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Proteomic Analysis of Bovine Axonemes Exposed to Acute Alcohol: Role of eNOS and HSP90 in Cilia Stimulation

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Abstract

Background—Cilia are fingerlike motor-driven organelles, which propel inhaled particles and mucus from the lung and airways. We have previously shown that brief alcohol exposure stimulates ciliary motility through an endothelial nitric oxide (eNOS)-dependent pathway localized in the ciliary metabolon. However, the signaling molecules of the ciliary metabolon involved in alcohol-triggered cilia beat frequency (CBF) stimulation upstream of eNOS activation are unknown.

Methods and Results—We hypothesized that brief alcohol exposure alters threonine and serine phosphorylation of proteins involved in stimulating ciliary beat frequency. Two-dimensional electrophoresis indicated both increases and decreases in the serine and threonine phosphorylation states of several proteins. One of the proteins identified was heat shock protein 90 (HSP90), which undergoes increased threonine phosphorylation after brief alcohol exposure. Because HSP90 has been shown to associate with eNOS in lung tissue, we hypothesized that HSP90 is a key component in alcohol-triggered eNOS activation and that these two proteins co-localize within the ciliary metabolon. Immunofluorescence experiments demonstrate that eNOS and HSP90 co-localize within basal bodies of the ciliary metabolon and partially translocate to the axoneme upon brief alcohol exposure. Pretreatment with geldanamycin, which disrupts HSP90 chaperone functions, prevented eNOS-HSP90 association and prevented the translocation of eNOS from the ciliary metabolon to the axoneme. Functional cilia motility studies revealed that geldanamycin blocked alcohol-stimulated ciliary motility in bovine bronchial epithelial cells and mouse tracheal rings.

Conclusions—Based on the HSP90 localization with eNOS, alcohol activation of HSP90 phosphorylation, and geldanamycin's ability inhibit HSP90-eNOS association, prevent eNOS translocation to the axoneme, and block alcohol-stimulated ciliary motility, we conclude that alcohol-induced cilia stimulation occurs through the increased association of HSP90 with eNOS. These data help further elucidate the mechanism through which brief alcohol exposure stimulates CBF.

Introduction

Cilia are finger-like projections that propel inhaled particles and mucus from the lung and airways. We have previously shown that brief alcohol intake stimulates ciliary motility through activation of endothelial nitric oxide (eNOS), which subsequently stimulates guanylyl cyclase, sequentially activating protein kinase G (PKG) followed by protein kinase A (PKA) activation (Sisson, 1995; 2009; Stout et al., 2007; Wyatt et al., 2003).

The rapid stimulation of ciliary beat frequency (CBF) by these essential alcohol-triggered signaling molecules indicates that they are tightly associated with the axoneme and localize to the attached axonemal basal body (BB) of each cilium. Although we have been able to dissect the downstream signaling effect of alcohol-triggered eNOS activation on cilia function, we do not know what signaling molecules are activated by alcohol upstream of eNOS. We hypothesize that acute alcohol exposure alters threonine and serine phosphorylation of a select few proteins involved in regulating ciliary beat frequency. Two-dimensional electrophoresis indicated both increases and decreases in the serine and threonine phosphorylation states of a few proteins in isolated cilia + BB preparations. One of the proteins identified was heat shock protein 90 (HSP90), which undergoes *increased* threonine phosphorylation under brief alcohol exposure of isolated cilia. HSP90 has been shown to associate with eNOS in a variety of systems including the lung (Mount et al., 2007; Polikandriotis et al., 2007; Takahashi and Mendelsohn, 2003). HSP90, like other heat shock proteins, acts as a molecular chaperone and is involved in the folding of proteins. However, HSP90 may also act as a signal transducer by activating proteins such as protein kinases and transcription factors (Richter and Buchner, 2001; Wandinger et al., 2008).

Within the axoneme + BB organelle preparation, we suspect HSP90 is involved in regulating ciliary beat frequency (CBF) through its interaction with eNOS. It is likely, based on research in other organ systems that HSP90 can function as a molecular chaperone in the ciliary metabolon by enhancing eNOS function. Therefore, we hypothesized that HSP90 is a key component in alcohol-triggered eNOS activation and that these two proteins co-localize within the ciliary metabolon. Furthermore, we hypothesized that alcohol-triggered increased CBF is blocked by pretreatment with geldanamycin, an HSP90 disruptor.

Our results demonstrate that HSP90 undergoes increased threonine phosphorylation upon brief alcohol exposure and co-localizes with eNOS in the ciliary metabolon. Also upon brief alcohol exposure both HSP90 and eNOS partially translocate to the axoneme. Ciliary motility assays revealed that alcohol-triggered increased CBF was blocked by pretreatment with geldanamycin, which blocks HSP90's interaction with eNOS. These results demonstrate that alcohol activates eNOS by increasing threonine phosphorylation of HSP90, resulting in increased HSP90-eNOS association, leading to the observed increases in CBF triggered by alcohol.

Methods

Trachea cilia + BB extraction and preparation

Demembranated ciliary axonemes + BB were isolated from bovine trachea ciliated epithelium as previously described (Sisson et al., 2009). Briefly, fresh bovine tracheas were obtained from a local abattoir. Soft tissue and adipose were removed from the outside of the trachea followed by washing in cold (4°C) phosphate buffered saline (PBS). Both proximal and distal tracheal ends were closed using large hemostats following the addition of 15 ml of extraction buffer (20 mM Tris-HCl, 10 mM calcium chloride, 1 mM EDTA, 50 mM NaCl, 7 mM 2-mercaptoethanol, 100 mM Triton X-100, and 1 mM dithiothreitol). Next, tracheas were shaken for 90 seconds and extraction buffer containing released axonemes + BB was filtered through 100 µm polypropylene mesh and centrifuged at 17,250g for 10 min. Supernatant was discarded and axonemes + BB were resuspended to a concentration of 1 mg/ml in resuspension buffer (20 mM Tris HCl, 50 mM KCl, 4 mM MgCl₂, 0.5 mM EDTA, 1 mM dithiothreitol, 10 mM soybean trypsin inhibitor, and 25% sucrose [w/v]). Isolated axonemes were used immediately in proteomics studies. Excess axonemes + BB were stored in aliquots at -80°C for use up to six months after isolation.

2D-Gel electrophoresis, Western blot and protein identification

Isolated axonemes + BB exposed to alcohol (10 mM) for 10 min were centrifuged, supernatant removed, and flash frozen in liquid nitrogen. Axoneme samples were stimulated with alcohol and assessed using Sisson-Ammons Video Analysis (SAVA, see method below) to ensure that alcohol triggered ciliary beating before samples were submitted to Applied Biomics (Hayward, CA) for 2D DIGE analysis, phospho-westerns, and protein identification. Briefly, protein concentrations were determined and adjusted to the desired concentration. Samples were labeled with either Cy2 or Cy3 dye and run for comparative analysis on two separate preparations. Following electrophoresis, gels were scanned using a Typhoon image scanner (Piscataway, NJ). ImageQuant software (Piscataway, NJ) was used to generate gel image data. Proteins from the 2D gel were then transferred to PVDF membranes and membranes scanned. Western blots were performed using mouse anti-phospho-serine and rabbit anti-phospho-threonine.

Isolation of ciliated bovine bronchial epithelial cells

Primary bovine bronchial epithelial cells (BBECs) were prepared from bovine lung obtained from a local abattoir, as described previously (Stout et al., 2007). Briefly, bronchi were dissected from the lung and digested overnight at 4° C in 0.1% bacterial protease type IV in minimum essential media (M199 with Earl's salts; Gibco, Carlsbad, CA). The following day, the bronchi were repeatedly rinsed in M199 containing 10% FBS (Gibco) to collect a mixture of >95% viable ciliated and basal epithelial cells lining the lumen. Clumped, ciliated primary cells attached to a confluent basal monolayer were collected by a 40 µm mesh filter and grown on collagen coated tissue culture dishes. This preparation resembles the ciliated, goblet, and basal cells that populate the normal airway. BBECs were washed in M199 media and counted with a hemacytometer, and 10⁵ cells were cytospun (1200 rpm for 4 min) onto concanavalin A (5 mg/ml; Sigma-Aldrich, St. Louis, MO)–coated slides in preparation for immunohistochemistry (IHC).

Immunofluorescence

Bovine bronchial airway epithelial cells were isolated from fresh bovine lungs and cytospun onto slides for indirect immunofluorescence as follows: cells were fixed in 4% paraformaldehyde then permeabilized and blocked for 15 min (0.1% Triton X-100, 1% BSA, 0.02% Sodium Azide). Cells were incubated in primary antibodies (mouse anti-HSP90 and rabbit anti eNOS; Invitrogen by Life Technologies, Carlsbad, CA) overnight at 4°C. Following incubation with the primary antibody, cells were gently rinsed in PBS (5 min) and then incubated with secondary antibodies (Alexa 488 and Alexa 647 against rabbit or mouse; Invitrogen by Life Technologies, Carlsbad, CA) for 1 hr. at room temperature in a humidified chamber. Following incubation, cells were rinsed in PBS and mounted using ProLong Gold with DAPI (Invitrogen by Life Technologies, Carlsbad, CA). Imaging was performed using an LSM 510 Meta Laser Scanning Confocal Microscope (Zeiss, Thornwood, New York). All images were collected as Z-stacks using the set parameters (laser strength, gain, etc.). Z sections were obtained through cells at 0.5 µm.

Treatment of ciliated bovine bronchial epithelial cells

Primary BBECs were pretreated with 1µM geldanamycin for 24 hours. Following pretreatment, BBECs were exposed to 100 mM alcohol for 1 hour. We have tested lower concentrations of alcohol, which also stimulate ciliated BBECs, however treatment with 100mM alcohol results in maximum CBF and signaling activation of ciliated BBECs. Brief alcohol stimulation of CBF in cultured BBECs maximizes between 1–2 hours of exposure. CBF measurements were made using SAVA (see method below).

Immunoprecipitation of HSP90 and eNOS

Primary ciliated BBECs were pretreated with 1 μ M geldanamycin for 24 hours. Following pretreatment BBECs were exposed to 100 mM alcohol for 1 hour. Cells were lysed with RIPA buffer, centrifuged and supernatant saved. Next, cells were treated for 1 hour at room temperature with 0.05 mM EDC (Pierce, Thermo Fisher, Rockford, IL) made in 100 mM Hepes (pH 7.5). The reaction was terminated by 5-fold molar excess addition of 2-mecaptoethanol. Samples were then diluted 20 fold with 1% Nonident P-40 in Tris-buffered Saline (10 mM Tris-HCL; pH 7.5, 0.15 NaCl) and rotated for 24 hours at 4° C in the presence HSP90 (1 μ g/ml) or eNOS (1 μ g/ml) monoclonal antibodies (BD Biosciences). After 24 hours protein A/G Plus-Agarose beads (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) were added to the samples and rotated for 24 hours at 4°C. Immunoprecipitates were centrifuged at 2,500 rpm (~1000g) for 5 min at 4°C. Supernatant was removed and pellet was washed in Nonident P-40 Tween and centrifuged. The wash was repeated 3 times. After the final wash supernatant was discarded and the pellet was resuspended in 40 μ l of SDS-comprising sample buffer and boiled for 10 min. Samples were centrifuged to remove agarose bead followed by SDS-PAGE analysis.

Assay for Protein Content

Protein concentrations were measured using the NanoDrop 1000 (Thermo Scientific) according to the manufacturer's instructions.

Mouse tracheal rings

Tracheas were isolated from C57BL/6 mice. Upon extraction, tracheas were immediately sliced into 1mm sections under a dissection scope using a sharp scalpel. Tracheal rings were placed into M199 media (Gibco) supplemented with 10% FBS (Gibco). Slices were kept in a 37 °C incubator with 5% CO₂ until needed. Tracheal rings were exposed to alcohol (10 μ M) for 6 hours $\pm$ pretreatment with geldanamycin (1 μ M) for 24 hours. CBF was measured using SAVA.

Ciliary beat frequency measurement and analysis

Measurement of ciliary beat frequency (CBF) of tracheal rings and ciliated BBECs was derived using the Sisson-Ammons Video analysis (SAVA) system (Sisson et al., 2003). Experiments were captured as previously described (Wyatt et al., 2005). Temperatures were controlled with a thermostatic stage to remain constant at 26°C. The sampling rate was set at 85 frames per second for all experimental conditions. Cilia with a frequency of ≥ 2 Hz were not analyzed and considered non-motile, as were data points with less than 10% of the original numbers of beating points. A minimal number of six separate fields were captured, analyzed, and expressed as mean $\pm$ SEM for each data point. Significance for paired samples was determined using the Student's t test with P value <0.05. Significance for more than two conditions was determined using a one-way ANOVA with P value <0.05.

Results

Brief alcohol exposure enhances threonine and serine phosphorylation in isolated bovine tracheal cilia + BB

The mechanism of eNOS activation by brief alcohol exposure is unknown. We hypothesized that a limited number of ciliary +BB proteins are phosphorylated by threonine or serine upon acute alcohol exposure.

To determine alcohol-triggered changes in threonine and serine phosphorylation, control and brief alcohol-exposed axoneme + BB samples (10 mM for 10 min) were separated by gel

electrophoresis in two dimensions (2D) by size and pH and stained for all detectable proteins (Figure 1 A, B). Control and alcohol-exposed 2D separated samples were also transferred to nitrocellulose membranes and probed for phospho-serine (data not shown) and phospho-threonine (Figure 1 C, D). Changes in protein phosphorylation states were observed between control and axonemes + BB exposed to brief alcohol. Serine phosphorylation was decreased in spots #1–3 (data not shown) in alcohol-exposed axonemes. Both increases and decreases in threonine phosphorylation were observed in alcohol-exposed axonemes + BB. Overall, phosphorylation changes were detected in approximately 14 different peptides (Table 1).

Protein identification

Mass spectrometry was performed to identify proteins undergoing alterations in protein phosphorylation, which were identified on the blots. Twelve of the 14 spots of interest were selected for analysis, which identified the phosphorylated proteins as keratin, tektin-3, tubulin, KRT5, and HSP90 β (Table 1). While a number of proteins were identified, we were particularly interested in HSP90 because it has been shown to interact with eNOS in other systems resulting in increased eNOS activity (Mount et al., 2007; Takahashi and Mendelsohn, 2003) and we have previously shown that eNOS is present in the basal body of ciliated cells (Stout et al., 2007). We have shown that eNOS is involved in regulating CBF, however the upstream signaling mechanism that activates eNOS is unknown. We hypothesized that HSP90 is a key component in alcohol-triggered eNOS activation and that these two proteins co-localize within the ciliary metabolon.

HSP90 co-localizes with eNOS

To determine the localization of HSP90 in relation to eNOS, we performed indirect immunofluorescence on unstimulated ciliated bovine bronchial epithelial cells (ciliated BBECs). Immunofluorescence revealed that HSP90 co-localized with eNOS to the ciliary basal body of intact ciliated BBECs (Figure 2A). We have previously shown that eNOS localized with pericentrin, a basal body marker, in ciliated epithelial cells (Stout et al., 2007). HSP90 also localized with pericentrin in ciliated epithelial cells, confirming that both eNOS and HSP90 both localize to the basal body (Figure 2B) in the unstimulated state.

HSP90 and eNOS translocates from the ciliary metabolon to the axoneme following brief alcohol exposure

Because we suspect that HSP90 and eNOS association increases CBF, we hypothesized that HSP90-eNOS, that under resting conditions localize to the basal body, translocates to the axoneme upon brief alcohol-stimulation (100 mM alcohol for 1 hour). As indicated by immunofluorescence, some HSP90 and eNOS are still localized to the basal body however, a significant amount of the HSP90-eNOS complex translocates to the axoneme (Figure 3).

Geldanamycin blunts HSP90-eNOS association

The close localization of HSP90-eNOS, and the partial translocation of these two proteins upon acute alcohol exposure, suggests that these two proteins form a complex. We hypothesized that pre-treatment of BBECs with geldanamycin disrupts alcohol-induced HSP90-eNOS association. To determine the association between HSP90 and eNOS during acute alcohol exposure (100 mM alcohol for 1 hour) and pretreatment with geldanamycin, ciliated BBECs were exposed to alcohol and immunoprecipitations were performed by pulling down HSP90 or eNOS. Western blots of HSP90 pull down assays analyzed for eNOS show that eNOS is not readily expressed in control cells, while BBECs treated with 1 hour of alcohol have increased eNOS associated with HSP90. This increased HSP90-eNOS association was blunted by the pretreatment with geldanamycin (Figure 4). Analogous

results were seen in ciliated BBECs immunoprecipitated for eNOS and stained for HSP90 (data not shown). These results demonstrate that acute alcohol increases the association of HSP90 with eNOS and that this association was prevented by pretreatment with geldanamycin, an HSP90 disruptor.

Geldanamycin prevents eNOS translocation from the ciliary metabolon to the axoneme following brief alcohol exposure

Because geldanamycin was able to partially inhibit eNOS and HSP90 association, we hypothesized that geldanamycin would prevent alcohol-triggered transport of eNOS from the ciliary metabolon to the axoneme. Indeed, our results show that eNOS remained in the cytoplasm and ciliary metabolon in ciliated BBECs pretreated with geldanamycin + 1 hour of alcohol. Interestingly, geldanamycin did not prevent the translocation of HSP90 to the axoneme (Figure 5).

Geldanamycin inhibits alcohol-stimulated increased ciliary beat frequency in BBECs and mouse tracheas

Based on our immunoprecipitation data showing that geldanamycin blocks HSP90-eNOS association, we hypothesize that HSP90's association with eNOS during acute alcohol exposure leads to alcohol-triggered CBF stimulation, but pre-treatment with geldanamycin blocks this alcohol-triggered CBF stimulation. To determine the functional role of HSP90 in alcohol-triggered increased CBF, ciliated BBECs were pretreated with geldanamycin (1 μ M), which blocks HSP90-eNOS interaction, for 24 hours. Cells were then exposed to 100mM alcohol for 1 hour and CBF was measured. As expected, alcohol exposure significantly increased CBF ($P < 0.01$ vs. control), while pretreatment with geldanamycin prevented the alcohol-triggered CBF ($P < 0.05$ vs. geldanamycin control) in both BBECs and mouse tracheal ring slices (Figure 6). This supports that HSP90 association with eNOS is required for alcohol to stimulate NO-dependent stimulation of CBF in both cell culture and *ex vivo* animal models.

Discussion

Cilia are specialized motor-driven organelles that expel inhaled particles and mucus from the lung and airways. Essential signaling molecules involved in CBF regulation are tightly associated within the axoneme and attached basal body. These regulator molecules are activated by brief alcohol exposure resulting in increased CBF. We have previously shown that these alcohol-triggered increases on CBF are dependent upon eNOS activation and NO release, which stimulates a guanylyl cyclase and activates protein kinase G (PKG) (PKA; Sisson et al., 2009; Wyatt et al., 2003). However, the signaling molecules activated by brief alcohol exposure that are upstream of eNOS are unknown. 2D Western blots of isolated cilia + BB activated by ATP detected 14 proteins that underwent either increases or decreases in serine or threonine phosphorylation following brief alcohol exposure. One of the key proteins we identified whose phosphorylation state was enhanced by alcohol was HSP90, which has been shown to regulate eNOS activation in other systems (Polikandriotis et al., 2005). Indeed, our localization studies of HSP90 and eNOS illustrate the co-localization of these two proteins within the ciliary metabolon at the base of the cilia under normal conditions and the partial translocation of these proteins when cells are exposed to alcohol. This close association between HSP90 and eNOS within the ciliary metabolon make the likelihood of HSP90-eNOS interactions likely.

Indeed immunoprecipitation assays demonstrate the increased association of HSP90 and eNOS in cells briefly exposed to alcohol. We also demonstrated that pretreatment with

geldanamycin, an HSP90 disruptor, blocked alcohol-triggered HSP-eNOS association and prevented the translocation of eNOS to the axoneme.

To determine if HSP90-eNOS interactions are needed to drive the dual signaling cascade for increased CBF, both BBECs and mouse tracheal rings slices were treated with geldanamycin for 24 hours prior to acute alcohol exposure. CBF significantly increased in both BBECs and mouse tracheal rings with brief alcohol exposure, while pretreatment with geldanamycin prevented alcohol-stimulated CBF.

Overall our data demonstrate that under normal conditions HSP90 and eNOS localize to the ciliary metabolon. Upon brief alcohol exposure HSP90 becomes phosphorylated and increases its association with eNOS leading to increased ciliary beat frequency likely representing an alcohol-driven chaperone effect of HSP90 on eNOS. Pretreatment with geldanamycin blunts the alcohol-triggered HSP90-eNOS association, prevents the translocation of eNOS but not HSP90 to the axoneme, and inhibits alcohol-stimulated CBF.

These observations give us a better understanding of which signaling molecules are being activated in alcohol-stimulated CBF. We speculate that activation of HSP90's chaperone function of eNOS is an important triggering effect of alcohol on cilia and may be important in other cilia stimulatory situations. It known that heavy and prolonged alcohol intake causes non-responsive cilia or cilia desensitization. This non-responsiveness prevents cilia from properly responding to inhaled debris and pathogens, making one more susceptible to disease and lung injury. We suspect that the acute stimulatory pathway is down regulated during heavy and prolonged alcohol intake. Future studies investigating HSP90-eNOS interactions in chronic alcohol exposure may elucidate why chronic alcohol exposure results in non-responsive cilia.

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Figure 1. Two dimensional electrophoresis of control and Ethanol treated axonemes (A & B) and membrane blots stained for phospho-Threonine (C & D)

Total protein of control axonemes and axonemes exposed to Ethanol (10 mM) for 10 min (A & B). Membranes were stained for phospho-threonine (C & D). White circles indicate the regions of interest (ROI) where phospho-threonine increased. Threonine phosphorylation increased in ROIs 7–14 upon alcohol exposure. Arrow indicates HSP90.

Figure 2. Immunofluorescence of eNOS, HSP90, and pericentrin in ciliated bovine airway epithelial cells

A) HSP90 (green) and eNOS (red) are co-localized (yellow) within the basal body of ciliated cells. B) Immunofluorescence of pericentrin, a basal body marker, and HSP90 in ciliated bovine airway epithelial cells. HSP90 (green) and pericentrin (red) are co-localized (yellow) within the basal body of ciliated cells.

Figure 3. Immunofluorescence of eNOS and HSP90 in ciliated bovine airway epithelial cells treated with 1 hour 100 mM Ethanol
HSP90 (green) and eNOS (red) are co-localized (yellow) within the basal body of ciliated cells. Some of the HSP90 + eNOS complexes upon brief alcohol exposure translocate from the basal body out to the axonemes compared to Figure 2A.

Figure 4. Immunoprecipitation eNOS and analyzed for HSP90 in ciliated BBECs treated $\pm$ geldanamycin pretreatment (24hrs) and $\pm$ Ethanol (1hr)

A) Graph of HSP90 integrated density normalized to eNOS. B) A small amount of HSP90 precipitated with eNOS in the absence of alcohol. In contrast, brief alcohol exposure triggered a five-fold increase (compared to media) in HSP90s association with eNOS. This alcohol-enhanced association of HSP90 with eNOS was decreased 2.5-fold (compared to ethanol) by pretreatment with geldanamycin (Geld), an HSP90 disruptor.

Figure 5. Immunofluorescence of eNOS and HSP90 in ciliated bovine airway epithelial cells pre-treated with geldanamycin and then with 1 hour 100 mM Ethanol

HSP90 (green) and eNOS (red) are co-localized (yellow) within the cytoplasm of ciliated cells. Some HSP90 translocates from the basal body out to the axonemes, whereas eNOS is only localized in the cytoplasm.

Figure 6. Ciliary beat frequency of ciliated BBECs (A) exposed to Ethanol (100mM) for 1 hour $\pm$ 24 hours pretreatment with geldanamycin (1 μ M)

CBF of mouse tracheal ring slices exposed to alcohol for 6 hours $\pm$ pretreatment with geldanamycin (1 μ M) for 24 hours. Alcohol significantly increases CBF in both BBECs and mouse tracheal rings. Geldanamycin, which disrupts HSP90 association with eNOS, significantly blocks alcohol-induced stimulated ciliary motility in BBECs and mouse tracheal rings.

Table 1
Protein identification of regions of interest of alcohol-driven changes in phosphorylation
Regions of interest (ROI) numbers correspond to white circles observed on the blots in Figure 1.

Roi#	Protein Name	Accession No	Protein MW	Δ EtOH	Phosphorylation type
1	Leucine-rich repeat-containing protein 23	296487129	39586.8	decreased	Serine
2	Keratin, type II cytoskeletal	114051865	51546.5	decreased	Serine
3	Keratin, type II cytoskeletal	114051865	51546.5	decreased	Serine
4	Tubulin beta-2C chain	298351865	31895.9	decreased	Threonine
5	Tektin-3	149773556	56645.5	decreased	Threonine
6	Tektin-3	296476587	56606.6	decreased	Threonine
7	Heat shock protein HSP-90 beta	296474454	78552.9	increased	Threonine
8	Tubulin-alpha-ID chain	114051854	50250.6	increased	Threonine
9	EF-hand domain-containing protein 1	300795327	73984.5	increased	Threonine
10	EF-hand domain-containing protein 1	300795327	73984.5	increased	Threonine
11	Tektin 3	296476587	56606.6	increased	Threonine
14	KRT5 protein	146186887	62644.2	increased	Threonine