Heat Shock Cognate 70 Regulates the Translocation and Angiogenic Function of Nucleolin

Yanping Ding, Nan Song, Chang Liu, Ting He, Wei Zhuo, Xiaoli He, Yang Chen, Xiaomin Song, Yan Fu, Yongzhang Luo

Objective—Cell surface nucleolin (NCL) plays fundamental roles in tumor angiogenesis. However, the mechanism underlying its surface translocation remains obscure. The present study discovered that heat shock cognate 70 (Hsc70) is essential in both the surface translocation and the angiogenic function of NCL.

Methods and Results—We identified that Hsc70 interacted with NCL in endothelial cells via the peptide-binding domain of Hsc70 and the RNA-binding domain of NCL. Functional knockdown of Hsc70 remarkably inhibited the expression of surface NCL, which was rescued by wild-type Hsc70 rather than its truncations. Phosphorylation of NCL by either protein kinase C-ξ or casein kinase 2 mediated its interaction with Hsc70 and the surface expression. Hsc70 regulated NCL translocation via stabilizing NCL and enhancing its interaction with nonmuscle myosin heavy chain 9. Moreover, Hsc70 was associated with NCL-induced endothelial cell migration and tubule formation in vitro and angiogenesis in both matrigel plugs and xenograft tumors. Tissue array analysis revealed that the expression levels of NCL and Hsc70 were intimately correlated in human lung adenocarcinomas.

Conclusion—Our study demonstrates that Hsc70 is a prerequisite for the surface translocation and angiogenic function of NCL, which suggests strategies to target both Hsc70 and NCL for more effective antiangiogenic therapies. (Arterioscler Thromb Vasc Biol. 2012;32:00-00.)

Key Words: angiogenesis  heat shock cognate 70  nucleolin  translocation  tumor

Nucleolin (NCL) is a ubiquitous, nonhistone phosphoprotein with remarkably versatile functions in transcription, cell proliferation, and virus infection. It is predominantly expressed in the nucleus and regulates fundamental aspects of ribosome biogenesis. Many studies have reported that NCL is also localized on the cell surface and serves as a receptor for multiple ligands, such as virus, cytokines, and DNA nanoparticles. Surface NCL form clusters on ligand binding and the ligand–NCL complex is considered to be actively internalized. Since the last decade, the role of surface NCL in angiogenesis and tumor growth has been extensively studied. Surface NCL has been identified as a marker in angiogenic blood vessels. The blockade of surface NCL can impede angiogenesis, normalize tumor vasculature, and suppress tumor growth. NCL is an endostatin receptor that mediates both the antiangiogenic and antilymphangiogenic functions of endostatin. These studies reveal the crucial roles of surface NCL in angiogenesis and tumorigenesis, which has emerged as a promising target for cancer diagnosis and therapy. Consequently, it is essential to understand the mechanism of NCL expression on the cell surface.

Accumulating evidences have suggested that the expression of surface NCL is attributed to its active translocation. Vascular endothelial growth factor and extracellular matrix can mobilize NCL to the surface of endothelial cells, whereas nonmuscle myosin heavy chain 9 (MyH9) provides an anchorage for NCL translocation along cytoskeletons. In proliferating cells, cytoplasmic NCL translocates to the cell surface via small vesicles, which is independent of the endoplasmic reticulum–Golgi pathway but requires N-linked glycosylation. Hovanessian et al have reported that the surface NCL is de novo synthesized and constantly induced in both endothelial and tumor cells. Nevertheless, the molecular mechanism regarding the surface translocation of NCL is still poorly understood.

Here, we identified that heat shock cognate 70 (Hsc70), a molecular chaperone, is a novel NCL-interacting partner in endothelial cells. The interaction involved the RNA-binding domain (RBD) 3–4 of NCL and the peptide-binding domain (PBD) of Hsc70. Hsc70 regulated the surface translocation of NCL via enhancing its stability and its interaction with MyH9. Meanwhile, phosphorylation of NCL by protein kinase C-ξ (PKC-ξ) and casein kinase 2 (CK2), respectively, mediated its interaction with Hsc70 and influenced its surface expression. Moreover, Hsc70 was associated with the proangiogenic function of surface NCL. Taken together, our results shed light on the mechanism of NCL translocation coordinated by molecular chaperones and reveal that Hsc70 is critical for the expression and angiogenic function of cell surface NCL.
Methods

Cell Lines
Human umbilical vein endothelial cells (HUVECs) were purchased from ATCC (CRL-1730). Human microvascular endothelial cells (HMECs), a human dermal microvascular endothelial cell line (Sciencell) transfected with simian vacuolating virus 40 large T antigen,16 were cultured by DMEM and 10% fetal bovine serum (Wisent).

Separation of Nucleus, Cytosol, and Membrane Fractions
HMECs were homogenized on ice in buffer A (25 mmol/L HEPES, 250 mmol/L sucrose, pH 7.4, protease, and phosphatase inhibitor cocktails). Nucleus was pelleted by centrifugation at 1500g for 10 minutes and organelle components were removed by spinning at 8000g for 10 minutes. The membrane pellet was obtained by ultracentrifugation at 100 000g for 1 hour, and the supernatant was the cytosol fraction. Both nucleus and membrane pellets were dissolved in buffer B (50 mmol/L Tris, pH 7.5, 150 mmol/L NaCl, 1% Nonidet-P40, 1 mmol/L dithiothreitol, protease, and phosphatase inhibitor cocktails).

Coinmunoprecipitation
Cell lysates or different fractions were preincubated with protein A/G agarose to reduce nonspecific binding. The cleared lysates were incubated with the antibody or isotype control IgG (1 μg/mg cell lysate) for 1 hour and subsequently with protein A/G beads (20 μL) for 6 hours at 4°C. After washing 3 times, the coinmunoprecipitation (co-IP) samples were subjected to SDS-PAGE or immunoblotting. Studies were repeated 3 times.

Liquid Chromatography–Mass Spectrometry
After NCL in the nucleus, cytosol, and membrane fractions of HMECs were immunoprecipitated, the co-IP samples were applied to SDS-PAGE and stained with Coomassie blue. Whole cell lysates immunoprecipitated with rabbit IgG served as negative control. Gel slices containing protein bands of great variation compared with control were excised and digested by sequencing grade modified trypsin. Peptide mixture was analyzed by liquid chromatography–mass spectrometry (Agilent 6300 Series Ion Trap Liquid Chromatography/ Mass Systems). Mass data were piloted by the Swiss-Prot database.

Immunofluorescence of Cell Surface Proteins
According to a previous study,6 at 20°C, cells were incubated with primary antibodies for 1 hour, fixed with 4% formaldehyde for 30 minutes, blocked by 10% goat serum/PBS for 1 hour, and subsequently incubated with relevant fluorescein isothiocyanate- or TRITC-conjugated secondary antibodies for 1 hour. Nuclei were stained with 4′,6-diamidino-2-phenylindole. Images were captured by a Nikon A1 confocal microscope.

Flow Cytometry
As previously described,11 cells were blocked with 5% goat serum/PBS, incubated with NCL antibody (5 μg/mL) or IgG isotype control, and stained with fluorescein isothiocyanate–conjugated goat anti-rabbit IgG (1 μg/mL). Cells were washed 3 times by PBS and analyzed by a FACSCalibur flow cytometry system (Becton Dickinson, San Jose, CA).

Transwell Migration
According to a previous study,17 2×10⁵ cells were seeded into the upper compartment of the transwell chambers and allowed to migrate for 6 hours. The lower compartment contained 500 μL complete growth medium. Cells were fixed and stained by crystal violet. All the transwell experiments were repeated 3 times and 3 wells for each treatment. The number of migrated cells was calculated in 5 random fields (×100).

Xenograft Tumor Model
According to a previous study,18 human lung adenocarcinoma cells A549 (1×10⁷) were inoculated subcutaneously into 6-week-old nude mice. When tumors reached ∼50mm³, mice were separated into 2 groups, and, respectively, treated with dimethyl sulfoxide or 50 μg/g KNK437 every other day for 2 weeks.

Results

NCL Interacts With Hsc70 in Endothelial Cells
We and others have previously reported that cell surface NCL is a marker in angiogenic blood vessels and regulates tumor angiogenesis.7,8,10 To investigate the regulatory mechanism underlying the surface translocation of NCL in endothelial cells, we searched for potential NCL-interacting proteins from the nucleus, cytosol, and membrane fractions. Immunoprecipitation and mass spectrometry analysis revealed that several proteins could bind with NCL; among them, a novel binding partner Hsc70 and MyH9 were simultaneously detected in both the cytosol and membrane compartments (Figure 1A). Thirteen distinct peptides covering most of the Hsc70 sequences were identified by mass spectrometry (Figure 1A in the online-only Data Supplement). We therefore explored whether Hsc70 interacts with NCL. We first examined their interaction in HUVECs by co-IP. NCL and Hsc70 were found to bind with each other when either NCL or Hsc70 was immunoprecipitated (Figure 1B). Next, we performed the pull-down experiment using purified green fluorescent protein–NCL to incubate with HUVEC lysates and immunoblotted the pull-down proteins with anti-Hsc70 antibody. Green fluorescent protein-NCL but not green fluorescent protein was able to pull-down Hsc70 (Figure 1B in the online-only Data Supplement). By immunofluorescence assay, we found that NCL colocalized with Hsc70 in endothelial cells (Figure 1C; Figure 1C in the online-only Data Supplement). Moreover, the colocalization between NCL and Hsc70 was also observed in the vascular-like regions of human tumor tissues (Figure 1C). Taken together, these results demonstrate that NCL forms a complex with Hsc70 in endothelial cells.

The RBD of NCL and the PBD of Hsc70 Are Indispensable for the Interaction Between NCL and Hsc70
NCL is composed of 3 structural domains, including the N-terminal domain comprising 4 acidic stretches (N-domain), the central globular region containing 4 RBD (RBD 1–4), and the C-terminal domain rich in arginine and glycine residues (RGG domain).19 To determine the interacting domain of NCL with Hsc70, we constructed a series of green fluorescent protein–tagged truncations (NCLWT, NCLA1–303, NCLA307–707, NCLA469–707, and NCLA467–707) encompassing various functional domains of NCL (Figure 1D, top). After transfecting HMECs with different NCL truncations, we detected their interactions with Hsc70 by co-IP. Compared with NCLWT, the interaction between NCLA307–707 and Hsc70 was dramatically disrupted, whereas that of NCLKA1–303 was not influenced (Figure 1D, bottom). Meanwhile, NCLA467–707 rather than NCLA469–707 could interact with Hsc70 (Figure 1D, bottom), indicating that the regulatory domain resides in the
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amino acid region 469 to 647 of NCL, which is defined as the RBD 3–4. Hsc70 contains an ATP-binding domain, a PBD, and a C-terminal domain. To investigate the binding domain of Hsc70 with NCL, we performed the co-IP assay with HA-tagged Hsc70 constructs covering the entire length of Hsc70 (Figure 1E, top). Immunoblotting revealed that the PBD of Hsc70 was required for its interaction with NCL (Figure 1E, bottom). Likewise, clusters of surface NCL were found to colocalize with Hsc70 and Hsc70 ΔATP rather than Hsc70ΔPBD in HMECs (Figure 1F). The aforementioned results demonstrate that the RBD 3–4 of NCL and the PBD of Hsc70 are responsible for their interaction.

**Hsc70 Regulates the Surface Translocation of NCL**

Hsc70 has been shown to regulate the trafficking of several proteins, including aquaporin-2, ASIC2, CFTR, and melanocortin-4 receptor.20–24 Because Hsc70 interacts with NCL, we speculated that Hsc70 participates in the NCL translocation. First, we treated HUVECs with an Hsc70 inhibitor KNK437 for different times. KNK437 significantly inhibited the expression of Hsc70 as early as 3 hours compared with heat shock protein 70 (Hsp70), Hsp90α, and Hsp90β (Figure IIA in the online-only Data Supplement). Then, we incubated HUVECs with KNK437 at different concentrations and found that the expression of membrane NCL was receded when the KNK437 concentration was increased (Figure 2A). The inhibitory effect of KNK437 on the surface NCL was further verified by flow cytometry (Figure 2B) and immunofluorescence (Figure 2C), respectively. Second, we knocked down Hsc70 in proliferating HUVECs or overexpressed Hsc70 in serum-starved HUVECs (Figure IIB in the online-only Data Supplement), and subsequently detected the expression of surface NCL by flow cytometry. The absence of Hsc70 led to a decrease of surface NCL, whereas the overexpression of Hsc70 resulted in the induction of surface NCL (Figure 2D). To further elucidate the effect of Hsc70 on NCL translocation, we detected the expression of NCL in plasma membrane, cytosol, and nucleus

**Figure 1.** Heat shock cognate 70 (Hsc70) interacts with nucleolin (NCL) in endothelial cells. A, Human microvascular endothelial cells (HMECs) were separated into nucleus (N), cytosol (C), and membrane (M) fractions and incubated with anti-NCL antibody (1 µg/mg lysate) for 1 hour and then with protein A agarose for 6 hours. Whole cell lysate (WCL) with rabbit IgG served as negative control. The coimmunoprecipitation (co-IP) samples were subjected to SDS-PAGE and stained with Coomassie blue. Protein bands of great variation compared with control were excised and analyzed by liquid chromatography–mass spectrometry. B, Human umbilical vein endothelial cell (HUVEC) lysates were immunoprecipitated with anti-NCL, anti-Hsc70 antibody, or relevant IgG isotype controls and subsequently immunoblotted with anti-NCL or anti-Hsc70 antibodies. C, HUVECs were incubated with anti-NCL and anti-Hsc70 antibodies at 20°C, fixed with formaldehyde, blocked by 10% goat serum, and stained with related fluorescein isothiocyanate (FITC)- and TRITC-conjugated secondary antibodies. Bars, 50 µm (4 left panels) and 10 µm (magnified). Human lung adenocarcinoma tissue chip was also stained with NCL and Hsc70. Bars, 200 µm (4 left panels) and 50 µm (magnified). Nuclei were indicated by 4′,6-diamidino-2-phenylindole (DAPI) staining. D, Green fluorescent protein (GFP)-fusion NCL truncations encompassing various functional domains were constructed (top). HMECs were transfected by GFP vehicle plasmid or NCL truncations and immunoprecipitated with GFP. The co-IP samples were immunoblotted against Hsc70 and GFP. Transfected HMEC lysates were immunoblotted against Hsc70 and GAPDH as input controls. E, HA-tagged Hsc70 truncations covering different domains were constructed (top). HMECs were transfected with Hsc70 truncations and immunoprecipitated with HA. Transfected HMEC lysates were immunoblotted against NCL and HA. Transfected HMEC lysates were immunoblotted against NCL and HA, and GAPDH as input controls. F, HMECs were transfected with HA-tagged Hsc70 truncations. Cell surface NCL and HA were detected by immunofluorescence. Nuclei were indicated by DAPI staining. Bars, 20 µm.
fractions. After knocking down Hsc70, membrane NCL was downregulated and cytosolic NCL was slightly accumulated, whereas nuclear NCL was marginally changed (Figure 2E). Then, we immunostained and detected surface NCL by confocal microscopy after altering the expression of Hsc70. As expected, a dramatic reduction of surface NCL occurred as a result of the loss of Hsc70, whereas surface NCL accumulated as a result of Hsc70 overexpression (Figure 2F). Moreover, the expression of surface NCL in Hsc70 knockdown cells was rescued by the transfection of Hsc70, but neither Hsc70ΔATP nor Hsc70ΔPBD (Figure 2G). Therefore, both the ATP-binding domain and PBD of Hsc70 are required for the surface translocation of NCL, which, respectively, provide ATPase activity and NCL-binding region. Collectively, these results show that Hsc70 is involved in the surface translocation of NCL.

Phosphorylation of NCL Mediates Its Interaction With Hsc70 and the Surface Translocation

Because NCL is massively phosphorylated in vivo, we wondered whether phosphorylation regulates the interaction between NCL and Hsc70. Endogenous NCL was immunoprecipitated from HUVEC extracts and immunoprecipitates were treated with λ protein phosphatase to inhibit protein phosphorylation. λ protein phosphatase impaired the complex between NCL and Hsc70, which suggests that NCL binds to Hsc70 in a phosphorylation-dependent manner (Figure IIIA in the online-only Data Supplement). NCL has been reported to be phosphorylated by PKC-ξ, CK2, and cell division control protein 2 in vivo. To examine whether the phosphorylation of NCL by these kinases impaired the complex between NCL and Hsc70, which suggests that NCL binds to Hsc70 in a phosphorylation-dependent manner (Figure IIIA in the online-only Data Supplement). NCL and Hsc70 were detected by immunofluorescence. Scale bar=20 μm. N.C.

Figure 2. Heat shock cognate 70 (Hsc70) mediates the surface translocation of nucleolin (NCL). A, Human umbilical vein endothelial cells (HUVECs) were serum-starved for 24 hours, pretreated by KNK437 at different concentrations (0, 25, 50, and 100 μmol/L) for 1 hour, and then serum-rescued for 6 hours to restore surface NCL expression. NCL and Hsc70 in both membrane fractions and whole cell lysates (WCL) were immunoblotted. CD31 and GAPDH were loading controls. The ratio of membrane NCL/CD31 was quantified in each group. B and C, HUVECs were serum-starved for 24 hours, pretreated by dimethyl sulfoxide (DMSO) (control) or KNK437 (100 μmol/L) for 1 hour, and then serum-rescued for 6 hours. B, Surface NCL was monitored by flow cytometry. C, Cell surface NCL and Hsc70 in HUVECs were detected by immunofluorescence. Nuclei were indicated by 4′,6-diamidino-2-phenylindole (DAPI) staining. Scale bar=20 μm. D, Proliferating HUVECs were transfected with scrambled siRNA (S.C.) or Hsc70 siRNA (Si-Hsc70). HUVECs transfected with pcDNA3.1 (vehicle) or pcDNA3.1-Hsc70 (Hsc70) plasmids were serum-starved for 24 hours. Surface NCL was analyzed by immunofluorescence. Scale bar=10 μm. E, Hsc70 was knocked down in HUVECs and rescued by transfection of full-length Hsc70 (FL), Hsc70ΔATP, and Hsc70ΔPBD constructs. Surface NCL was detected by flow cytometry. N.C. indicated cells incubated with rabbit IgG; MFI, mean fluorescence intensity.
by immunofluorescence. Nuclei were indicated by DAPI staining. Scale bar=20 μm. Cdc2 indicates cell division control protein 2; PKC-ζ, protein kinase C-ζ; S.C., scrambled siRNA.

Supplement) and attenuated the NCL cluster formation on the cell surface (Figure 3B). Next, we knocked down PKC-ζ, CK2, and cell division control protein 2 and then immunoprecipitated NCL. Consistently, the interaction between NCL and Hsc70 was inhibited after knocking down PKC-ζ or CK2 (Figure 3C). Meanwhile, the expression of surface NCL was decreased as a result of knocking down either PKC-ζ or CK2 (Figure 3D; Figure IIIID in the online-only Data Supplement). In summary, these results suggest that the phosphorylation of NCL by PKC-ζ and CK2 mediates the surface translocation of NCL via regulating its interaction with Hsc70.

**Hsc70 Stabilizes NCL and Enhances the Interaction Between NCL and MyH9 in Endothelial Cells**

Previous studies have shown that the stability of NCL is positively correlated with the cell proliferation status. Meanwhile, the expression of surface NCL is much higher in proliferating cells compared with that in quiescent cells, which indicates that the integrity of NCL is related to its surface expression. Thus, we wondered whether Hsc70 influences the stability of NCL. We first detected whether the interaction between NCL and Hsc70 alters under different cell proliferation status. As expected, the interaction was reduced in quiescent cells compared with that in proliferating cells (Figure 4A), which suggests that the interaction with Hsc70 may be related to the stability of NCL. To further assess this issue, we knocked down Hsc70 and detected NCL in the whole cell lysates by immunoblotting. Degradation of NCL was increased as a result of knocking down Hsc70 (Figure 4B). Intriguingly, the stability of NCL was restored when the expression of Hsc70 was rescued by transfection of Hsc70 rather than Hsc70ΔATP or Hsc70ΔPBD (Figure 4B). Moreover, Huang et al have reported that MyH9 serves as a motor for the surface translocation of NCL. Here, we found that the RGG domain of NCL regulates its interaction with MyH9 (Figure IV in the online-only Data Supplement). Consistently, the interaction between NCL and MyH9 was correlated with the cell proliferation status. Meanwhile, the expression of surface NCL was decreased as a result of knocking down Hsc70 (Figure 4B). These results demonstrate that Hsc70 stabilizes NCL and mediates the interaction between NCL and MyH9.

**Hsc70 Regulates the Angiogenic Function of Cell Surface NCL**

Because surface NCL has been well documented in angiogenesis, we thus hypothesized that the angiogenic function of NCL can be regulated by Hsc70. It has been reported that surface NCL regulates endothelial cell migration while only marginally affecting cell adhesion and proliferation. To evaluate the effect of Hsc70 on surface NCL-induced cell migration, we overexpressed NCL in HMECs to enhance the level of surface NCL and simultaneously knocked down Hsc70. In the transwell migration assay, we found that the upregulation of NCL led to enhanced cell migration compared with the vehicle group. However, lack
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mortality induced by surface NCL. To extend this finding, we

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expression (Figure 5A). A similar result was observed in the

vessels (Figure 5C). Meanwhile, the colocalization between

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Hsc70 Participates in NCI-Associated Tumor Angiogenesis

Considering that Hsc70 regulates NCL-induced angiogenesis, we further investigated their relationship in tumor angiogenesis. Mice bearing A549 xenograft tumors were treated with dimethyl sulfoxide or KNK437. After injection for 2 weeks, tumors were sectioned and immunostained with the endothelial cell marker CD31, NCL, and Hsc70. Consistent with previous results, either the blood vessel density or the expression of NCL on blood vessels was much lower in the KNK437-treated group (Figure 6A). Moreover, KNK437 ultimately inhibited the colocalization between NCL and Hsc70 as a result of the reduction of both NCL on blood vessels and Hsc70 expression (Figure 6B). To further confirm the association between Hsc70 and NCL in tumor angiogenesis, we investigated their expression in both normal lung and non-

small-cell lung cancer tissues. Immunostaining analysis represented that there was almost no expression of Hsc70 and NCL in normal lung tissues, whereas their expression varied in different tumor specimens. Among 40 tumor specimens, blood vessels of 27 cases lacked Hsc70 and NCL (low Hsc70-low NCL). In 10 specimens, the expression of both Hsc70 and NCL on blood vessels was >50% (high Hsc70-high NCL). Hsc70 but no NCL was detected in the blood vessels of another 3 cases (Figure 6C). Therefore, Hsc70 and NCL are mostly coexpressed and colocalized on the blood vessels of human lung tumor tissues. Intriguingly, none of the 40 human tumor tissues showed low Hsc70 but high NCL on blood vessels, which again supports that Hsc70 is a prerequisite for the surface translocation of NCL in endothelial cells. Taken together, these results demonstrate that Hsc70 participates in NCL-associated tumor angiogenesis.

Discussion

Although NCL is primarily characterized as a nucleolar protein, it is also localized on the cell surface. During its surface translocation, NCL must interact with a host of accessory proteins. Understanding these interactions is crucial for unraveling the mysteries of the translocation, postmodification, and functions of NCL. Here, we provide direct evidences regarding the interaction between NCL and the chaperone system. We investigated the relationship between Hsc70 and NCL, in that Hsc70 was identified to interact with NCL in both the plasma membrane and the cytosol using mass spectrometry. Further, by co-IP and immunofluorescence, we first demonstrate that Hsc70 interacts with NCL in endothelial cells. The RBD 3–4 of NCL and the PBD of Hsc70 are responsible for their interaction. Interestingly, among NCL truncations, NCLΔ1–303 has shown the robust binding with Hsc70 (Figure 1D), indicating that the N domain of NCL plays a regulatory role in their interaction. The N domain of NCL is rich in lengthy acidic amino acids. Because the PBD of Hsc70 is inclined to bind with
hydrophobic and basic amino acids, but few or no acidic residues, the N domain of NCL is likely to prohibit its interaction with Hsc70. Moreover, the phosphorylation status of NCL by PKC-ε and CK2 also mediates the complex formation. The exact structural characteristics of the NCL–Hsc70 complex merit further investigation.

A key question, therefore, is how this interaction regulates NCL translocation. We found that suppression of Hsc70 impedes the expression of surface NCL, which can be restored by the rescue of Hsc70 (Figure 2). These observations illustrate that interacting with Hsc70 is crucial for the surface translocation of NCL. It is noteworthy that Hsc70 is a critical regulator of protein trafficking. On one hand, several studies have reported that Hsc70 participates in the clathrin-mediated endocytosis and functions as an ATPase in the disassembly of clathrin-coated vesicles. In this respect, Hsc70 restrains the expression of membrane aquaporin-2, ASIC2, and CFTR. On the other hand, Hsc70 is a chaperone, which can facilitate the correct folding and the stability of client proteins. For instance, Hsc70 promotes the surface translocation of melanocortin-4 receptor by improving its folding. Therefore, Hsc70 regulates the trafficking of various proteins via distinct mechanisms. Currently, we demonstrate that Hsc70 enhances the surface translocation of NCL. Because NCL is found to colocalize with Hsc70 both in the cytosol and on the surface of endothelial cells, it is reasonable to deduce that Hsc70 accompanies NCL during its surface translocation process.

Our mass spectrometry analysis reveals several other potential NCL-binding proteins in cytosol, including Hsp90α and enolase-1 (Figure 1A). Interestingly, Hsc70, Hsp90α, and enolase-1 are all exosome components, which are crucial for protein secretion. More investigations are required to characterize whether the surface translocation of NCL is associated with exosome.

NCL undergoes multiple postmodifications intracellularly, including phosphorylation, glycosylation, methylation, and proteolysis. N-glycosylation has been identified essential for the expression of surface NCL. The phosphorylation of NCL has been mostly studied and demonstrated to be regulated by PKC-ε, CK2, and cell division control protein 2. Particularly, suppression of PKC-ε and CK2 impedes the interaction between Hsc70 and NCL, which causes the decrease of surface NCL. Further explorations are needed to elucidate the exact phosphorylation sites, which are indispensable for the surface translocation of NCL, as well as the variations of NCL phosphorylation status in different cellular compartments. Meanwhile, our group has reported that the phosphorylation of Thr90 on Hsp90α facilitates its secretion in tumor cells, which stabilizes matrix metalloproteinase-2. Therefore, that the phosphorylation of Hsc70 may also mediate the surface translocation of NCL cannot be ruled out and merits further investigation.
Cell surface NCL has been implicated in angiogenesis via regulating endothelial cell migration.1,2 During the surface mobilization of NCL, stress fibers and focal adhesion complex form simultaneously.3 Because the dynamic of focal adhesions plays a central role in cell migration, it is likely that the proangiogenic role of surface NCL is correlated with focal adhesions. Intriguingly, our mass spectrometry analysis reveals that talin, an essential focal adhesion molecule, potentially interacts with NCL. Surface NCL can be diminished by knocking down talin (Figure VI in the online-only Data Supplement). Previously, we have reported that NCL mediates the internalization of endostatin.11,35 In a further study, only Hsc70, but not Hsp70, was identified as a potential NCL-interacting protein in endothelial cells. We propose that Hsc70 and Hsp70 share high homology in both structure and physiological functions as chaperones. Recent studies have suggested that Hsc70 and Hsp70 are distinguishably different under certain circumstances.38–40 Hsc70 is constitutively expressed and participates in cellular processes of normal cells, such as endothelial cells. It serves as a scaffold protein and coordinates the regulation of signal transduction32 and cell survival.41 In contrast, Hsp70 is expressed at significantly low levels in most unstressed cells but can be rapidly induced by physical and chemical stresses. In our present study, only Hsc70, but not Hsp70, was identified as a potential NCL-interacting protein in endothelial cells. We propose that Hsc70 and Hsp70 have differential roles in regulating NCL translocation. Particularly, it is reported that Hsc70 regulates the trafficking of NCL in normal cells, whereas Hsp70 may participate in this process under stress conditions.

In conclusion, our present study identifies a novel mechanism underlying the surface translocation of NCL (Figure VII in the online-only Data Supplement). Hsc70 interacts with NCL and modulates the surface translocation of NCL via enhancing its stability and the interaction with MyH9. Phosphorylation of NCL by PKC-ζ and CK2 remarkably influences its interaction with Hsc70 and consequently regulates its surface expression. Furthermore, Hsc70 is intimately associated with the angiogenic function of surface NCL. This study, for the first time, demonstrates that Hsc70 is a prerequisite for the expression and angiogenic function of surface NCL.
NCL, which suggests strategies to target both Hsc70 and NCL for more effective antiangiogenic therapies.

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Disclosures
None.

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Supplemental Material

Supplemental methods

Reagents - Rabbit anti-nucleolin and rat anti-Hsc70 antibodies were obtained from Abcam (Cambridge, MA, USA). Rabbit anti-MyH9 and rabbit anti-CK2 were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Mouse anti-GFP was purchased from Origene (Rockville, MD, USA). Mouse anti-HA was from Abmart (Arlington, MA, USA). Mouse anti-CD31 was purchased from Sino Biological Inc. (Beijing, China). Rabbit anti-PKC-ζ and anti-Cdc2 were from Bioworld (Dublin, OH, USA). Staurosporine, CK2 inhibitor 4, olomoucine, and N-formyl-3, 4-methylenedioxy-benzylidene-γ-butyrolactam (KNK437) were from Calbiochem (Merck, Darmstadt, Germany). Mouse anti-GAPDH, goat anti-lamin B, FITC- and TRITC-conjugated secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Protein A agarose and protein G agarose were obtained from Roche Applied Biosciences (Indianapolis, IN, USA). Lambda phosphatase (λPPase) was got from New England Biolabs (Ipswich, MA, USA). Matrigel was purchased from BD Biosciences (San Diego, CA, USA). Sequencing grade modified Trypsin was got from Promega (Madison, WI, USA).

Preparation of cell lysates - HUVECs or HMECs were homogenized with cell lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% NP-40, 1 mM DTT, protease and phosphatase inhibitor cocktails) on ice and then centrifuged at 13000 g for 15 min. Pellets were removed and the supernatant was the cell lysates.

RNA interference (RNAi) - According to the manufacturer’s instructions, HUVECs or HMECs were transfected using lipofectamine 2000 (Invitrogen, Grand Island, NY, USA)
with following siRNAs targeting Hsc70: (1) 5'-CCUAAAUUCGUAGCAAAUU-3', (2) 5'-UAAUUCUAAGUACAUUGAGACCAGC-3'. The siRNA sequences targeting PKC-ζ, CK2, and Cdc2 were 5'-CUCUCUUUGGGGUCUUAUU-3', 5'-UCAAGAUGACUACCAGCUG-3', and 5'-UGUACCAGAGUUCUACCUCUAUG-3', respectively. Scrambled siRNA served as a negative control. All the siRNAs were synthesized by GenePharma (Shanghai, China).

Plasmid construction and Transfection – Full length nucleolin coding sequence was subcloned into pCMV6-AN-GFP vector (Origene, Rockville, MD, USA). Human Hsc70 was subcloned into pcDNA3.1(+) vector with Hemagglutinin (HA) epitope at the N-terminus. N-terminal GFP-tagged nucleolin mutants (NCLΔ1-303, NCLΔ307-707, NCLΔ469-707, and NCLΔ647-707) and HA-tagged Hsc70 mutants (Hsc70ΔATP and Hsc70ΔPBD) were constructed by the Quick Change Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA, USA). Plasmids were transfected to HUVECs by Nanojuice transfection kit (Novagen, Darmstadt, Germany) according to the instructions. HMECs were transfected with the Turbofect in vitro transfection reagent (Fermentas, Harrington, Canada) according to the manufacturer’s instructions.

Pull-down assay – Approximately 500 μg cell lysates were pre-cleared on 20 μl protein A beads at 4 °C for 1 h. Protein A or protein G beads were incubated with 1 μg GFP antibody, 20 μg GFP or GFP-nucleolin protein and the pre-cleared cell lysates at 4°C with gentle rocking for 6 h. Then the beads were washed twice with lysis buffer. The pulled-down proteins were analyzed by SDS-PAGE and immunoblotting analysis. Assays were performed at least three times.
**Immunofluorescence** – Cells were grown on glass coverslips for 24 h. To detect cell-surface proteins, coverslips were incubated with primary antibodies for 1 h at 20°C before fixation with 4% formaldehyde. For intracellular proteins, cells were fixed in 4% formaldehyde/PBS for 30 min and permeabilized by 0.2% Triton X-100/PBS for 10 min at room temperature. Non-specific binding was blocked by incubating the coverslips in 10% goat serum/PBS for 1 h at room temperature. Permeabilized cells were then incubated with relevant primary antibodies (2 μg/ml in PBS) at 4°C overnight. Coverslips were finally incubated with FITC- or TRITC-conjugated secondary antibodies for 1 h at room temperature. Nuclei were stained with DAPI. Images were captured by a Nikon A1 confocal microscope.

**Immunoblotting** – Cell lysates or cell fractions were harvested, denatured, resolved by SDS-PAGE and transferred to PVDF membrane. The protein blots were blocked by 10% non-fat milk and incubated with appropriate primary antibodies and secondary antibodies. Signals were developed using the SuperSignal West Pico chemiluminescent substrate from Pierce (Rockford, IL, USA). All the immunoblotting experiments were conducted at least three times.

**Scratch migration** – Cells were seeded in a 24-well plate and cultured until confluency. A straight scratch was made by a pipette tip to stimulate a wound. Cells were allowed to migrate for 24 h at 37°C 5% CO₂. All the scratch migration experiments were repeated at least three times and more than 3 wells for each treatment. The wound edge images were captured by microscopy (×40) in five random fields for each group. The relative wound closure width was calculated in each group.

**Tubule formation** – In brief, 24-well plates were coated with 100 μl/well of matrigel (Becton
Dickinson Pharmingen, San Diego, CA, USA). HUVECs (5×10^4) were seeded in each well of the matrigel-coated plates and incubated for 6 h in DMEM and 5% new-born calf serum. Wells were then fixed by 4% paraformaldehyde and visualized by microscope (×100). The experiment was repeated three times and each treatment was in triplicate. Relative tubule length was calculated in three randomly selected fields each well using the software Scion Image (Scion, Frederick, MA, USA).

**Matrigel plug assay** - Matrigel (400 μl, mixed with DMSO or 50 μg/g KNK437) was inoculated subcutaneously in the midventral abdominal region of 5 nude mice. Mice were intraperitoneally injected with DMSO or 50 μg/g KNK437 every other day for 7 days.

**Immunostaining for frozen sections** – After mice were anesthetized to death by 100 mg/kg pentobarbital sodium, matrigel plugs or tumor tissues were excised, embedded, and frozen in O.C.T. compound. Ten-μm-frozen sections were prepared and fixed in acetone for 5 min. The sections were blocked by 10% goat serum, incubated with primary antibodies overnight, and stained with FITC- or TRITC- conjugated secondary antibodies for 1 h at room temperature. Nuclei were stained with DAPI. Images were captured by a Nikon A1 confocal microscope.

**Tissue array analysis** – The tissue array containing 5 normal lung tissues and 40 cases of non-small cell lung cancer tissues was purchased from Outdo (Shanghai, China). The tissue specimen were blocked by 10% goat serum/PBS, incubated with anti-nucleolin, anti-Hsc70, and anti-CD31 antibodies, and subsequently stained with FITC-, TRITC-, and Cy5-conjugated secondary antibodies. DAPI was stained for nuclei. Images were captured by laser scanning confocal microscopy (Nikon, Tokyo, Japan).

**Statistical Analysis**- All quantitative data were shown as mean ± SD. The t-test was used for
comparisons between two groups. Differences were considered statistically significant when \( p<0.05 \).
Supplemental Figure I

**A**

Hsc70

| 1 | __ | __ | __ | __ | __ | __ | 646 |

| 26 | VEIIANDQGNR |
| 37 | TTPSYVAFTDTER |
| 138 | TVTNAVVTVPAYFNDSQR |
| 160 | DAGTIAGLNVLR |
| 172 | IINEPTAAAIAYGLDK |
| 221 | STAGDTHLGGEDFDNR |
| 300 | ARFEELNADLFR |
| 302 | FEELNADLFR |
| 329 | SQIHDIVLVGGSTR |
| 362 | SINPDEAVAYGAAVQAAAILSGDK |
| 424 | QTTQTFTTYSNQPGVLIQVYEGER |
| 510 | LSKEDIER |
| 584 | NQTAEKEEEFEHQQK |

**B**

IP: GFP

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Input

<table>
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Hsc70

GFP-NCL

IgG

**C**

DAPI NCL Hsc70 Merge Mag.

PB-HUVEC

Supplemental Figure I Hsc70 interacts with nucleolin (NCL) in endothelial cells.

(A) Thirteen peptides that span the sequence of Hsc70 are identified by mass spectrometry.

(B) Hsc70 is pulled down by NCL in endothelial cells. Purified GFP or GFP-NCL proteins were incubated with anti-GFP antibody, protein A agarose, and pre-cleared HUVEC lysates at 4°C with rocking for 6 h. The pull-down samples were subjected to SDS-PAGE and immunoblotted with anti-Hsc70 and anti-GFP antibodies. The whole cell lysates were immunoblotted against GFP as input control.

(C) Hsc70 co-localizes with NCL intracellularly in HUVECs. HUVECs were fixed with 4% formaldehyde, permeabilized by 0.2% Triton X-100, blocked by 10% goat serum/PBS, incubated with anti-NCL and anti-Hsc70 antibodies, and finally stained with FITC-conjugated goat-anti-rabbit IgG and TRITC-conjugated goat-anti-rat IgG. Nuclei were stained by DAPI. Bars, 50 μm (four left panels) and 10 μm (Magnified).
Supplemental Figure II

Supplemental Figure II Inhibition of Hsc70 by KNK437 and RNA interference or overexpression of Hsc70 in HUVECs.
(A) The inhibitory effect of KNK437 on heat shock proteins. HUVECs were treated by 100 μM KNK437 for different time (0, 1.5, 3, 4.5, 6 h). The expression of Hsc70, Hsp70, Hsp90α, and Hsp90β were detected by immunoblotting. GAPDH served as loading control.
(B) Knock-down and overexpression of Hsc70 in HUVECs. HUVECs were transfected with scrambled siRNA (S.C.), Hsc70 siRNA-1 (Si-Hsc70-1) and Hsc70 siRNA-2 (Si-Hsc70-2), respectively. For overexpression of Hsc70, HUVECs were transfected with pcDNA3.1 (Vehicle) and pcDNA3.1-Hsc70 (Hsc70) plasmids, respectively. The efficiency was determined by immunoblotting against the anti-Hsc70 antibody. GAPDH served as loading control.
Supplemental Figure III

Phosphorylation of NCL regulates its surface expression.

(A) NCL binds to Hsc70 in a phosphorylation-dependent manner. NCL was immunoprecipitated from HUVEC extracts. The immunoprecipitants were treated with λPPase for 2 h and applied to immunoblotting assay with relevant antibodies. Whole cell lysates were immunoblotted against Hsc70 and GAPDH as input controls.

(B) Inhibition of PKC-ξ, CK2, and Cdc2 suppresses the phosphorylation of NCL. After HUVECs were respectively treated with Staurosporine (iPKC-ξ), CK2 inhibitor IV (iCK2), and olomoucine (iCdc2) for 12 h, cells were immunoprecipitated by anti-NCL antibody. The immunoprecipitants were immunoblotted against anti-phosphothreonine (p-Thr), anti-phosphoserine (p-Ser), and anti-NCL antibodies, respectively.

(C) Suppression of PKC-ξ or CK2 down-regulates the expression of surface NCL by flow cytometry. HUVECs were serum-starved for 24 h, pretreated with iPKC-ξ, iCK2, or iCdc2 for 1 h, and serum-rescued for 12 h. Surface NCL was detected by flow cytometry. DMSO served as a solvent control. N.C. indicated cells incubated with rabbit IgG.

(D) Knocking down PKC-ξ or CK2 suppresses the expression of surface NCL by flow cytometry. After PKC-ξ, CK2 or Cdc2 were respectively knocked down in HUVECs, surface NCL was detected by flow cytometry. N.C. indicated cells incubated with rabbit IgG. S.C. indicated the control group transfected with scrambled siRNA.
Supplemental Figure IV MyH9 interacts with the RGG domain of NCL. HMECs were transfected with various nucleolin truncations and immunoprecipitated with non-specific IgG or MyH9. The co-IP samples were immunoblotted against GFP and MyH9. Whole cell lysates were immunoblotted against GFP and GAPDH as input controls.
Supplemental Figure V

Hsc70 regulates the cell motility induced by NCL. HMECs were transfected with pCDNA3.1 (Vehicle) or pCDNA3.1-NCL (NCL) to overexpress NCL, and simultaneously transfected with scrambled siRNA (S.C.) or Hsc70 siRNA (Si-Hsc70) to knock down Hsc70. Cells were seeded in a 24-well plate and allowed to migrate towards the scratched wounds. The wound edge images were captured by microscopy (×40) in five random fields for each group. The relative wound closure width was calculated.
Supplemental Figure VI Knock-down of talin influences the expression of surface NCL. Talin was knocked down in HMECs by transfection of talin siRNA (top). HMECs transfected with scrambled siRNA (S.C.) and Hsc70 siRNA (Si-Hsc70) were respectively incubated with rabbit anti-NCL antibody (5 μg/ml) for 1 h and subsequently with FITC-conjugated goat anti-rabbit IgG for 1 h, and analyzed by flow cytometry (bottom). Negative control (N.C.) antibody was rabbit IgG at the same concentration.
Supplemental Figure VII Schematic model of NCL translocation regulated by Hsc70. Hsc70 interacts with NCL in endothelial cells, which is mediated by both the PBD of Hsc70 and the RBD3-4 of NCL. Hsc70 modulates the surface translocation of NCL via stabilizing NCL and enhancing the interaction between NCL and MyH9. Phosphorylation of NCL by PKC-ξ and CK2 remarkably influences the interaction between NCL and Hsc70, and subsequently affects the expression of cell-surface NCL. The cell-surface NCL modulates endothelial cell migration and tumor angiogenesis.