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Regulation of Embryonic Stem Cell Pluripotency By Heat Shock Protein 90

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Abstract

Deciphering the molecular basis of stem cell pluripotency is fundamental to the understanding of stem cell biology, early embryonic development, and to the clinical application of regenerative medicine. We report here that the molecular chaperone heat shock protein 90 (Hsp90) is essential for mouse embryonic stem cell (ESC) pluripotency through regulating multiple pluripotency factors, including Oct4, Nanog and signal transducer and activator of transcription 3 (Stat3). Inhibition of Hsp90 by either 17-*N*-Allylamino-17-demethoxygeldanamycin (17-AAG) or miRNA led to ESC differentiation. Over-expression of Hsp90 β partially rescued the phenotype; in particular, the levels of Oct4 and Nanog were restored. Notably, Hsp90 associated with Oct4 and Nanog in the same cellular complex and protected them from degradation by the ubiquitin proteasome pathway, suggesting that Oct4 and Nanog are potential novel Hsp90 client proteins. In addition, Hsp90 inhibition reduced the mRNA level of Oct4, but not that of Nanog, indicating Hsp90 participates in Oct4 mRNA processing or maturation. Hsp90 inhibition also increased expression of some protein markers for mesodermal lineages, implying that Hsp90 suppresses mesodermal differentiation from ESCs. These findings support a new role for Hsp90 in maintaining ESC pluripotency by sustaining the level of multiple pluripotency factors, particularly Oct4 and Nanog.

Keywords

embryonic stem cells; pluripotency; Hsp90; Oct4; Nanog; Hop; mesoderm

Introduction

The two fundamental characteristics of ESCs are self renewal and pluripotency^{1–2}. Recent advances in the field, especially those of induced pluripotent stem cells (iPSCs), have discovered that these characteristics are regulated by a complex network of pluripotency factors, including: Oct4^{3–5}, Nanog⁶, Stat3^{7–8}, c-Myc^{9–10}, Sox2^{9–10}, and epigenetic

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Competing Interest None

processes¹¹, etc. Among them, the POU transcription factor Oct4 is a central player, whose cellular level must be tightly controlled to maintain ESC pluripotency status. Up- or down-regulation by 50% leads to ESC differentiation *in vitro*^{12–13}. *In vivo*, Oct4 deletion leads to inner cell mass failure in mice⁴. The homeoprotein Nanog is another central factor for ESCs to maintain their identity¹⁴. Nanog functions by inhibiting NFκB and cooperating with Stat3^{14–15}. Stat3 plays an important role in ESC pluripotency since target deletion of Stat3 resulted in early embryonic lethality, and ectopic expression of a dominant-negative Stat3 in ESCs leads to loss of pluripotency^{7–8}. The successful generation of iPSCs from terminally differentiated cells also demonstrates that multiple of the pluripotency factors need to be present in cells simultaneously in order to sustain their pluripotency^{16–27}, the molecular mechanism of which is still elusive and is of particular interest in the field.

ESCs appear to have higher stress tolerance than differentiated cells^{28–30} and have a chaperone profile that is similar to cancer cells^{30–31}. Molecular chaperones play an important role in the regulation of proteostasis, a process that cells utilize to preserve the integrity of proteins^{32–33}. They facilitate newly synthesized proteins to adopt their biologically active confirmation³⁴, assemble and disassemble macromolecular complexes, and to mediate refolding of misfolded proteins and break up protein aggregates^{34–37}.

Hsp90 is a group of highly conserved and crucial stress proteins that are expressed in all eukaryotic cells³⁸. Two isoforms are present in the cytosol, Hsp90α and Hsp90β. Hsp90β is expressed constitutively to a high level in most tissues and is generally more abundant than Hsp90α, whereas Hsp90α is stress-inducible and over-expressed in many cancer cells³⁸. Apart from its housekeeping function as an integral component of the chaperone complex to regulate proteostasis^{36, 39–40}, Hsp90 regulates many cell signaling processes through its client proteins including kinases v-Src, Wee1, and c-Raf, transcriptional regulators such as p53 and steroid receptors, and the polymerases of the hepatitis B virus and telomerase^{541–42}. When bound to ATP, Hsp90 interacts with co-chaperones Cdc37, p23, and an assortment of immunophilin-like proteins, forming a complex that stabilizes and protects target proteins from proteasomal degradation. In most cases, Hsp90-interacting proteins have been shown to co-precipitate with Hsp90 when carrying out immunoprecipitation studies, and to exist in cytosolic heterocomplexes with it. In a number of cases, variations in Hsp90 expression or Hsp90 mutation has been shown to degrade signaling function via the protein or to impair a specific function of the protein (such as steroid binding, kinase activity) *in vivo*^{34, 36–37}. Ansamycin antibiotics, such as Geldanamycin (GA) and radicicol, inhibit Hsp90 function^{43–44}. It is rational to assume that the pluripotency factors are regulated by the molecular chaperones in some way, in particular Hsp90. However, their precise role and the underlying mechanism have not been systematically investigated.

Mouse ESCs are cultured under strict conditions to sustain self renewal and pluripotency, for which the cytokine leukemia inhibitory factor (LIF) is required^{7, 45}. The key action of LIF in maintaining mouse ESCs pluripotency is the activation (phosphorylation) and subsequent nuclear translocation of Stat3 via an IL-6 pathway^{46–48}. In differentiated cells, inhibition of Hsp90 by GA suppresses the IL-6 induced activation of Stat3, suggesting potential involvement of Hsp90 in IL-6 signaling pathway⁴⁹. In breast cancer cells, Stat3 physically associates with Hsp90 and Hsp27⁵⁰. Additionally, the activation and nuclear translocation of Stat3 is facilitated by a co-chaperone Hsp70/Hsp90-organizing protein (Hop)^{47–48} and LIF promotes Hsp90 association with Stat3 in mouse ESCs⁵¹. In spite of such previous findings, the role of Hsp90 in regulating stem cell pluripotency, and its underlying mechanism, have yet been thoroughly investigated and established.

In this report, we present evidence to demonstrate that Hsp90 is required for pluripotency in mouse ESCs by using multiple approaches. We also show that Oct4 and Nanog are potential

Hsp90 client proteins because Hsp90 associates with Oct4 and Nanog and protects them from degradation by the ubiquitin proteasomal pathway.

Materials and Methods

Materials

ES-J1 mouse ESCs and feeder fibroblasts were purchased from the ES cell core facility (Dr. Ali Eroglu), Medical College of Georgia, Georgia Health Sciences University. 17-AAG was from LC laboratories (Woburn, MA). Hoechst 33258, goat anti-rabbit IgG, and horseradish peroxidase conjugate were obtained from Sigma (St. Louis, MO). Antibodies against cleaved caspase 3, phospho-Stat3, and Stat3 were from Cell Signaling (Beverly, MA). Donkey anti-mouse and anti-rabbit IgG Cy2 and Cy3 were from Jackson ImmunoResearch (West Grove, PA). Knock-out Dulbecco's modified Eagle's medium (DMEM), knock-out serum replacement (KSR), MEM non-essential amino acid, β -Mercaptoethanol, and ES qualified fetal bovine serum were from Invitrogen (Grand Island, NY). Antibody against Nanog was from ReproCELL (San Jose, CA). Hop antibody was from Enzo Life Sciences International, Inc (Plymouth Meeting, PA). Esgro LIF, Hsp90 α and Hsp90 β antibodies were from Millipore (Billerica, MA). Antibodies against Oct4 and AFP were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against Desmin and Vimentin were from BD Biosciences (San Jose, CA). MG132 was from Cayman Chemical (Ann Arbor, MI).

Generation of Hsp90 miRNA

Hsp90 α and Hsp90 β miRNA plasmid constructs were generated using the BLOCK-iT Pol II miRNAi Expression Vector Kits according to manufacturer's instructions (Invitrogen, Carlsbad, CA). Oligonucleotide sequences were as follows: for 90 α -2139: 5'- ATC TTG CTG TAT GAA ACT GCA -3'; 90 α -2161: 5'- TCC TAT CTT CTG GCT TCA GTC-3'; 90 β -1201: 5'-TGA TAC CTG AGT ACC TCA ACT-3'; and 90 β -1626: 5'- GTG TAT ATG ACT GAG CCT ATT-3'.

Generation of Hsp90 β lentiviral particles and titer measurement

A full length Mouse Hsp90 β cDNA was cloned into pSMPUW-IRES-GFP lentiviral vector using the ViraSafe Lentiviral Bicistronic Expression System (Pantropic) kit (Cell Biolabs, San Diego, CA) according to the manufacturer's instructions. Hsp90 β lentiviral particles were generated in 293LTV cell lines by co-transfection of the plasmids pSMPUW-IRES-GFP Hsp90, pRSV-REV, pCMV-VSV-G, and pCgpV in a 3:1:1:1 ratio. A control (LacZ) viral particle was also generated. The titer of the viral particles was measured using the QuickTiter Lentivirus Titer Kit (Cell Biolabs, San Diego, CA) according to the manufacturer's instructions.

ES cell culture, treatment and electroporation

Mouse ESCs (ES-J1) were cultured following a protocol as previously published⁵²⁻⁵⁴. Briefly, undifferentiated ES-J1 cells were first grown on a feeder layer of irradiated mouse embryonic fibroblasts (MEF) in Knockout DMEM containing 15% KSR, pen/strep, and 1,000 U/ml of Leukemia Inhibitory Factor (LIF). They were passed onto gelatin-coated tissue culture dishes without feeder MEF in Knockout DMEM containing 15% heat-inactivated ES-qualified Fetal Bovine Serum, pen/strep, and LIF. Human ESCs BG01 were maintained on irradiated MEF. They were passed onto feeder-free dishes in feeder-conditioned medium (MEF-CM). MEF-CM was produced by conditioning of irradiated MEFs for 24–36 hours in DMEM/ F-12 medium containing 20% KSR, 8ng/ml human recombinant fibroblast growth factor 2 (FGF2, R&D Systems), 1 mM nonessential amino acids, L-glutamine, pen/strep, 1000U/ml LIF and 0.1 mM 2-mercaptoethanol⁵⁵⁻⁵⁶. Hsp90

miRNAs are electroporated using a nucleofactor II device (Lonza, Walkersville, MD, USA). For the proteasomal inhibition, 0.2 μ M MG132 was supplemented to the ES cell medium 0.5 hour prior to 250nM 17-AAG addition. Cell lysates were collected after another 48 hours.

Co-Immunoprecipitation (Co-IP), immunofluorescence and Western blot analyses

For co-IP experiments, ESCs were lysed in NP-40 lysis buffer (137mM NaCl, 20mM Tris-HCl, pH 8.0, 1.5mM MgCl₂, 1mM EDTA, 0.2% NP-40) containing protease and phosphatase inhibitors. Cell debris was removed by centrifugation, and equal amounts of supernatant protein were used for the control and target antibody immunoprecipitation experiments. The lysate was pre-cleared by incubating with protein A/G-conjugated agarose beads and 1–2 μ g of primary antibody was added. The mixture was incubated at 4 °C for 2 hours on a shaker. Protein A-conjugated agarose beads were then added to the mixture and incubated overnight. The beads were pelleted by centrifugation and washed with the lysis buffer three times. The washed beads were then mixed with Laemmli SDS buffer and processed for immunoblot analysis.

Immunostaining of fixed ESCs was performed following procedures described previously⁵⁴. Briefly, the cells fixed on coverslips were permeabilized by 0.2% Triton X-100 and non-specific antigen binding sites were saturated by incubation with 3% ovalbumin in phosphate-buffered saline (PBS). The specimens were incubated with primary antibodies and then secondary antibodies with different fluorophores. Epifluorescence microscopy was performed with an Axiophot microscope (Carl Zeiss MicroImaging, Inc., Thornwood, NY) equipped with a Spot II CCD camera. Phase contrast micrographs were taken using Nikon Eclipse TE300 (Melville, NY, USA). Micrographic images were taken at the same settings for laser intensity and signal amplification. Images obtained with secondary antibody only were used as negative controls representing the background intensity in a particular laser channel.

Western blot analyses were performed following previously published protocols^{52–54}.

AP Assay and colony counting

AP staining was performed using an Alkaline Phosphatase Detection Kit (Millipore) according to the manufacturer's instructions. Briefly, ESCs (on cover slips) were fixed with 4% paraformaldehyde for 1–2 minutes, rinsed once with TBST buffer (20mM Tris-HCl, pH 7.4, 0.15M NaCl, 0.05% Tween-20), and stained with Naphthol/Fast Red Violet Solution (Fast Red Violet, Naphthol AS-BI phosphate and water were mixed in a 2:1:1 ratio). The cover slips were rinsed with TBST, and covered with PBS. The number of red and colorless colonies was counted double blindly under a microscope. Undifferentiated ES colonies were stained red because they expressed AP; while differentiated colonies were not stained.

Reverse transcription, RT PCR and Quantitative PCR

Total RNA was prepared from control and 17-AAG-treated ESCs using TRIzol reagent following the manufacturer's protocol (Invitrogen, Grand Island, NY). First-strand cDNA was synthesized using an Omniscript RT kit according to the manufacturer's protocol (Qiagen, Valencia, CA). The amount of template from each sample was adjusted until PCR yielded equal intensities of amplification for β -actin or glyceraldehyde-3-phosphate dehydrogenase mRNA. All real-time PCR reactions were performed using an iQ SYBR Green Supermix and an iCycler real-time PCR detection system (Bio-Rad, Hercules, CA). Fluorescence measurements during the extension steps of PCR cycles were used to calculate threshold cycle values. Fold changes in Oct4 mRNA abundance were calculated by a comparative threshold cycle method⁵⁷ using β -actin mRNA as an internal control in each sample. The following primers were used for RT-PCR or real-time PCR: mOct4 (sense): 5'-

TGC CCC CAG GTC CCC ACT TTG-3'; mOct4(antisense): 5'CAG TTT GAA TGC ATG GGA GAG-3'; hOct4 (sense): 5'-TGC AGC AGA TCA GCC ACA TCG-3'; hOct4(antisense): 5'-ACC CAG CAG CCT CAA AAT C-3'; β -actin (sense) 5'-CAT CGA GCA CGG CAT CGT CA-3'; β -actin (antisense) 5'-TAG CAC AGC CTG GAT AGC AAC-3'; mdesmin (sense): 5'- GAA TAC CGA CAC CAG ATC CAG-3'; mdesmin (antisense): 5'- TCT CCA TCC CGG GTC TCA ATG-3'; mKlf4 (sense): 5'-AGA TTA AGC AAG AGG CGG TCC-3'; mKLF4(antisense): 5'-TTA AAA GTG CCT CTT CAT GTG-3'; mc-Myc (sense): 5'-ACT CAC CAG CAC AAC TAC GCC-3'; mc-Myc (antisense): 5'-TTA TGC ACC AGA GTT TCG AAG-3'; mNanog (sense): 5'-AGG GTC TGC TAC TGA GAT GCTCTG-3'; mNanog(antisense): 5'-CAA CCA CTG GTT TTT CTG CCA CCG-3'.

Animal handling and ESC injection

Mice were handled according to Institutional Animal Care and Use Committee of Georgia Health Sciences University (GHSU) guidelines. The 250nM 17-AAG-treated or untreated ESCs were non-enzymatically dissociated and counted. Equal number (500,000) of viable cells as determined by trypan blue staining were transplanted into the striatum of 10-day-old C57CLB6 mice by intracranial injection (bregma 1 mm, right hemisphere 2 mm off suture, 2mm deep) in 5 μ l of 0.9% sterile saline solution⁵². The mice were housed in the GHSU Laboratory Animal Service facility for 4 weeks until further experimentation.

Statistical analysis

The mean, standard deviation and statistical significance of control and treatment samples were calculated using Microsoft Excel 2007 following published procedures^{58–59}. Data were presented as mean $\pm$ SD. A value of $p < 0.05$ was considered statistically significant.

Results

Hsp90 is required for ESC pluripotency

To investigate the role of Hsp90 in ESC pluripotency, we inhibited Hsp90 using 17-AAG in J1 mouse ESCs. Multiple approaches were used to determine whether pluripotency was maintained after 17-AAG treatment: 1) morphology examination; 2) AP staining; 3) teratoma formation; and 4) pluripotency protein markers (Oct4, Nanog, and phosphorylated Stat3 (pStat3)) measurement. Pluripotent mouse ESCs typically exhibit a dome-shaped morphology with compact colonies and high nucleus-cytoplasm ratios; are AP positive; form teratomas; and express Oct4, Nanog, and activated Stat3 (pStat3)^{10, 60}. Figure 1 shows that all the four tested features of pluripotent mouse ESCs were lost after Hsp90 inhibition by 17-AAG. Instead of a dome-shaped morphology, the 17-AAG treated cells displayed a more extended and flatter shape (Fig.1A). The number of AP positive colonies was reduced by more than 90% in 17-AAG treated cells when compared to vehicle control (Fig.1B). The teratoma size was decreased by more than 50% by 17-AAG treatment (Fig.1C). And finally, the levels of the pluripotency factors Oct4, Nanog, and pStat3 were greatly diminished in the 17-AAG treated samples (Fig.1D). Similar phenotypes were observed when the ESCs were treated by another Hsp90 inhibitor GA (Fig.S1 A and S1B), indicating that Hsp90 inhibition renders mouse ESC differentiation. To further validate Hsp90's role in ESC pluripotency maintenance, we measured the Hsp90 levels during differentiation into embryoid bodies (EB). Figure S2 shows that the levels of both Hsp90 α and Hsp90 β were down-regulated dramatically after 48 hours (EB1-2 in Fig.S2), the time point when EBs were completely formed and further differentiation proceeded. Their levels stayed low after the EBs were attached to tissue culture dish surface and further differentiate (EB2-1 and EB2-2 in Fig.S2).

To establish a genetic link between ESC pluripotency with Hsp90, we generated miRNAs that specifically inhibit Hsp90 α or Hsp90 β . We first used NIH3T3 cells to determine the

efficacy of these miRNAs. Figure S3 shows that miRNA A2139 and A2161 effectively reduced the Hsp90 α level; and miRNA B1201 and B1626 reduced that of Hsp90 β . When miRNA A2139 and B1201 were electroporated into mouse ESCs, we found that these miRNAs effectively reduced the protein levels of Hsp90 α and Hsp90 β , respectively, and a mixture of A2139 and B1201 reduced both (Fig.2A). To evaluate these miRNAs' effect on ESC pluripotency, we measured the levels of Oct4 and Nanog, and found that these pluripotency markers were reduced by Hsp90 miRNAs (Fig.2A). We then counted the AP positive colonies and found that these Hsp90 miRNAs significantly reduced the number of AP positive colonies, with the double transfection of both Hsp90 α and Hsp90 β miRNA being the most potent (Fig.2B).

Next, we performed rescue assays. Over-expression of Hsp90 β by a lentiviral vector partially restored the loss of the ESC pluripotency as determined by AP staining (Fig. 2C), and the levels of pluripotency factors Oct4, Nanog, and pStat3 (Fig.2D). The above data strongly support the conclusion that Hsp90 is required for mouse ESC pluripotency.

Hsp90 associates with Oct4 and Nanog and protects them from degradation

Our data show that Hsp90 modulates multiple pluripotency factors simultaneously, especially Oct4 and Nanog. To characterize the mechanism of Hsp90 mediated Oct4 and Nanog protein expression, we determined whether Hsp90 physically associates with Oct4 and Nanog by co-IP. Figure 3A shows that both Hsp90 α and Hsp90 β pulled down endogenous Oct4 from ESC lysates. In the reciprocal immunoprecipitation reaction, we found that Oct4 antibody pulled down both endogenous Hsp90 α and Hsp90 β (Fig.3B). Similarly, endogenous Nanog and Hsp90 were found to be pulled down by each other (Figs. 3C and 3D). These data indicate that Hsp90 associates with Oct4 and Nanog in the same cellular complex.

Molecular chaperone machineries associate with client proteins to assist with protein folding and maturation, and protect them from degradation through the proteasomal pathway^{34, 37}. To determine whether Hsp90 protects Oct4 and Nanog from degradation, we inhibited the ubiquitin proteasomal degradation pathway by MG132 in 17-AAG treated ESCs. Figure 3E shows that MG132 treatment prevented the Nanog and Oct4 down-regulation mediated by Hsp90 inhibition. The above data demonstrate that Oct4 and Nanog are potential novel Hsp90 client proteins, through which Hsp90 participates in the maintenance of ESC pluripotency.

Hsp90 inhibition reduced Oct4 mRNA level in both mouse and human ESCs

To further understand how Hsp90 regulates the pluripotency of ESCs, we measured the mRNA levels of some major pluripotency factors, namely Oct4, Nanog, Sox2, c-Myc, and Klf-4. Surprisingly, we found that Oct4 mRNA level was reduced by 17-AAG in mouse ESCs in a dose-dependent manner, while Nanog, Sox2, c-Myc, and Klf-4 mRNA levels remained intact (Fig. 4A). Quantitative real time PCR indicated that Hsp90 inhibition by 17-AAG reduced the Oct4 mRNA level to $17\pm3.6\%$ of control in mouse ESCs (Fig. 4B). To test whether Hsp90 inhibition mediated similar phenotype in human ESCs, we treated human ESCs with 17-AAG. Quantitative real time PCR showed that Hsp90 inhibition reduced Oct4 level to $8.8\pm0.5\%$ of control level in human ESCs (Fig. 4C). These data indicate that Hsp90 regulates Oct4 mRNA transcription, maturation, or stability in ESCs. The results in Figure 4A also imply that the effect of 17-AAG is specific.

Hsp90 inhibition reduced Oct4 mRNA in embryoid bodies (EBs)

The ESC-derived EBs is a bona fide *in vitro* model of early embryo morphogenesis^{61–62}. EBs form an outer primitive endoderm layer and the underlying primitive ectoderm,

recapitulating a pregastrulation embryo^{61–62}. To further validate that Hsp90 is a direct modulator of Oct4 mRNA transcription, maturation, or stability, we measured its mRNA level in EBs treated with 17-AAG. Figure 4D shows that the Oct4 mRNA level was down-regulated by 17-AAG.

To determine which lineage of differentiation was affected by Hsp90 inhibition in the EBs, we analyzed the primitive ectoderm and primitive endoderm for differentiation markers and found that only the primitive ectoderm marker Fgf5 was reduced, while the primitive endoderm marker GATA4 remained unchanged (Fig.4D and 4E).

Hsp90 inhibition by 17-AAG reduces multiple pluripotency factors in a dose and time-dependent manner

To gain further insight into the kinetics of Hsp90 inhibition mediated down-regulation of the pluripotency factors, we used 17-AAG to perform a dose response study. Figure 5A shows that 17-AAG treatment of ESCs for 48 hours significantly reduced the level of pStat3 and Nanog at a concentration of 50nM or higher. However, it was only when 17-AAG concentration reached 250nM that the levels of Oct4 were significantly reduced (Fig.5A) and the pluripotency of the mouse ESCs was significantly altered (Fig. S4), indicating that 250nM of 17-AAG is an optimal concentration to fully repress Hsp90 in mouse ESCs.

On the other hand, the cochaperone Hsp70/Hsp90 organizing protein (Hop), which was implicated to be required for mouse ESC pluripotency⁴⁸, was upregulated by increasing 17-AAG concentrations (Fig. 5A). We hypothesize that this is a stress response to compensate for Hsp90 inhibition by 17-AAG.

We have also observed that Caspase 3, a marker for cell apoptosis, was activated by increasing concentrations of 17-AAG (Fig. 5A). TUNEL staining showed that 17-AAG treatment resulted in more TUNEL+ cells (Fig. S5A), which was quantified by flowcytometry (Fig. S5B). Western blot analysis showed that 17-AAG increased the level of cytochrome C and reduced the level of a pro-survival factor Survivin (Fig. S5C), confirming that apoptosis was induced by 17-AAG in mouse ESCs. Furthermore, Hsp90 knock down by miRNA also activated caspase3 (Fig. S5D). These observations are consistent with data from previous reports since Hsp90, Oct4, Nanog, and Stat3 are all factors that favor cell survival^{36, 63–66}. Therefore, we tested whether the observed pluripotency loss mediated by Hsp90 inhibition was a result of increased cell death. We determined the time course of pluripotency loss versus cell death using 250nM 17-AAG. Figure 5B shows that 17-AAG significantly repressed pStat3 and Nanog within 24 hours and Oct4 protein within 36 hours, which occurred before the activation of caspase3 that showed a significant increase at 48 hours. These observations exclude the possibility that the ESC pluripotency loss induced by Hsp90 inhibition is a secondary effect of cell death/apoptosis. As in the dose studies shown in Figure 5A, the co-chaperone Hop was increasingly upregulated with time (Fig. 5B), representing a potential compensatory response.

Hsp90 inhibition increased markers for mesoderm lineage

The above data demonstrated that Hsp90 modulates multiple proteins simultaneously to maintain mouse ESC pluripotency. Next, we investigated what cell type (or types) the ESCs differentiate into after Hsp90 inhibition. We first immunostained cells using markers for different cell lineages: α -fetoprotein (AFP) for endoderm, vimentin for ectoderm, and desmin and protein T (Brachyury) for mesoderm^{52, 67}. Figure 6A shows that 17-AAG treated mouse ESCs stained positive for desmin. The immunofluorescence data was validated by RT-PCR, real time PCR and Western blot analysis (Fig. 6 B–D). AFP and vimentin were determined by Western blot analysis and RT-PCR, however their levels were

too low to be detected (data not shown). Another mesoderm marker, protein T (Brachyury)^{67–68}, was also increased by Hsp90 inhibition (Fig. 6D).

Discussion

Both human and mouse ESCs have been shown to have similar chaperone profiles as cancer cells^{30–31}. However, what role the molecular chaperones play in stem cell pluripotency maintenance has yet to be determined in depth. Hsp90 is an evolutionarily conserved major molecular chaperone that participates in stabilizing and activating more than 100 proteins — referred to as Hsp90 'client proteins' — many of which are crucial for constitutive cell signaling and adaptive responses to stress^{36, 69}. In order to carry out these functions, Hsp90, Hsp70, and other co-chaperones, such as Hop, form a dynamic complex known as the Hsp90 chaperone machinery. Cancer cells use this machinery to prevent degradation and misfolding of mutated and over-expressed oncoproteins⁴⁰. Therefore, Hsp90 is now regarded as a key facilitator for oncogene tolerance and cancer cell survival by facilitating numerous transient low-affinity protein–protein interactions^{36, 43, 70}.

Loss of function studies of Hsp90 have been performed in several organisms^{71–73}. In *S. cerevisiae*, mutation of one of the two Hsp90 genes leads to retarded growth at elevated temperatures, whereas mutation of both genes leads to impaired growth at any temperature⁷¹. In *D. melanogaster*, mutation of the one Hsp90 gene (Hsp83) was found to be lethal⁷³. And in mice, knockout of Hsp90 β causes early embryonic lethality⁷². Hsp90 α knockout mice have not been reported but are believed to be embryonic lethal, too. These previous findings suggest that Hsp90 is required for early embryonic development and possibly stem cell self renewal and pluripotency.

To maintain mouse ESC pluripotency, LIF is generally used as an extrinsic factor^{7, 45}. However LIF (and hence the LIF/Stat3 pathway) is not sufficient to maintain human ES cell pluripotency⁷⁴, probably due to the lower expression level of LIF receptor in human ESCs^{75–76}. Instead, BFGF is required as an extrinsic factor to grow pluripotent human ESCs. One important finding of this manuscript is that Hsp90 not only influences STAT3 activity, but also the protein levels of Oct4 and Nanog, which are essential for the pluripotency of both human and mouse ESCs.

Using both Hsp90 inhibitors and Hsp90 miRNAs, we show for the first time that Hsp90 is required to maintain the pluripotency in mouse ESCs by modulating the levels of Oct4, Nanog, and pStat3 (Fig. 1 and Fig. 2). Furthermore, we demonstrate Hsp90 associates with Oct4 and Nanog in the same cellular complex and protects them from proteasomal degradation (Figs. 3A–3E and 5A–5B), suggesting that Oct4 and Nanog are potential novel Hsp90 client proteins.

In addition, Hsp90 inhibition reduced the level of Oct4 mRNA in both mouse ESCs and EBs, and in human ESCs (Fig. 4), indicating that Hsp90 modulates Oct4 on the mRNA level, too. This is plausible since approximately 3% of the intracellular pool of Hsp90 is located in the nucleus and regulates nuclear events³⁶. Another possible interpretation of this phenomenon is that ESCs differentiate into mesoderm lineages after Hsp90 inhibition, leading to reduced Oct4 transcription.

Many of the pluripotency factors, such as c-Myc, Oct4 and Nanog, are oncoproteins and/or associate with cancer stem-like cells^{77–79} and Hsp90 inhibitors have been extensively studied for anticancer therapies^{36, 65}. Consistent with these studies, our data show that prolonged Hsp90 inhibition eventually led to apoptosis (Figs. 5A, 5B, and S5A–S5D). So the essential question is, was the observed pluripotency loss a result of cell death? Our time course study shows that 17-AAG down-regulated the pluripotency factors prior to the

activation of apoptotic marker caspase3, indicating that the cells start to lose pluripotency prior to the apoptosis onset (Fig.5B). These data demonstrated that Hsp90 is required for both ESC pluripotency and survival.

Hsp90 inhibition by 17-AAG seemed to increase the level of pStat3 and Nanog, and decrease cleaved caspase3, at a lower concentration of 10nM (Fig.5A). This is a very interesting phenomenon and will be addressed in a separate study.

Intriguingly, after Hsp90 inhibition by 17-AAG, the ESCs increased the expression of mesoderm markers desmin and protein T, implying that they might preferentially differentiate into mesoderm lineages. The mesoderm cells are essential for the development of the coelom and the inner cavity in which the major organs develop. In addition, mesoderm cells give rise to muscles and bones, the vascular and lymphatic systems, as well as the kidneys, gonads, and urogenital ducts⁸⁰. In our future studies, we will further investigate the mesoderm differentiation by using more model systems, such as the teratoma system⁶⁸.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ESCs	embryonic stem cells
Hsp90	Heat shock protein 90
Stat3	signal transducer and activator of transcription
iPSCs	induced pluripotent stem cells
LIF	leukemia inhibitory factor
Hop	Hsp70/Hsp90-organizing protein
17-AAG	17- <i>N</i> -Allylamino-17-demethoxygeldanamycin
EB	embryoid body

Reference

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Figure 1. Hsp90 is required for stem cell pluripotency- 17-AAG induced ESC differentiation

A. Bright field images of mouse ESC colony morphology, feeder-free J1 mouse ESCs were treated with vehicle control or 250 nM 17-AAG for 48 hours. Con, vehicle treated; AAG, 250nM 17-AAG, scale bar: 30 μ m. **B.** AP staining of the mouse ESC colonies. Feeder-free mouse ESCs grown on cover slips were treated with vehicle control or 250 nM 17-AAG for 48 hours, fixed and stained with alkaline phosphatase. Left panel, typical images, scale bar: 20 μ m; right panel, statistic analysis of the number of AP positive colonies per cover slip. N=4, **, $p < 0.01$. **C.** Teratoma formation (arrow on the inset). 17-AAG treated or untreated cells were injected intracranially into P3 mouse brains. Mice were sacrificed and relative teratoma weight was measured four weeks later, N=4, *, $p < 0.05$. Inset shows a set of typical images of the teratoma. **D.** Western blot analysis of pluripotency factors. Phosphorylated Stat3 (pStat3), total Stat3 (tStat3), Nanog, and Oct4 were detected using 250nM 17-AAG-treated mouse ESC samples; β -Actin was used as loading control. Results from two different sets of samples were shown.

Figure 2. Hsp90 is required for stem cell pluripotency - Hsp90 miRNA led to ESC differentiation and cell death and Hsp90β prevented 17-AAG mediated pluripotency loss

A. Hsp90 miRNA reduced Oct4 and Nanog protein levels in mouse ESCs. The Hsp90α miRNA2139 and Hsp90β miRNA1201 construct, which have been proved to effectively repress Hsp90α and Hsp90β expression (Fig. S3), were electroporated into mouse ESCs. Cell lysates were assayed by Westernblot analysis for Hsp90α, Hsp90β, Oct4, and Nanog. β-actin was used as loading control. **B.** Hsp90 inhibition by miRNA reduced ESC differentiation. Mouse ESCs were treated as in A except that the cells were fixed for AP staining and the number of AP positive colonies was counted. Y axis represents the number of AP positive colonies per cover slip. N=4, *, p<0.05; **, p<0.01. **C.** Overexpression of Hsp90β prevented 17-AAG mediated ESC pluripotency loss. Feeder free mouse ESCs were infected with an Hsp90β lentiviral vector or a control (LacZ) lentiviral vector. Twenty four hours later, the culture media were supplemented with 250 nM 17-AAG and the cells were incubated for another 48 hours. Then the cells were fixed, stained, and AP positive counted. Y axis represents the number of AP positive colonies per cover slip. N=3. **D.** Cells were treated as in C except that cell lysates were examined by Westernblot analysis for the proteins indicated.

Figure 3. Hsp90 associates with Oct4 and Nanog and prevented them from degradation by the ubiquitin proteasome pathway

ESC lysates were subjected to co-IP assays. **A.** Cell lysate was immunoprecipitated with antibodies against Hsp90β and Hsp90α, and immuno-blotted with Oct4 antibody. Beads only and pre-immune serum were used as control. **B.** Cell lysate was immunoprecipitated with Oct4 antibody, and immuno-blotted with antibodies against Hsp90α and Hsp90β. Beads alone was used as control. **C.** Cell lysate was immunoprecipitated with antibodies against Hsp90α and Hsp90β, and immuno-blotted with Nanog antibody. **D.** Cell lysate was immunoprecipitated with Nanog antibody, and immuno-blotted with antibodies against Hsp90α and Hsp90β. Beads alone was used as control. **E.** Feeder free ESCs were treated with 0.2μM MG132 0.5 hour prior to 250nM 17-AAG addition. Another 48 hours later, cell lysates were collected and Western blot analysis performed. Note proteasomal inhibition prevented Oct4 and Nanog protein loss mediated by 17-AAG. MG, 0.2μM MG132 treatment.

Figure 4.

Hsp90 inhibition reduced the level of Oct4 mRNA. **A.** Feeder free mouse ESCs were treated for 48 hours with 17-AAG of concentrations indicated. Total RNA was collected and RT-PCR performed with primers for the mRNA of indicated genes. **B.** Real time quantitative PCR of Oct4 mRNA using mouse ESCs. **C.** Real time quantitative PCR of Oct4 mRNA on human ESCs. **D.** RT-PCR of Oct4 mRNA on floating EBs treated with 17-AAG for 24 hours and 48 hours. **E.** Densitometry quantification of the Oct4 and Fgf5 bands in D, values shown are normalized to β -actin. N=3.

Figure 5. Dose and time course study of the effect of Hsp90 inhibition on ESC pluripotency factors

A. Feeder free mouse ESCs were treated for 48 hours with 17-AAG of concentrations indicated. Cell lysates were collected and Western blot analysis performed for proteins indicated. Casp3, cleaved (activated) caspase 3. **B.** Feeder free ESCs were treated with 250 nM 17-AAG for the time period indicated. Cell lysates were subjected to Western blot analysis for proteins as in A.

Figure 6. Hsp90 inhibition increases markers for mesoderm differentiation

A. Mouse ESCs were treated with 250nM 17-AAG or vehicle for 48 hours, fixed or collected for immunofluorescence for markers of endoderm (AFP), ectoderm (vimentin), and mesoderm (desmin). Hoe, nuclei staining with Hoechst. Scale bar, 30 μ m. **B.** RT-PCR for Desmin and SM-Actin mRNA; **C.** Real time PCR for Desmin mRNA. **D.** Western blot analysis for Desmin protein and another mesoderm marker protein T.