraction and retraction at the trailing edge (Lauffenburger and Horwitz, 1996). Within each cycle, the initiation, progression and completion of the sequential events are regulated mainly by two types of extracellular

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cues, the extracellular matrices (ECMs) and soluble growth factors (GFs) (Li *et al*, 2004b). Hypoxia has recently emerged as the third environmental regulator of skin cell motility, following ECMs and GFs. In the current study, we investigated the mechanism by which hypoxia enhances primary HDF migration *in vitro* and wound healing *in vivo*. Results of our study demonstrated the following mechanism: hypoxia > HIF-1 accumulation > secretion of hsp90α > hsp90α action > enhanced skin cell migration and wound healing. Identification of the important extracellular function of hsp90α may provide insights into new treatments of non-healing wounds.

Results

Hypoxia enhances dermal fibroblast migration only when there is no supply of growth factors

We wished to establish the correlation between environmental oxygen content and HDF motility, and then to determine if hypoxia-enhanced HDF migration depends upon simultaneous presence of an ECM or a GF or both. To study these questions, the individual cell-based colloidal gold cell migration assay was performed (Albrecht-Buehler, 1977). In this assay, cells are allowed to attach and migrate on a colloidal gold surface precoated with an ECM in the absence or presence of GFs. As the cells move, they leave behind migration tracks that can be visualized under dark field microscopy and summed as a 'migration index' (MI) by a computer-assisted analysis (Woodley *et al*, 1988). The presence of a cell-cycle inhibitor in the assay excludes potential contribution of cell proliferation. We performed this assay under either normoxia or hypoxia, with cells apposed to a type I collagen matrix and in the absence or presence of PDGF-BB. The combined effect of type I collagen and PDGF-BB maximally promotes HDF migration (Li *et al*, 2004c). As shown in Figure 1A, hypoxia clearly stimulated HDF migration on collagen, but under serum-free conditions in an oxygen content-dependent manner, reaching the maximum pro-motility effect at 1% O_2 content (panels a–e). We found that 0.5% or lower oxygen content caused significant detachment and death of the cells. The average size of the migration tracks under each experimental condition is highlighted with an open circle. As expected, the addition of PDGF-BB to the cells under normoxia greatly stimulated HDF migration

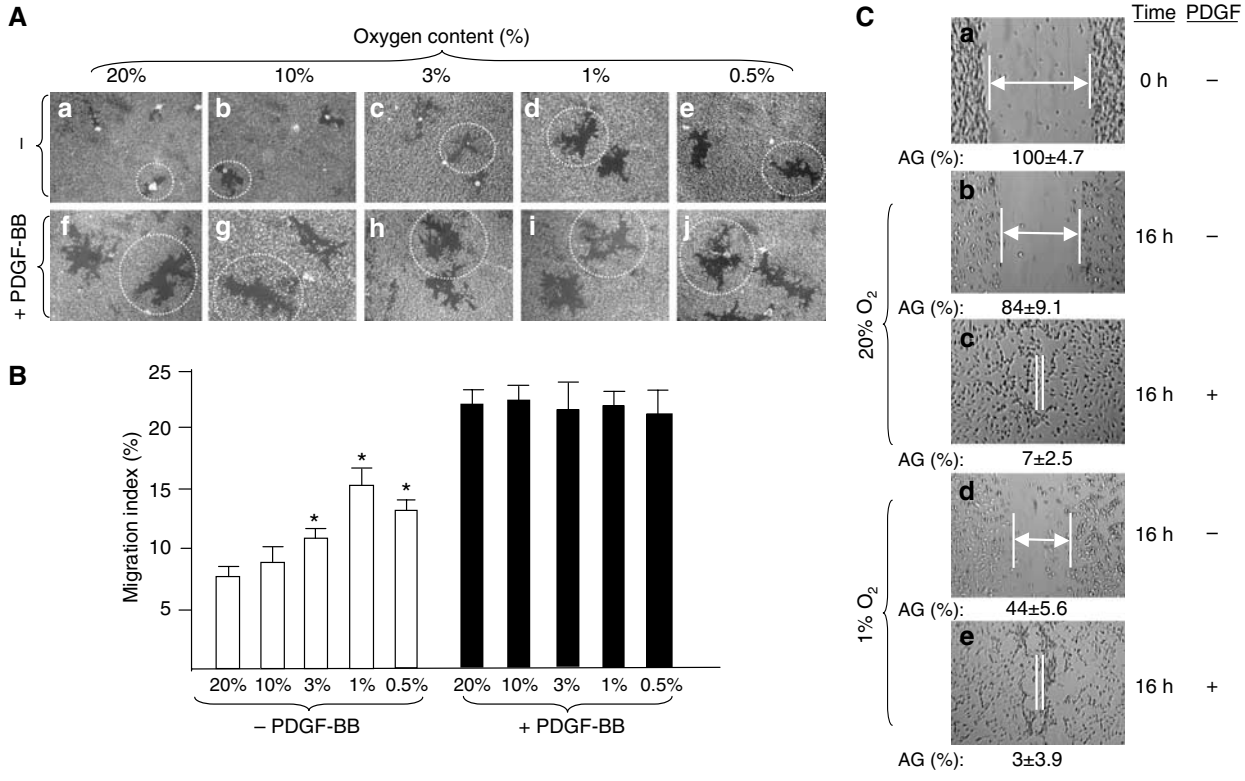

Figure 1 Hypoxia selectively promotes HDF migration on ECM in the absence of GFs. HDFs were serum-starved and subjected to colloidal gold migration assays under two defined conditions, (1) on a GF-free (−), collagen-coated surface and (2) on a collagen-coated surface in the presence of PDGF-BB (+), in response to the indicated environmental oxygen contents. Cell migration in all experiments was stopped at 16 h, analyzed and presented in two ways: (**A**) representative images of the migration tracks on collagen without PDGF-BB (panels a–e) or on collagen in the presence of PDGF-BB in media (panels f–j) are shown, in which the average size of migration tracks under each experimental condition was highlighted by dotted circles for the purpose of visual comparisons; (**B**) computer-assisted quantitation of the migration tracks is shown as MI, which measures the percentage area of 15 randomly selected microscopic fields consumed by cell migration tracks over the total area under the microscope. Bars with * are statistically significant over normoxia (20% oxygen) without PDGF-BB, $P<0.01$. (**C**) Hypoxia promotes HDF motility in 'scratch' assay. HDFs were cultured to a subconfluent monolayer in six-well tissue culture plates precoated with collagen to a subconfluent monolayer. The cells were serum-starved overnight and subjected to scratch assays (1) on collagen without GF (panels a, b and d) and (2) on collagen plus PDGF-BB (panels c and e), under either normoxia (20% O_2) or hypoxia (1% O_2). Experiments were stopped at 16 h and the remained cell-free space was analyzed in two ways: (1) the images of cell migration were photographed and (2) the remained cell-free space was quantitated as average gap (AG), which was carried out as previously described (Li *et al*, 2004a). The values shown are the means ± s.e.m. of three independent experiments.

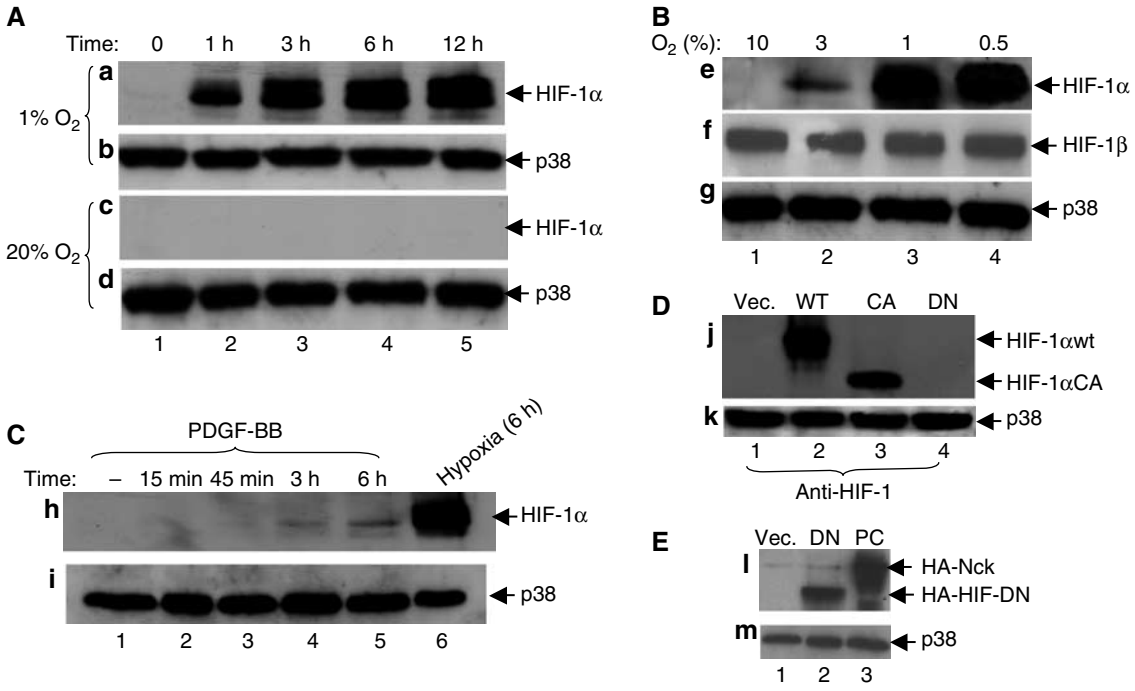

Figure 2 Induction of HIF-1α in HDFs by hypoxia versus PDGF-BB and establishment of HIF-1α constitutively expressing cells. The lysates of HDFs, which were subjected to (**A**) hypoxia (1% O_2) or normoxia (20% O_2) for the indicated time, (**B**) various levels of hypoxia or (**C**) stimulation with PDGF-BB (15 ng/ml, under 20% O_2) for the indicated times, were analyzed by Western immunoblotting analysis using antibodies specifically against HIF-1α (panels a, c, e and h) or HIF-1β (panel f). Anti-p38 antibody blotting of the lower portions of the same or duplicate membranes was used as sample loading control (panels b, d, g and i). (D, E) HDFs were infected with lentivirus carrying vector (vec.), HIF-1αwt, constitutively activated HIF-1α (CA) or dominant-negative HIF-1α (DN). After 48 h following infection, the lysates of the cells were immunoblotted with anti-HIF-1α antibodies (**D**, panel j) or anti-HA tag antibody (**E**, panel l). Anti-p38 antibody blotting of the lower portions of the same membranes was used as a sample loading and subsequent densitometry scan control (panels k and m).

(panel f versus a). Interestingly, hypoxia showed little further enhancement of HDF migration over normorxia, when PDGF-BB was present in the medium (panels g–j versus panel f). Quantitation of the migration tracks shows that hypoxia's pro-motility effect is limited to the cells apposed to an ECM matrix but without GFs, such as PDGF-BB (Figure 1B, left versus right panel).

The above finding was confirmed by a second cell motility assay, the 'scratch assay', in the presence of DNA replication inhibitor mitomycin C. Cells in this assay as a population share more closely their intercellular relationships *in vivo*. As shown in Figure 1C, hypoxia only enhanced the closure of 'scratched area' of the cells on collagen in the absence of PDGF-BB (panel d versus b). In the presence of PDGF-BB, the hypoxia-specific pro-motility effect could not be quantitatively distinguished (panel e versus c). The increased space among the cells, which underwent significant migration toward the scratched area, was likely due to the fact that the cell number remained the same before and after cell migration into the cell-free area. Third, similar results were also reproduced using a transwell cell migration assay (see Figure 7B, bar 2 versus bar 1). These findings suggest that *in vivo* hypoxia partially compensates for the absence of soluble GFs to promote cell motility.

Induction and function of HIF-1 is essential for transmitting hypoxia's pro-motility signal to HDFs

To investigate the mechanism of hypoxia-induced HDF motility, we examined the HIF-1α level, the rate-limiting factor in formation of the functional HIF-1α–HIF-1β transcription complex, in HDFs on collagen under either normoxia or hypoxia. As shown in Figure 2A, hypoxia caused increased protein levels of HIF-1α in a time-dependent manner, maximally between 3 and 6 h after exposure to hypoxia (panel a, lanes 3 and 4). In contrast, under normoxia, little HIF-1α protein could be detected (panel c). The lower portions of the membranes were blotted with anti-p38 MAPK antibodies to assure equal protein loading (panels b and d). The hypoxia-induced increase in HIF-1α correlated with the decrease in environmental oxygen content. As shown in Figure 2B, 1% O_2 caused the maximum induction of HIF-1α protein in the absence of GFs (panel e, lane 3). As expected, the cellular levels of HIF-1β remained unchanged (panel f). Interestingly, as shown in Figure 2C, PDGF-BB stimulation of HDFs under normoxia only slightly increased the cellular level of HIF-1α and it took a much slower time course compared with hypoxia-induced HIF-1 levels (panel h, lane 5 versus 6). In addition, the presence of PDGF-BB did not further enhance hypoxia-induced HIF-1α levels (data not shown).

To investigate whether increased HIF-1α levels are responsible for hypoxia-enhanced HDF migration, we constructed lentiviral viruses carrying (1) HIF-1αwt (Jiang *et al*, 1996), (2) a constitutively activated HIF-1α (HIF-1αCA, non-degradable form) (Kelly *et al*, 2003) and (3) a dominant-negative HIF-1α (HIF-1αDN) (Jiang *et al*, 1996). We have previously shown that the lentiviral gene transduction efficiency is more than 90% in primary HDFs (Chen *et al*, 2003; Li *et al*, 2004c; Fan *et al*, 2006). Following a single infection, the protein products of the exogenously expressed HIF-1α genes under normoxia were analyzed by Western immunoblotting analysis with an

anti-HIF-1α antibody. As shown in Figure 2D, endogenous HIF-1α was undetectable under normoxia (panel j, lane 1). As expected, the HIF-1αCA5 was readily detected owing to its non-degradable nature (lane 3). In addition, a strong expression of the HIF-1αwt was also detected (lane 2). It was likely due to the amount of overexpression that saturates HIF-1α degradation machinery. However, as HIF-1αDN lacks the N-terminal region of HIF-1α (amino acids 391–826), which contains the epitopes for all currently available anti-HIF-1α antibodies, its expression could not be detected by blotting with the anti-HIF-1α antibodies (lane 4). Therefore, we probed with an anti-HA tag antibody against the HA tag that is fused to the HIF-1αDN. As shown in Figure 2E, the expected 45-kDa protein was detected in HIF-1αDN-infected HDFs (panel l, lane 2), but not in the vector-infected cells (lane 1). HA-Nck was included as a positive control for anti-HA antibodies (lane 3).

These HIF-1αwt, HIF-1αCA- and HIF-1αDN-expressing cells, and the vector control HDFs, were then subjected to cell migration assays under either normoxia or hypoxia in the

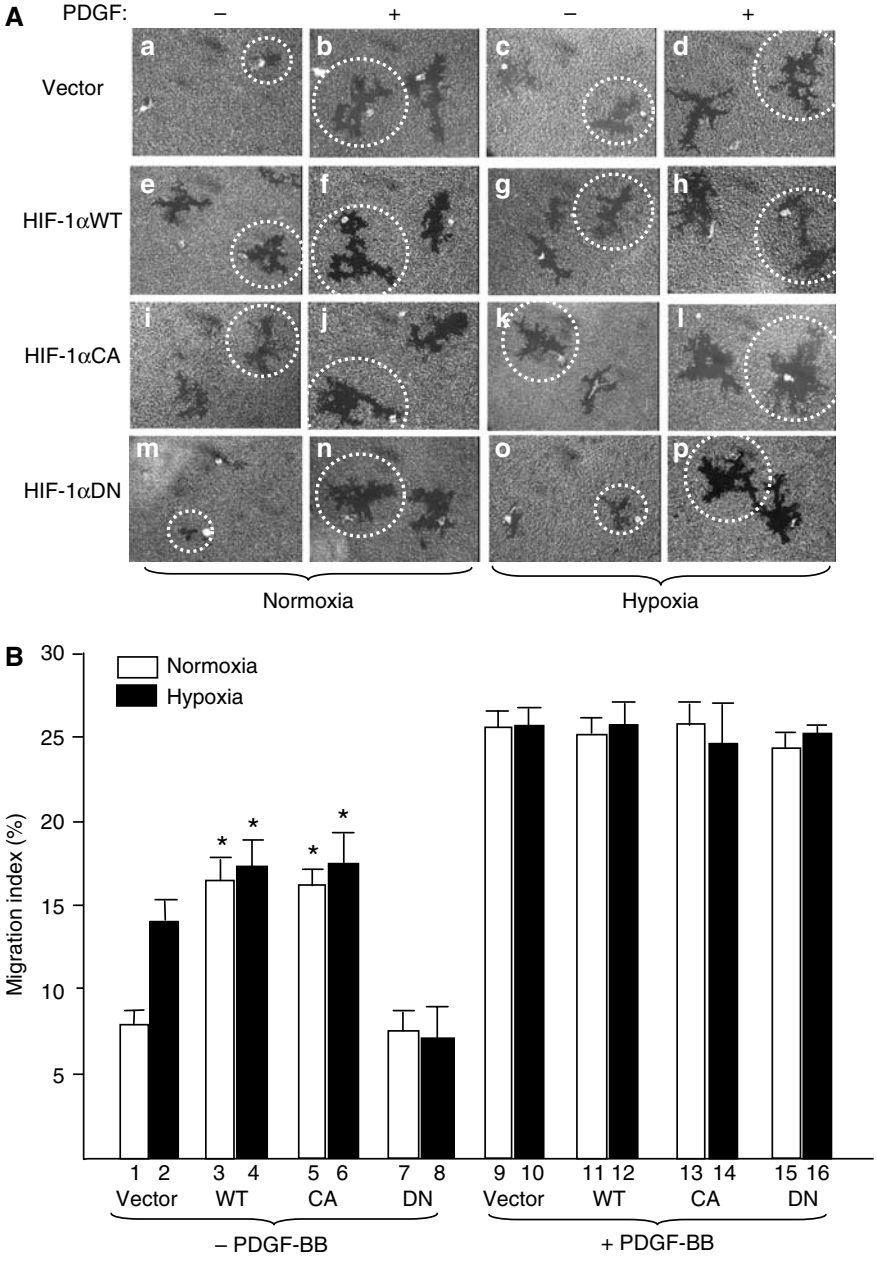

Figure 3 Forcefully maintaining HIF-1α expression mimics the full pro-motility effect of hypoxia. HDFs were infected with lentiviruses carrying vector, HIF-1αwt, HIF-1α-CA or HIF-1α-DN. After 48 h following infection, the cells were subjected to colloidal gold migration assays under either hypoxia (1% O_2) or normoxia (20% O_2) in the absence or presence of PDGF-BB (15 ng/ml). (**A**) The representative images of the migration tracks under the indicated conditions are shown. The average size of migration tracks is highlighted with a dotted circle for visual comparisons. (**B**) Computer-assisted quantitation of the migration is shown as MI, as previously described. Bars with * are statistically significant over their control under the same experimental condition (such as bars 3–6 versus bar 1), $P<0.05$. In the presence of PDFGF-BB, data under hypoxia were statistically insignificant over the normoxia control (such as bars 11 and 12 versus 9 and 10), $P>0.05$. Four repeated experiments showed similar results.

presence or absence of PDGF-BB. As shown in Figure 3A, overexpression of HIF-1αwt or HIF-1αCA enhanced HDF migration in the absence of PDGF-BB even under normoxia (panels e and i versus panel a), just like the hypoxia's effect on parental HDFs (panel c). Exposing the cells to hypoxia did not further increase migration of these cells (panels g and k versus e and i). In contrast, the HIF-1αDN-expressing HDFs failed to show enhanced migration under either normoxia (panel m versus a) or hypoxia (panel o versus c). Consistent with earlier observations, in the presence of PDGF-BB, we could not detect any significant difference in the migration of any of these cells (panels b, f, j and n versus d, h, l and p). Quantitation of the migration data is shown in Figure 3B. These results clearly indicate that induction of HIF-1α is essential for transmitting the pro-motility effect of hypoxia in HDFs (bars 3 and 5 versus bar 2). In contrast, HIF-1α does not mediate PDGF-BB-stimulated HDF migration, as the HIF-1αDN showed little inhibitory effect (bars 15 and 16).

To confirm the essential role of HIF-1α in hypoxia-enhanced HDF migration, we designed siRNA against human HIF-1α and delivered it into HDFs with the lentiviral vector-derived siRNA-expressing system, FG-12 (Qin *et al*, 2003). Our previous studies demonstrated that the FG-12 system offers more than 94% gene transduction efficiency to HDFs (Bandyopadhyay *et al*, 2006; Fan *et al*, 2006). To demonstrate the effectiveness of our designed siRNA, the total cellular proteins of HDFs, after being infected with the virus carrying HIF-1α-siRNA or control lacZ siRNA under either normoxia or hypoxia, were immunoblotted with an anti-HIF-1α antibody. As shown in Figure 4A, under hypoxia, HIF-1α protein was induced in parental HDFs (panel a, lane 1) and in HDFs infected with control lacZ siRNA (lane 3). However, this induction was completely suppressed in HDFs infected with HIF-1α-siRNA (lane 2). As expected, under normoxia, little HIF-1α protein could be detected in any of these cells (panel b). Anti-p38 antibody blotting of the same membrane was used as a control for sample equal loading (panel c). When these cells were subjected to migration assays under normoxia or hypoxia, as shown in Figure 4B, we found that HDFs infected with HIF-1α-siRNA were no longer able to migrate in response to hypoxia (panel d versus c). As expected, both parental HDFs and the HDFs expressing lacZ siRNA exhibited enhanced migration in response to hypoxia (panels b, f versus a, e). The quantitation of the cellular migration (MI) is presented below each of the representative images. Therefore, induction of HIF-1α is essential for hypoxia-induced HDF migration.

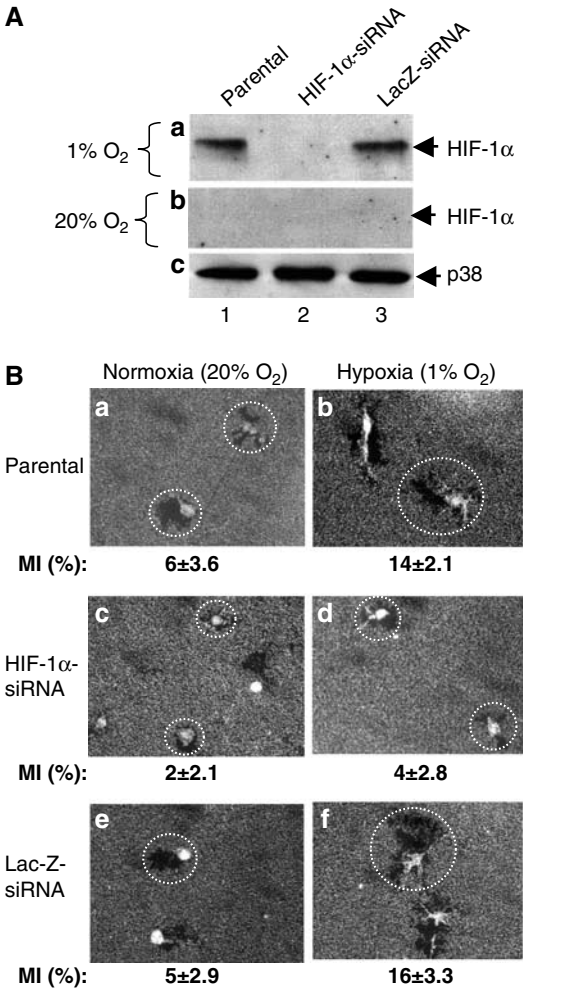

Figure 4 Preventing HIF-1α from accumulation blocks hypoxia's pro-motility effect on HDFs. HDFs were infected with FG-12 lentivirus carrying siRNA against HIF-1α (Materials and methods). (**A**) Lysates of cells under either hypoxia (panel a) or normoxia (panel b) were immunoblotted with anti-HIF-1α antibodies. The lower part of the membrane was blotted with anti-p38 antibody as a loading and densitometry scan control. (**B**) After 48 h following infection, the cells were subjected to colloidal gold migration assays under either hypoxia (1% O_2) or normoxia (20% O_2) in the absence of any GFs. Representative images of the migration tracks under the indicated conditions are shown. The average size of the migration tracks under each experimental condition is highlighted with dotted circles. Computer-assisted quantitation of the migration tracks, MI, is shown below each image. The increased motility in the controls (panel b versus a, and f versus e) and the decreased motility owing to downregulation of HIF-1α (panel c versus a) are statistically significant, $P<0.01$. The values shown are the means ± s.e.m. of the MIs from three independent experiments.

Secretion of hypoxic HDFs retains the full hypoxia's pro-motility effect

We asked the critical question: what is the downstream effector of HIF-1 that mediates hypoxia-enhanced HDF migration? We postulated that hypoxia triggers, via HIF-1, secretion of one or more key pro-motility factor(s) into the extracellular environment. The secreted factor(s) in turn acts to promote HDF migration. Therefore, serum-free conditioned medium (CM) from HDFs cultured under either normoxia or hypoxia, as well as CM from HDFs that overexpress HIF-1αCA, HIF-1αDN or HIF-1α-siRNA, was generated and analyzed. The procedure for preparation of serum-free CM is schematically shown in Figure 5A, in which the key point is that the procedure prevents any exogenous serum factors from being left in the CM and any polypeptides in the CM must come from secretion of the cells. As shown in Figure 5B, none of the concentrated normoxic CM from parental HDFs showed any significant enhancement of HDF migration over the control (bars 3–6 versus bar 1). In contrast, the hypoxic CM from parental HDFs enhanced HDF migration in a concentration-dependent manner (bars 7–10 versus bar 1). Furthermore, the induction of HIF-1 is fully responsible for the appearance of pro-motility factors in the CM, as even the normoxic CM from HDFs overexpressing HIF-1αCA was able to duplicate hypoxia's pro-motility effect (Figure 5C, bar 5

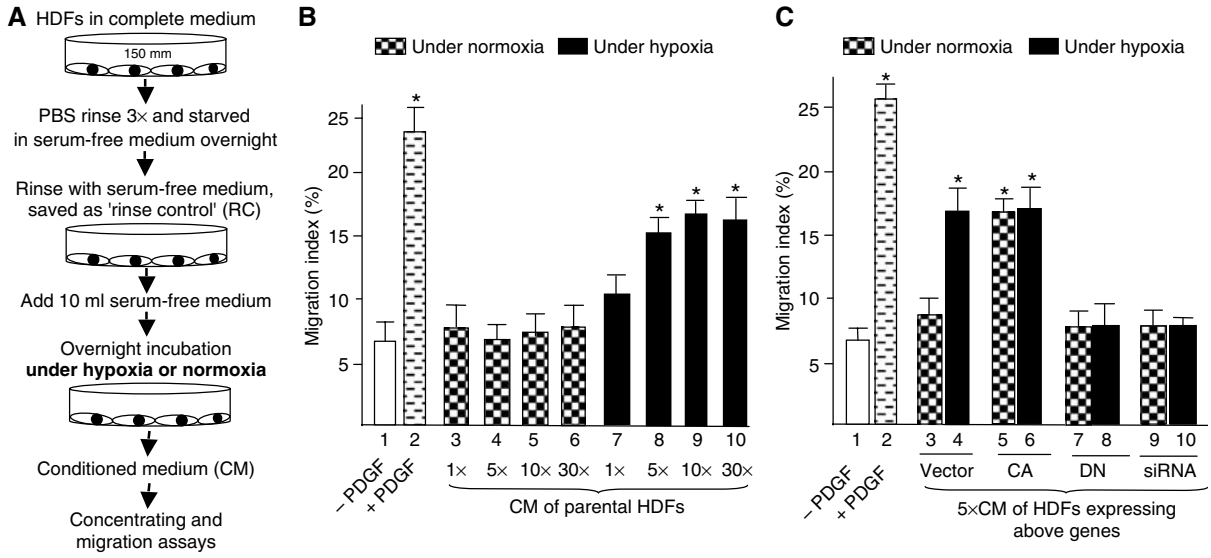

Figure 5 Hypoxia-triggered and HIF-1-mediated secretion of HDFs duplicates the full pro-motility effect of hypoxia. HDFs, wild type or HIF-1α-engineered, were cultured to subconfluence on collagen in serum-containing medium, washed in PBS and then serum-starved overnight. After rinsing with fresh serum-free media (RC, 10 ml/150-mm dish) and changing to a new set of serum-free media (10 ml/150 mm dish), the cells were incubated under either hypoxia (1% O_2) or normoxia (20% O_2) for 16 h. Cell-free medium (CM) was collected from each plate and concentrated by centrifugation. (**A**) A schematic summary of serum-free CM preparation procedure. (**B**) CM from parental HDFs under either normoxia or hypoxia were concentrated 5–30-fold (×) and tested for pro-motility effects on HDFs on collagen by colloidal gold migration assays. (**C**) CM from HDFs expressing vector alone (vector), HIF-1α-CA (CA), HIF-1α-DN (DN) or HIF-1α-siRNA (siRNA) were compared for their ability to stimulate HDF migration. Only the migration indices are shown. Bars with * are statistically significant over –PDGF-BB control, $P<0.02$. Unmarked bars are statistically insignificant over –PDGF-BB control, $P>0.05$. This experiment was repeated more than six times and the results were reproducible.

versus 4). Hypoxia added no further pro-motility activity to the CM from HDFs overexpressing HIF-1αCA (bar 6). In contrast, neither normoxia nor hypoxia CM from HIF-1αDN-overexpressing HDFs (bars 7 and 8) or from HIF-1α-siRNA-overexpressing HDFs (bars 9 and 10) showed any significant pro-motility effect on HDFs. These data suggest the following mechanism by which hypoxia promotes HDF migration: hypoxia > induction of HIF-1α > secretion of downstream factor(s) > enhancement of HDF migration.

Secreted heat-shock protein-90α executes hypoxia's pro-motility effect on HDFs

To identify the molecular nature of the motility-promoting activity in the hypoxic CM from HDFs, we compared the secreted proteins in the CM from HDFs cultured under normoxia and hypoxia by SDS–PAGE followed by silver staining (see Supplementary data). We were specifically interested in proteins that were only present in hypoxic CM. As shown in Figure 6A, surprisingly we observed that there were limited qualitative differences in the secreted proteins between normoxic CM (lane 1) and hypoxic CM (lane 2). The relatively distinguishable proteins were three with molecular masses of 40, 50 and 91 kDa (as indicated by arrows) in the hypoxia-CM (lane 2), but not in normoxic CM (lane 1). Mass spectrometry analyses of these proteins excised from the SDS–PAGE gel revealed that they are glyceraldehyde-3-phosphate dehydrogenase (GAPDH), matrix metalloproteinase 1 (MMP1) and heat-shock protein-90alpha (hsp90α).

We focused on hsp90α, as neither GAPDH nor MMP1 alone in its recombinant form showed any significant effect on HDF migration (data not shown). Western immunoblotting analyses of the CM (20 μl, 5 ×) confirmed the mass spectrometry data that the hypoxia-CM, but not normoxia-CM, contained hsp90α. As shown in Figure 6B, hsp90α was clearly detected from hypoxic CM (lane 2), but not normoxic CM (lane 3), by an anti-human hsp90α-specific antibody. More interestingly, similar amounts of hsp90α were also detected in CM of HDFs overexpressing the constitutively activated HIF-1αCA under either hypoxia (lane 4) or normoxia (lanes 5). In contrast, hsp90α was not detectable in CM from HDFs overexpressing the dominant-negative HIF-1αDN (lanes 6 and 7) or HIF-1α-siRNA (data not shown). Commercial recombinant hsp90α (50 ng) was included as the positive control (PC, lane 1). Based on a standard curve made from an increasing concentration of the commercially available hsp90α in SDS–PAGE and their densitometry scanning data, the working concentration of hsp90α in the hypoxia CM from HDFs (5 ×) was approximately 6–8 μg/ml.

The critical question then was whether the secreted hsp90α was responsible for the pro-motility activity in hypoxic CM from HDFs. First, we tested if the addition of an anti-hsp90α neutralizing antibody would inhibit the hypoxic CM-stimulated HDF migration under normoxia. As shown in Figure 6C, the hypoxic CM without any added antibodies stimulated HDF migration (bar 3 versus 1) and even the hypoxic CM with added control IgG showed a similar enhancement of HDF migration (bar 4). In contrast, the addition of anti-hsp90α neutralizing antibodies to the hypoxic CM blocked its pro-motility activity in a dose-dependent manner (bars 5–8). We then tested if recombinant hsp90α could duplicate the pro-motility effect of hypoxia or hypoxic CM. As shown in Figure 6D, recombinant hsp90α enhanced HDF migration in a dose-dependent manner (bars 4–7 versus 1),

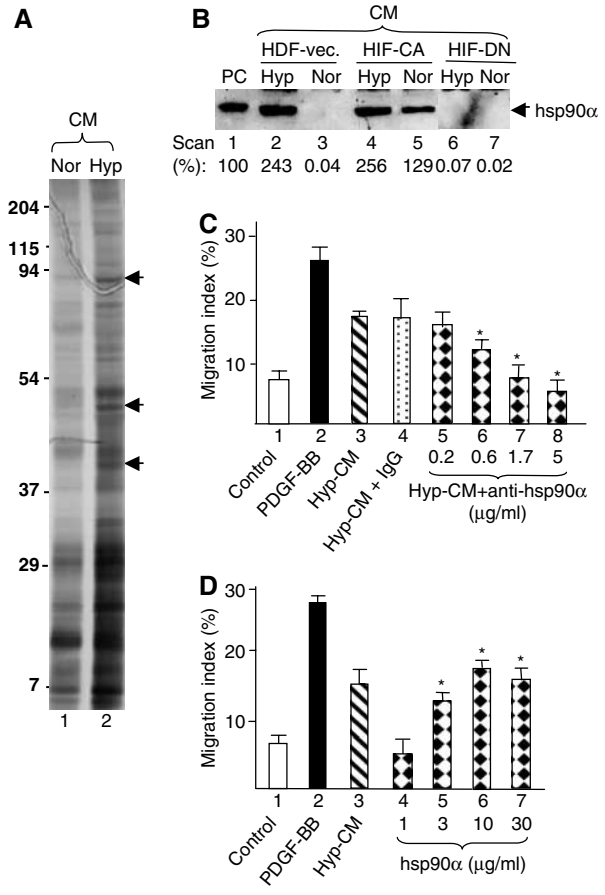

Figure 6 Secreted hsp90α is necessary and sufficient to replace hypoxia for promoting HDF migration. (**A**) The hypoxia-CM (Hyp-CM) and normoxia-CM (Nor-CM) of wild-type HDFs were concentrated to 50-fold (vol/vol) and equal volumes (that contain 148 and 96 μg of the total cellular proteins, respectively) were resolved in an SDS–PAGE and stained with Silver Stain Plus (Bio-Rad). The arrow indicates peptides that were excised, extracted and analyzed by mass spectrometry. (**B**) The Hyp-CM and Nor-CM of wild-type HDFs (vector alone) and HDFs expressing HIF-1α-CA or HIF-1α-DN were concentrated to 50-fold (vol/vol) and equal volumes of the CM (30 μl) were resolved in an SDS–PAGE, transferred onto a nitrocellulose membrane and immunoblotted with antibodies specific against hsp90α (CA1023). Commercial hsp90α (100 ng) was included as the positive control for both specificity and relative quantity (PC). (**C**) Anti-hsp90α neutralizing antibodies block Hyp-CM-stimulated HDF migration. HDFs were subjected to colloidal gold migration assays in the absence (bar 1) or presence of PDGF-BB (15 ng/ml, bar 2), Hyp-CM (5 ×, bar 3), Hyp-CM (5 ×) plus 5 μg of control IgG (bar 4) or Hyp-CM (5 ×) plus increasing amounts of anti-hsp90α neutralizing antibodies (SPS-771) (bars 5–8). The values shown are the means ± s.e.m. of the MIs from four independent experiments. Bars with * are statistically significant over their control under the same experimental condition (such as bars 6–8 versus bar 3), $P<0.05$. (**D**) Recombinant hsp90α duplicates the full pro-motility effect of Hyp-CM (5 ×) in HDFs. HDFs were subjected to colloidal gold migration assays in the absence (bar 1) or presence of PDGF-BB (15 ng/ml, bar 2), Hyp-CM (5 ×, bar 3) or increasing amounts of human recombinant hsp90α (bars 4–7). Only the migration indices are shown. The values shown (MI) are the means ± s.e.m. of three independent experiments. Bars with * are statistically significant over their control under the same experimental condition (such as bars 5–7 versus bar 1), $P<0.03$.

just like the hypoxic CM (bar 3). The optimal concentration of 10 μg/ml of hsp90α was similar to that of 5 × hypoxic CM (bar 6 versus 3), which was calculated to contain approximately 6–8 μg/ml (data not shown).

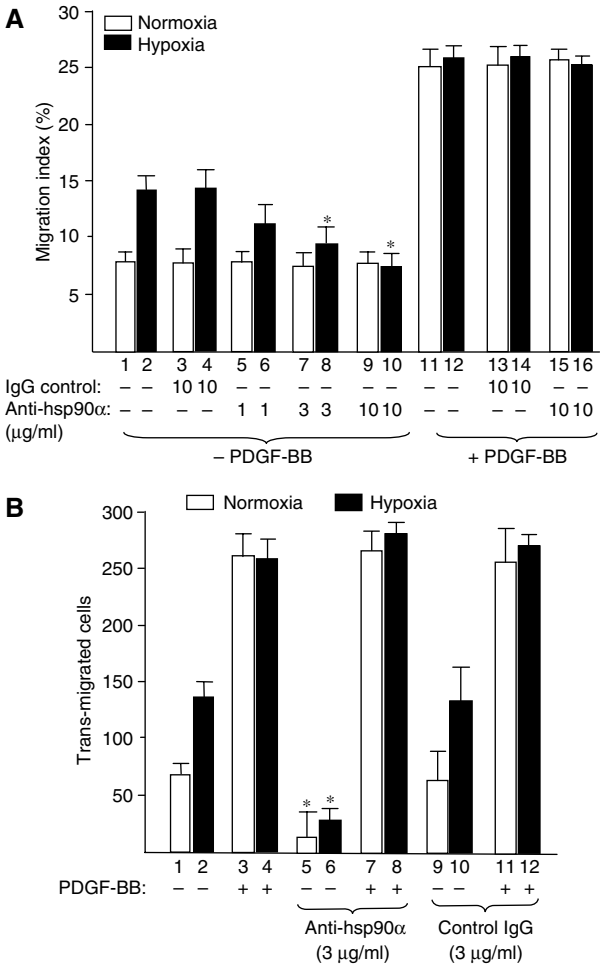

Figure 7 The extracellular function of the secreted hsp90α is essential for hypoxia-induced HDF migration. Serum-starved HDFs were subjected to colloidal gold (A) or transwell (B) migration assays under indicated conditions either with or without indicated amounts of anti-hsp90α neutralizing antibodies in the media. (**A**) Anti-hsp90α neutralizing antibodies selectively blocked hypoxia- (bars 6, 8 and 10), but not PDGF-BB- (bars 12, 14 and 16) stimulated HDF migration. The values shown are the means ± s.e.m. of MIs from four independent experiments. Bars with * are statistically significant over their control under the same experimental condition (such as bars 8 and 10 versus bars 2 and 4), $P<0.05$. (**B**) Anti-hsp90α neutralizing antibodies inhibited transmigration of HDFs from the upper chamber to the lower chamber. The values shown (number of transmigrated cells) are the means ± s.d. of triplicates used for each condition. These experiments were repeated four times and similar results were obtained. Bars with * are statistically significant over their controls (such as bar 5 versus 1 and bar 6 versus 2), $P<0.01$.

Extracellular function of hsp90α is essential for hypoxia to promote HDF migration

We, then, investigated whether secretion of hsp90α is necessary, like induction of HIF-1, for hypoxia-enhanced, but not PDGF-BB-stimulated, HDF migration. We added anti-hsp90α neutralizing antibodies to two independent cell migration assays. As clearly shown by colloidal gold migration assay in Figure 7A, hypoxia-enhanced HDF migration (bar 2 versus 1) was inhibited by the anti-hsp90α antibody in a dose-dependent manner (bars 4, 6, 8 and 10), but not by control IgG molecules (bars 3 and 4). This indicates an essential function for the secreted hsp90α in

mediating hypoxia's pro-motility signaling. In contrast, the addition of the anti-hsp90α neutralizing antibodies had little inhibitory effect on PDGF-BB-stimulated HDF migration (bars 15 and 16 versus 13 and 14). Similar results were obtained in transwell assays, in which PDGF-BB was kept in the lower chamber and anti-hsp90α neutralizing antibodies in the upper chamber. As shown in Figure 7B, hypoxia (bar 2) and PDGF-BB (bars 3 and 4) increased the cell transmigration into the lower chamber. However, presence of anti-hsp90α neutralizing antibodies selectively blocked hypoxia- (bar 6), but not PDGF-BB (bars 7 and 8)-, stimulated transmigration of HDFs. The antibodies also reduced the basal level of HDF migration under normoxia in transwell assays (lane 5 versus 1), although the reason remains unknown.

As PDGF-BB is the major serum factor that controls HDF migration (Li *et al*, 2004c), one might argue that the effect of hypoxia on HDF migration involves a PDGF-BB autocrine loop. To test this possibility, we designed siRNA against both human PDGFR-β and PDGFR-α and delivered them into HDFs with the FG-12 lentiviral vector-derived siRNA-expressing system, as previously described. To demonstrate the effectiveness of our designed siRNAs, the lysate of HDFs, after being infected with the virus carrying PDGFR-β-siRNA, PDGFR-α-siRNA or control lacZ siRNA, was immunoblotted with antibodies against either human PDGFR-β or human PDGFR-α. As shown in Figure 8A, both PDGFR-β (panel a, lane 1) and PDGFR-α (panel c, lane 1) were clearly detected in HDFs infected with control lacZ siRNA. However, both of these receptors were completely downregulated in HDFs infected with PDGFR-β-siRNA (panel a, lane 2) or PDGFR-α-siRNA (panel c, lane 2). When these cells were subjected to migration assays under normoxia or hypoxia, as shown in Figure 8B, we found that downregulation of PDGFR-β or PDGFR-α had little effect on hypoxia-induced HDF migration (bars 3–6 versus bars 1 and 2). As expected, downregulation of PDGFR-β (bars 9 and 10), but not PDGFR-α (bars 11 and 12), significantly decreased PDGF-BB-stimulated HDF migration. Therefore, a PDGF-BB autocrine loop is not involved in hypoxia-induced HDF migration.

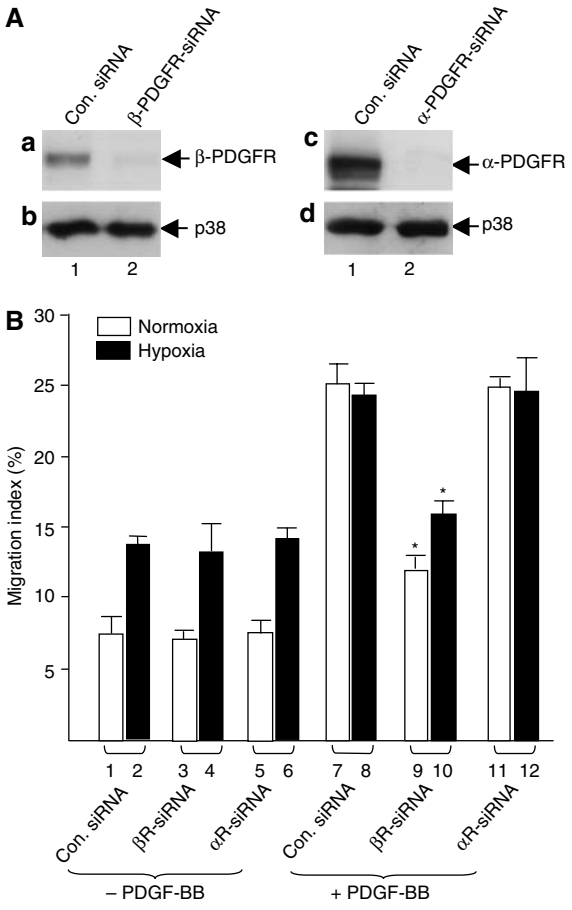

Figure 8 PDGF autocrine plays no role in hypoxia-induced HDF migration. HDFs were infected with FG-12 lentivirus carrying either a nonspecific siRNA or siRNA against human PDGFR-α, PDGFR-β. (**A**) Lysates of cells were immunoblotted with anti-PDGFR-β antibodies (panel a) or anti-PDGFR-α antibodies (panel c). The lower part of the same membrane was blotted with anti-p38 antibody as a loading and densitometry scan control. (**B**) After 48 h following infection, the cells were subjected to colloidal gold migration assays under either hypoxia (solid bars) or normoxia (open bars) in the absence (bars 1–6) or presence (bars 7–12) of PDGF-BB. Computer-assisted quantitation of the migration tracks, MI, is shown. Downregulation of PDGFR-β inhibited PDGF-BB signaling (bars 9 and 10) but not hypoxia-induced HDF migration (bars 3 and 4). Downregulation of PDGFR-α showed little effect on either hypoxia signaling (bars 3 and 4) or PDGF-BB signaling (bars 11 and 12), $^*P<0.01$. Results of this experiment are reproducible in three repeated experiments.

Hypoxia–HIF-1 pathway induces hsp90α secretion without new gene expression or protein synthesis

To study how hypoxia regulates hsp90α, we took several independent approaches. First, HDFs were untreated or treated with hypoxia for different periods of time, fixed and stained with anti-hsp90α antibodies, followed by a fluorescence-labeled secondary antibody. As shown in Figure 9A, we reproducibly detected hypoxia-induced hsp90α-clustered vesicles both inside and outside the cells (panels c–e versus a) (indicated by arrows). The percentage of 80 randomly selected cells, which showed a phenotype similar to that shown under each condition, is indicated. Second, to confirm this phenomenon, we constructed GFP-hsp90α in the lentiviral vector system and infected HDFs, as previously described. The exogenously expressed GFP-hsp90α could be easily distinguished from rest of the cellular proteins under a fluorescent microscope. As shown in Figure 9B, very similar results were obtained. Hypoxia drove GFP-hsp90α into clustered vesicles around the cell periphery and, then, these vesicles seemed to get released to the outside (panels c'–e' versus a') (indicated by arrows). Third, we found that hypoxia did not change the steady-state transcription level of the hsp90α promoter and, furthermore, the hypoxia-caused decrease in intracellular hsp90α was unaffected by the presence of cycloheximide, suggesting that hypoxia causes secretion of pre-existing hsp90α in the cells (as shown in Figure 1S in Supplementary data). Finally, RT–PCR analysis showed that hypoxia does not change the steady-state level of hsp90α mRNA in HDFs (data not shown).

Topical application of highly purified recombinant hsp90 enhances skin wound healing in vivo

The ultimate question was whether hsp90α alone is able to directly affect wound healing *in vivo*. We subcloned human hsp90α cDNA into the pET15b bacterium expression system

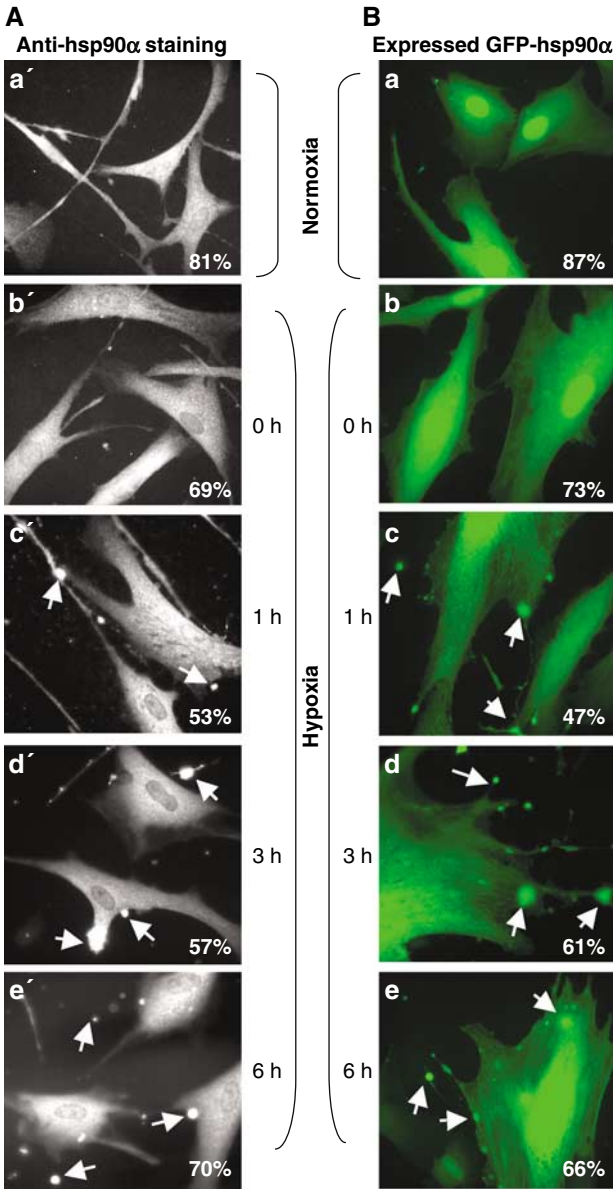

Figure 9 Hypoxia drives hsp90α clustering and export. (**A**) Serum-starved HDFs were subjected to normoxia or hypoxia for the indicated time. Cells were then fixed, permeabilized and immunostained with anti-hsp90α antibodies, followed by a fluorescence-conjugated secondary antibody. (**B**) HDFs, infected with lentivirus carrying a GFP-hsp90α fusion gene, were serum-starved and subjected to hypoxia (1% O_2) or normoxia (20% O_2) for the indicated time. Translocation of GFP-hsp90α was directly visualized under a fluorescent microscope. In both experiments, 80–120 cells in each condition were randomly selected for analysis. Percentages indicate the proportion of cells in each hypoxia time condition that is represented by the given image.

(Supplementary data), produced and purified recombinant human hsp90α in milligram quantities. sp90α (100 μg) in 100 μl of 10% carboxymethylcellulose cream or the cream alone was topically applied to the 1 × 1 cm wound on the back of nude mice (*Foxn1*^{nu}) daily for 5 days. These mice offer several advantages to our wound-healing studies: (1) their lack of hair evidently makes the wounding procedure and measurements easier; and (2) their lack of cell-based immunity reduces both potential immunological responses of the mice to foreign antigens and the inflammatory response that could complicate the specific effect of hsp90α (Wadman, 2005). Wound healing was analyzed every 2 days and selected wound images of a representative experiment are shown in Figure 10A. It can be seen that hsp90α significantly accelerated closure of the wounds on days 5, 9 and 13 (panel f versus b and panel g versus c). Quantitation of the data from four independent experiments is shown in Figure 10B, which indicates an overall ∼30% improvement of the wound healing time.

To further examine whether the newly healed wounds resulted from skin dermal contraction or re-epithelialization or both, sections of normal unwounded skin and the newly healed wounds were analyzed by H&E staining. As shown in Figure 10C, staining unwounded skin revealed the typical architecture of the epidermal and dermal junction full of hair follicles (pointed by arrows). In contrast, staining the newly healed wounds clearly showed a flat layer of re-epithelialized, hair follicle-free epidermis (indicated with dotted lines). Estimation based on the average distance in reference to the initial size of wounds showed ∼40–50% wound closure by re-epithelialization ($n = 17$). When the day-5 wounds, with or without topical treatments of hsp90α, were sectioned and analyzed by H&E staining, as shown in Figure 10D, significant more re-epithelialization was observed from hsp90α-treated than the control wounds (panel d versus c, indicated by arrows and dotted lines). The presence of hsp90α caused thicker and longer newly formed epidermis (see enlarged panel a versus b). Taken together, our study has established the following novel mechanism: hypoxia > HIF-1 accumulation > hsp90α secretion into the extracellular environment > promotion of HDF migration > wound healing.

Discussion

In this study, we investigated how hypoxia promotes primary HDF motility, a critical event for fibroplasia (migration of HDFs into the wound and deposition of ECMs) during wound healing. We found that the motility-enhancing effect of hypoxia is only detected when the cells are subjected to an environment of GF deprivation. Hypoxia has little detectable, negative or positive, effects on migration of HDFs in the presence of serum factors, such as PDGF-BB. As expected, we showed that the induced HIF-1α is necessary and sufficient for mediating hypoxia-induced HDF migration. Unexpectedly, however, we observed that the mechanism of the hypoxia-HIF-1 signaling to promote HDF migration is to drive the intracellular hsp90α to the extracellular environment. The secreted hsp90α in turn executes the pro-motility signal of hypoxia. Most intriguingly, topical application of highly purified recombinant human hsp90α significantly enhances wound healing in mice. As schematically illustrated in Figure 11, we propose that the secretion and extracellular action of hsp90α is a critical step downstream of the hypoxia–HIF-1 pathway, leading to enhanced skin cell migration and wound healing.

Our observation that hypoxia promotes HDF migration by triggering HIF-1-mediated post-translational secretion of hsp90α was entirely unexpected. Historically, Hsp90 is a highly conserved family of intracellular molecular chaperones that account for 1–2% of all cellular proteins in most cells.

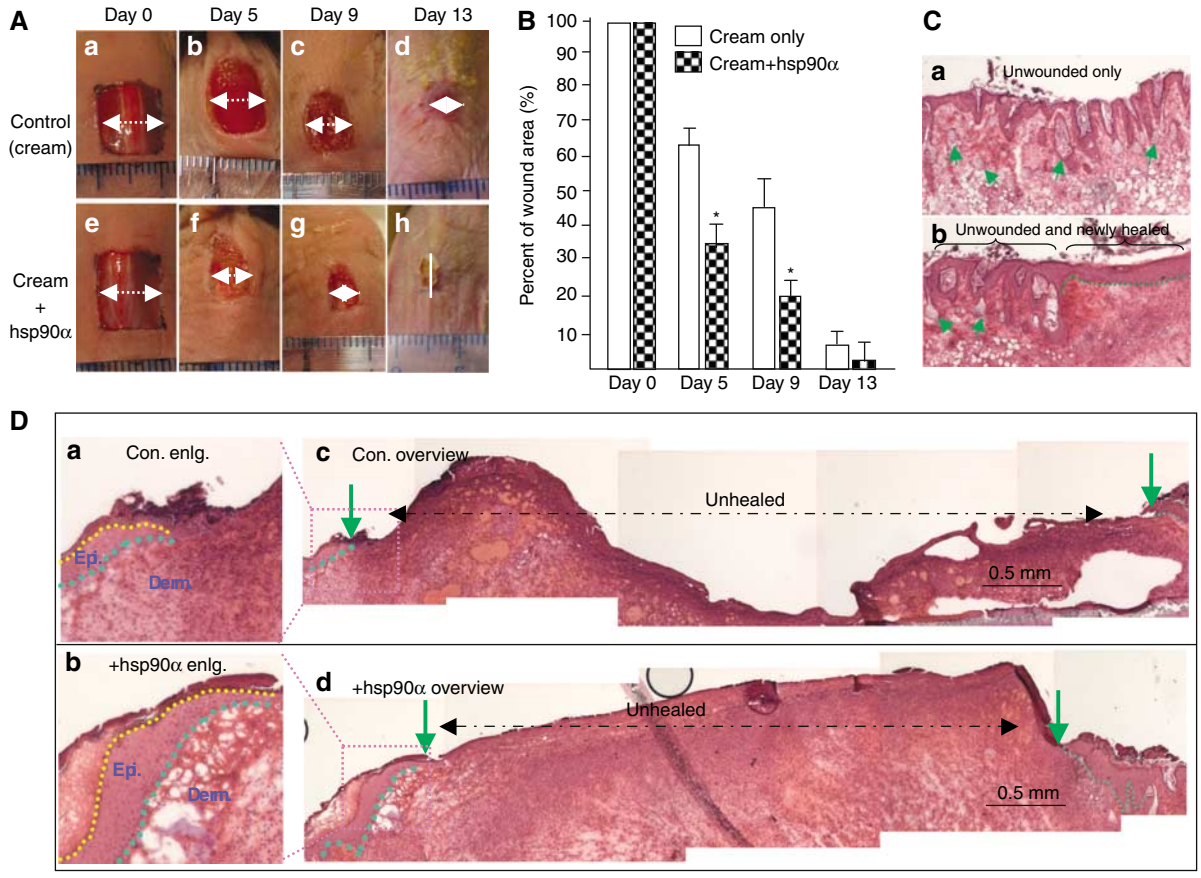

Figure 10 Topical application of hsp90α enhances wound healing in mice. A 1.0-cm^2 (1 × 1 cm) square full-thickness excision wound was made on the mid-back of 8- to 10-week-old athymic nude mice and human recombinant hsp90α was applied topically daily for 5 days ($n = 10$ mice per group). (**A**) Representative day 0, 5, 9 and 13 wounds are shown. Wound sizes were significantly reduced in mice topically treated with the cream containing hsp90α (lower panels), but not cream alone (upper panels). (**B**) Mean ± s.d. wound size measurements at day 0, 5, 9 and 13 post-wounding ($n = 10$ mice for each group). (**C**) H&E-stained sections of the day 13 wounds with or without hsp90α treatment were photographed to compare the epidermal structure of unwounded normal skin versus newly healed wounds ($n = 5$ for each group). (**D**) H&E staining of sections of the day 5 wounds with or without hsp90α treatment. Green arrows indicate the leading edges of the newly formed epidermis. Green dotted lines indicate the basement membrane zone. Purple dotted boxes (panels c and d) were enlarged (panels a and b) to visualize and compare the re-epithelization between control and hsp90α-treated wound ($n = 6$ mice for each group, five randomly selected wound sections/mouse). For each section, evidently multiple overlapping pictures had to be used to reconstitute the entire necessary part of skin/wounds. Epi, epidermis; Derm, dermis.

They regulate the folding, transport, maturation and degradation of a diverse set of client proteins, many of which are signaling proteins such as Akt, Her2 and HIF-1α (Richter and Buchner, 2001). Their levels of expression are normally much higher than required for basal function in order to buffer variation caused by environmental stress (Young *et al*, 2001; Workman, 2003; Whitesell and Lindquist, 2005). However, studies of the past decade showed that HSP proteins, such as Hsc70, are also actively secreted by the cells and have important extracellular functions, such as activation of the immune system and anticancer action (Binder *et al*, 2004; Schmitt *et al*, 2007). Liao *et al* (2000) showed that oxidative stress causes sustained release of hsp90α, which in turn stimulates activation of the ERK1/2 pathway in rat vascular smooth muscle cells. Jay and co-workers identified hsp90α, but not hsp90β, in the conditioned media of tumor cells (Eustace *et al*, 2004). Two groups showed that heat stress causes increased secretion of hsp70 and hsp90 (Clayton *et al*, 2005; Lancaster and Fabbraio, 2005). Yu *et al* (2006) recently reported that γ radiation causes secretion of hsp90β into CM in a p53-dependent manner. Hsp90α and hsp70 were exported from the cells by exocytosis via the nano-vesicles (30–90 nm in diameter) at the plasma membrane called exosomes (Hegmans *et al*, 2004; Clayton *et al*, 2005; Lancaster and Fabbraio, 2005; Yu *et al*, 2006). The exosome pathway (reviewed by Denzer *et al*, 2000) exports cellular proteins that cannot be secreted by the conventional endoplasmic reticulum/Golgi transport pathway, due to lack of a signal sequence. In fact, all of the proteins that have been identified in exosomes are located in the cell cytosol or endosomal compartments, never in endoplasmic reticulum, Golgi apparatus, mitochondria and nucleus (Théry *et al*, 2002). It will be of great interest to understand whether and how HIF-1 communicates with the exosome pathway and, more importantly, how Hsp90α drives cell migration.

Nonetheless, identification of hsp90α as a novel and essential mediator of hypoxia-induced HDF migration and a new factor for promoting skin wound re-epithelialization not only unveils a previously unrecognized mechanism of skin cell motility, but also provides a potential target for design of new treatments for chronic skin wounds.

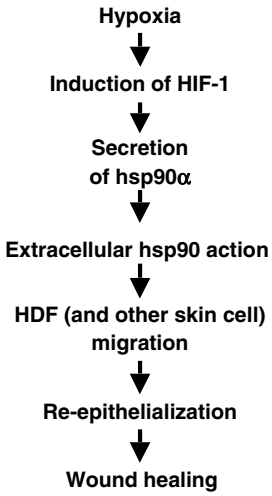

Figure 11 A schematic representation of how hypoxia signaling promotes HDF migration and wound healing. Hypoxia drives hsp90α secretion in the absence of new protein synthesis and promoter activity. Secreted hsp90α in turn promotes migration of HDFs (may be other types of skin cells, as well). The mechanisms by which hypoxia communicates with intracellular hsp90α and extracellular hsp90α promotes cell motility remain unknown.

Materials and methods

Primary HDFs were purchased from Cascade Biologics (Portland, OR) and cultured in DMEM supplemented with 10% fetal bovine serum (FBS), according to the manufacturer's instructions. HDFs at passages 4–5 were used in all the experiments throughout this study. Native rat-tail type I collagen was from BD Biosciences (Bedford, MA). Colloidal gold (gold chloride, G4022) was purchased from Sigma (St Louis, MI). The cDNAs that encode HIF-1αwt, HIF-1αCA5 (constitutively active) and HIF-1αΔNBΔAB (dominant negative) were gifts from Dr Gregg Semenza (Johns Hopkins University). The Hsp90α promoter (−1757 to +37) was provided by Dr Yu-Fei Shen (Peking Union Medical College, Beijing, China) (Zhang *et al*, 1999). Anti-HIF-1α antibody (#610958) and anti-HIF-1β antibody (#611078) were from BD Transduction Laboratories (Lexington, KY). Anti-hsp90α antibody (CA1023) for Western analysis was from Calbiochem and anti-hsp90α neutralizing antibody (SPS-771) was from Stressgen (Victoria, BC, Canada). Commercial recombinant hsp-90α was from Stressgen (SPP-776). Athymic nude mice (*Foxn1*^{nu}) were from Jackson Laboratory (Bar Harbor, ME). Antibodies against PDGFR-α and PDGFR-β were from Santa Cruz (SC-338) and Genzyme (1263-00), respectively. XL-10 Gold Ultra competent cells (XL-10 Gold) were from Stratagene (Kingsport, TN).

Hypoxia

The OxyCycler C42 from BioSpherix Ltd (Redfield, NY) was used as oxygen content controller in this study. This equipment allows creation of any profile with full-range oxygen control (0.1–99.9%) and/or CO_2 control (0.1–20.0%). All media used in hypoxia experiments were preincubated in the chambers with the designated oxygen content overnight.

Three independent cell migration assays

A recently updated protocol for the colloidal gold cell motility assay, including data and statistic analysis, was as described previously (Li *et al*, 2004a). A recently modified *in vitro* wound-healing assay, including procedures for precoating with ECMs, cell plating, scratching and quantitation of migration data, was detailed by Li *et al* (2004a) and Fan *et al* (2006). Transwell assays were performed using HDFs that are serum-starved overnight and pretreated with 5 μg/ml mitomycin C (Sigma, MO) for 2 h to stop cell proliferation. These cells were seeded onto the upper side of the microporous membrane (8.0 μm, Corning Inc., MA) and incubated in the presence or absence of anti-hsp90α neutralizing antibodies in both upper and lower chambers or control IgG, in the presence (lower chamber) or absence of PDGF-BB (15 ng/ml). The transwell plates were then incubated at 37°C for 6 h under either normoxia (20% O_2) or hypoxia (1% O_2). Cells were stained with crystal violet for 5 min. The cells that remained on the upper side of the microporous membrane were removed by wet Q-tips, whereas the cells on the lower side were fixed, photographed and counted under a microscope.

Subcloning, production of lentivirus stocks and infection

Wild-type or mutant HIF-1α cDNAs were inserted into the lentivirus-derived vector, pRRLsinhCMV, at *Bam*HI + *Eco*RI. These constructs were used to transfect 293T cells together with two packaging vectors, pCMVΔR8.2 and pMDG, to produce virus stocks as previously described (Li *et al*, 2004a). Conditioned media were collected, filtered and concentrated, if necessary, before being used to infect HDFs (Li *et al*, 2004c). Expression of the HIF-1α gene products was detected by immunoblotting lysates of the infected cells with corresponding antibodies (see Supplementary data).

FG-12 RNAi delivery system

We used the RNAi Selection Program as described to identify possible target sequences (Yuan *et al*, 2004). Four potential sites were selected and synthesized. The effectiveness of synthetic double-strand siRNA in downregulation of HIF-1α or PDGFR was measured in 293 cells by transfection and the cell lysates blotted with corresponding anti-HIF-1α or anti-PDGFR antibodies. The most effective RNAi was cloned into the lentiviral RNAi delivery vector, FG-12, as previously described (Qin *et al*, 2003). The gene transduction efficiency of infected HDFs was monitored based on the coexpressed GFP gene marker in the same vector under a fluorescent microscope, followed by FACS analysis. The selected RNAi sequence (sense) against human HIF-1α for FG12 cloning was GCCACAAUUGCACAAUAUA. The RNAis against PDGFR-β and PDGFR-α were GCCGTCAAGATGCTTAAAT and GGACTTGGTT GATGTTTAA, respectively. Downregulation of the endogenous gene products was detected by Western immunoblotting the lysates of infected cells with corresponding antibodies (see Supplementary data).

H&E staining and analyses

The Hsp90α-untreated or treated mice were euthanized 5 days after the surgery (wounding). The wounds, together with unwounded skin margins, were excised, and embedded in optimal cutting temperature (OCT) compound. The tissue was cut into 5-μm thickness sections. A series of sections covering the whole wound area were stained with H&E staining by using the H&E frozen tissue staining kit (Thermo Shandon, PA) and used to calculate the average re-epithelialized gap, which is defined as the distance between the advancing edges of epidermal keratinocyte migration. In order to show the entire 5-day wound, multiple overlapping pictures had to be taken under a microscope (Nikon, Eclipse TE2000-U, 4 ×) and used to reconstitute them.

In vivo wound-healing assay

Full-thickness excision wounds (1 × 1 cm) were made by lifting the skin with forceps and removing full-thickness skin with a pair of scissors in the mid-back of 8- to 10-week-old mice. The wounds were topically covered by 100 μl 10% carboxymethylcellulose either without (as a control) or with 100 μg recombinant hsp90α protein and then covered with bandaid and Coban, a self-adherent wrap, to prevent desiccation. Fresh recombinant hsp90α protein mix was applied daily to the wounds for 5 days. Standardized digital photographs were taken of the wounds at 0, 5, 9 and 13 days post-wounding and the open wound areas were determined with an image analyzer (AlphaEase FC version 4.1.0, Alpha Innotech Corporation). Percentage of wound area was defined by comparing areas of healing wounds to those of the original wounds. The Student's *t*-test was used for the statistical analysis. All animal studies were conducted using protocols approved by the University of Southern California Institutional Animal Use Committee.

Additional information on Materials and methods is available in Supplementary data.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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