 doi:10.20944/preprints202205.0089.v1

Article

HSPA1B is the Dominant Partner in the HSPA1A/HSPA1B Mediated Proteotoxic Stress Response in Placenta-Derived Stem Cells

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Abstract: Placenta-derived stem cells (PDSCs) offer the advantages of possessing mesenchymal and embryonic traits, broad differentiation potential, large-scale availability, and no ethical constraints in their utilization in therapeutic applications. Elevated protein synthesis and consequently enhanced protein maintenance networks become necessary both due to the requirement to maintain stemness and respond to different stresses. This study aimed to identify the primary determinants of proteotoxic stress response in PDSCs. We generated heat-induced dose-responsive proteotoxic stress models of three stem cell types DBMSCs, DPMSCs, and pMSCs, and measured stress induction through biochemical and cell proliferation assays. RT-PCR array analysis of 84 genes involved in protein folding and protein quality control led to the identification of Hsp70 isoforms HSPA1A and HSPA1B as the prominent ones among 17 significantly expressed genes and with further analysis at the protein level through western blotting. A 24-hours' time series analysis of stress-response allowed a detailed kinetic analysis of HSPA1A and HSPA1B gene and protein expression. More prominent differences between the two Hsp70 isoforms were detected at the translational level eluding to a potential higher requirement for HSPA1B during proteotoxic stress in PDSCs. To conclude, consideration should be given to the manipulation of definitely characterized chaperones at their expression or functional levels when utilizing PDSCs in therapeutic and regenerative applications.

Keywords: placenta-derived stem cells; placenta; stem cells; proteostasis; heat-shock; chaperones; HSPA1B; HSPA1A

1. Introduction

Placenta-derived stem cells (PDSCs) are a type of mesenchymal stem cells (MSCs) that possess characteristics of both embryonic and mesenchymal stem cells while possessing differentiating advantages of immune tolerance like non-carcinogenic status [1–4]. Despite having a mesodermal phenotype, PDSCs still display broad differentiation potential and can differentiate into all embryonic germ layers [3–5]. The dispensability of the placenta post-delivery helps resolve ethical concerns that are integral to embryonic stem cells [2,4,6] and affords the large-scale availability of placenta-derived tissues and stem-cell derivatives without the need for subjecting donors to invasive surgical procedures [2,5]. These features make PDSCs an attractive alternative in cell replacement therapies and regenerative medicine. The fetal membranes forming a specialized interface between mother and fetus are rich in different types of cells, including mesenchymal cells. With

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the growth of the fetus, fetal membranes expand, spreading these cells to different regions of the placenta: placental origin cells are placenta-derived mesenchymal stem cells *pMSCs*, maternal origin cells are decidua parietalis mesenchymal stem cells *DPMSCs, and* those from the maternal-fetal interface are decidua basalis mesenchymal stem cells, *DBMSCs* [7–9].

PDSCs like other stem cells require elevated protein synthesis emanating from their requirements to maintain stemness and differentiation potential and, consequently a need for continuous replication[10–13]. This requirement for dynamic protein synthesis in stem cells is compounded by their ability to sense and respond to varying conditions and stresses from different physiological and cell-habitat sources [11,14]. Consequently, the necessity for constant protein production exerts additional stress on the proteome regulating machinery to ensure dynamic adjustment of the proteome to endogenous needs of the cells in a Spatio-temporal manner. The protein homeostasis (proteostasis) network in the cells coordinates the proteome balance by regulating all steps of protein life cycle; synthesis, folding, conformational maintenance, and degradation [10,13,15–17]. Multiple recent studies point toward a close connection between proteostasis and stem cell function, highlighting the presence of stem cell intrinsic proteostasis mechanisms and their tight coupling to cellular properties and functions [11,12,18–21]. The determining goal for proteostasis is to ensure the operational levels of proteins in their native conformations while simultaneously reducing the presence of deleterious products like aggregates [10,16,17]. Proteostasis, as a result, is critically dependent upon a complex network of proteins called chaperones which function in different stages of the protein life cycle [15,16,20].

70-kDa heat shock proteins (Hsp70s) are a ubiquitous group of chaperones that assist in various processes, including folding of nascent proteins, refolding of aggregated proteins, protein trafficking, and degradation of irreversible aggregates [16,20]. Like other chaperones, Hsp70s display little specificity but form a critical component of the protein-folding machinery because of a high degree of adaptation in their functional properties largely attributed to their interactions with other function complementing proteins [16,22–26]. Because of these properties and their association with different phases of a protein life cycle, the stress inducibility of Hsp70s becomes a crucial factor in the maintenance of cellular health [20,27]. Here we report a quantitative gene expression analysis of 84 genes involved in protein-folding pathways in a proteotoxic stress model in placenta-derived stem cells. We follow this by a detailed analysis of the role of two of the gene- and protein-expression of Hsp70 isoforms HSPA1A and HSPA1B during proteotoxic stress response. Differences in proteostasis patterns have been observed in different stem cell types alluding to existence of cell-specific mechanisms to ensure their functionality according to their purpose and properties[11]. We hope the outcome of this study will shed significant and new light on how placenta-derived stem cells operate their proteostasis network leading to an understanding of their biology and identity. This knowledge, in return can help manipulate stress response pathways to enhance the utilization of these stem cells in cell therapy and other clinical applications.

2. Materials and Methods

2.1. Isolation and culture of placenta-derived Stem Cells

The three placenta-derived stem cells reside in different zones of placenta and the cell cultures of these three cell types were achieved utilizing the already established protocols for their isolation, characterization, and sub-culturing; pMSCs [7], DPMSCs [9], DBMSCs [8]. After harvesting from human placentae, the cells are at passage zero and were used for subsequent experiments at passage two. The cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air, in T25 flasks (Becton Dickinson, Saudi Arabia) in medium containing Dulbecco's Modified Eagle Medium nutrient mixture F-12 (DMEM-F12), 10% Mesenchymal Stem Cell Certified Fetal Bovine Serum (MSC FBS), 100 μg/mL L-glutamate, 100 μg/mL streptomycin, and 100 U/mL Penicillin. The placentae

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were obtained from uncomplicated pregnancies following normal vaginal delivery (38–40 weeks gestation). The placentae were utilized within 2-3 hours of delivery.

2.2. Heat stress experimentation

Heat stress in cells was induced by transferring ~ 60% confluent cells grown at 37˚C to the temperature to be tested. At zero time-point all dishes (with 60-65% confluent cells) will be transferred to the heat stress temperature other than the control that will continue to grow at 37˚C. The exposure temperatures tested were 42˚C for and 44˚C and the cells were initially exposed for 1, 2, and 3 hours. Cell physiology post stress i.e., during recovery phase was monitored by transferring cells back to ambient growth temperature i.e., 37˚C and cells were harvested at each time point and processed according to the next planned downstream step. Briefly, heat stressed, and control cells were washed twice with PBS, and trypsinized. The cells were centrifuged at 5000 rpm for 5 minutes and the supernatant is discarded, and the pellets were frozen for subsequent analyses.

2.3. RNA isolation and cDNA synthesis

Total RNA was isolated from frozen cell pellets using mini RNeasy Mini Kit (Qiagen, Germantown MD). RNA integrity and yield was be analyzed and quantified using the Nano Drop (Thermo Fischer, Wilmington, DE). 2 µg of the total RNA was transcribed into cDNA for all gene arrays and real time PCR experiments, using FastLane Cell cDNA Kit (Qiagen, Germantown MD).

2.4. Protein Aggregation Assay

Protein aggregation as an indicator of cellular stress was measured in the heat-stressed cells and tracked during the recovery phase using the 96-well Protein Aggregation Assay Kit (Cat No: ab234048) supplied by Abcam. This assay relies on binding of a fluorescent probe to the aggregated proteins involving excitation at 440nm and emission at 500nm. 50-100ug protein was required/well, and the samples were read in triplicates during each run. Cells from three experiments were assayed for presence of aggregates. The protein extraction for aggregation assay was accomplished through freezing and thawing cycles to avoid interference from the detergents present in the standard cell lysis buffers. Data was analyzed with reference to the control samples grown at 37°C and represented at percentage increase in fluorescence.

2.5. Cell Proliferation Assays

The heat-stress models were evaluated at cellular level for impact of heat-stress on viability and proliferation. The xCELLigence Real-Time Cell Analyser (RTCA-DP version; Roche Diagnostics, Mannheim, Germany), continuously monitors cellular adherence recording label-free changes in electrical impedance [28–30]. This system uses an electronic readout called *impedance* (resistance to alternating current) used to express the impeded electron flow generated by disruption of interaction between electrodes and bulk solution and is stated as arbitrary units called Cell Index (CI), the magnitude of which is dependent on cell number, morphology, size and on the strength of cell attachment to the plate surface. An initial titration of different cell densities (5, 10, and 20,000 cells/well) was performed and 10,000 cells was found to be the ideal cell density for seeding. Cells growing in the cell culture dishes were trypsinized, counted using trypan blue and then resuspended in the culture medium. Wells of the E-16 plates were equilibrated with the culture media and background measurements were taken. Cells were then plated at density of 10,000 cells/well in fresh medium to a final volume of 200 µl and incubated for 30 minutes at 37 °C and 5 % CO_2 in the RTCA cradle. The impedance signals were recorded for every 10 minutes over a period of 72 hours in control cells grown at 37˚C and heat exposed cells.

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2.6. Gene Arrays and Real time PCR

For gene expression analysis we initially monitored the expression of individual Hsp70 gene followed by a comprehensive analysis of 84 heat shock protein genes through use of PCR array RT² Profiler™ PCR Array Human Heat Shock Proteins & Chaperones (Cat. No: PAHS-076Z Qiagen). This array helps simultaneously profile the expression of 84 heat shock protein genes, in addition offering the capacity to simultaneously evaluate results utilizing five endogenous controls. The primer sequences for Hsp70 gene were obtained from the Harvard Primer Bank repository [31]. Primers used were as follows: HSPA1B, 5′- GCGAGGCGGACAAGAAGAA -3′ (forward) and 5′- GATGGGGTTACACACCTGCT -3′ (reverse); GAPDH, 5′- GGAGCGA-GATCCCTCCAAAAT -3′ (forward) and 5′- GGCTGTTGTCATACTTCTCATGG -3′ (reverse). Quantitative measurement of gene expression for individual gene and those in array was carried out with real time polymerase chain reaction using Platinum PCR SuperMix (Thermo Fisher Scientific) in triplicate with SYBR Green PCR Mix (Qiagen, Germantown MD).

2.7. Protein extraction and concentration determination

Protein extraction from frozen and stored cell pellets was performed by use of RIPA Lysis and Extraction Buffer (Thermo Scientific™ Cat.No: 89900). The cells were thoroughly resuspended in RIPA buffer, vortexed, and incubated on ice for 30minutes prior to centrifuging at 10000*g for 20-30 minutes at 4 ˚C to separate the cell debris. Protein quantification was accomplished by use of Thermo Scientific™ Pierce™ BCA Protein Assay Kit. Protein concentrations were determined using 96-well format and evaluated with reference to a standard like bovine serum albumin (BSA).

2.8. Immunoblotting

Equal quantities of extracted proteins (7-10 µg) were run on 10% Sodium Dodecyl Sulfate Polyacrylamide gel and subsequently transferred onto a nitrocellulose membrane using Mini transblot system (Bio-Rad, Hercules, CA, USA). The blots were probed overnight at 4˚C, with target protein specific antibodies using dilutions according to the antibody requirements. This was followed by probing with Specific horseradish peroxidase (HRP)-conjugated secondary antibodies and visualizing using SuperSignal™ West Femto Chemiluminescent Substrate (Thermo Fisher Scientific, Waltham MA) in a ChemiDoc visualization system (Bio-Rad, Hercules CA). Densitometry of the bands was performed by the image analyzing software ImageJ [32].

2.9. Data Analysis

All the data are presented as mean ± standard deviation (SD). Statistical differences among different groups were evaluated by one-way analysis of variance (ANOVA) using GraphPad Prism 7.0 software (GraphPad Software Inc. La Jolla, CA, USA). P-values <0.05 are considered as statistically significant.

3. Results and Discussion

3.1. Cellular Models of Proteotoxic Stress

Stem cells are perpetually in an enhanced state of translation and protein synthesis and thus expectedly have augmented proteostasis machinery[12,18]. Despite this, the requirement for sustained protein synthesis and potential exposure to different stresses necessitates the capacity to sense damaged proteins and respond to that damage in a coordinated manner. Therefore, we intended to induce proteotoxic stress in the cells in a controlled manner to identify the main protagonists of the proteotoxic stress response. Biological systems are adapted to grow at optimum temperatures which largely reflect the structural and functional stability limits of their proteins [27]. The folded three-dimensional structures of most proteins are not energetically ideal but present a balance between

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thermodynamic stability and conformational flexibility required for their optimal functioning [15,17,20]. As a result, most of the time proteins have a limited stability range in the physiological milieu and are susceptible to multiple structural perturbations [15,27]. Protein structural maintenance and stability is an outcome of the inter- and intra- molecular interactions, and anything with a potential to disturb these interactions, like temperature, oxidative stress, heavy metals etc., is a proteotoxic stressor [27]. Consequently, temperature fluctuations in growth conditions lead to protein misfolding and potentially aggregation [27,33].

We, therefore, relied on the use of heat shock as proteotoxic stress inducer. Optimum heat-stress condition termed as '*circumstance condition*' is the one that leads to the production of measurable protein aggregates without impacting cell morphology. Each 'circumstance condition' can be defined by Exposure Temperature (ET) and Exposure Duration (ED). Because stem cells already harbor elevated heat shock proteins, we opted for relatively higher temperatures than the optimum temperature of 37˚C. Exposure temperatures of 42˚C, 44 ˚C, and 46 ˚C, and exposure durations of 1, 2, and 3 Hours formed part of the heat-stress induction protocol in *decidua basalis mesenchymal stem cells (DBMSCs)*, *decidua parietalis mesenchymal stem cells (DPMSCs)*, and *placenta-derived mesenchymal stem cells (pMSCs)*. Exposing cells to heat-stress at 46˚C resulted in morphological changes, and all the three cells were tolerant to 44˚C, while as DBMSCs and DPMSCs stayed morphologically unaltered for up to 3 hours, pMSCs stayed stable only up to 2-hour exposure. Post-stress the cells were allowed to recover at 37˚C and samples were harvested at multiple time points up to 24 hours post stress exposure. This led to the development of a 'time-course' approach to comprehensively study the proteotoxic stress response.

We estimated protein aggregation in these heat-stressed cells, and these were calculated immediately at conclusion after heat-stress i.e., 0H and at 6 and 24 hours post heat stress (Figure 1). The presence of aggregates was detected immediately at 0H in all the three cell types with the highest around 55% aggregation detected in the whole proteome isolated from DPMSCs, followed by approximately 40% in DBMSCs, and a relatively less value of around 25% in pMSCs. One reason for less aggregation in pMSCs compared to DPMSCs and DBMSCs could be the exposure duration [ED] of two hours compared to ED of three hours in other two cell types. In all the three cell types, the protein aggregates seem to be having been resolved six hours post heat exposure. As this is the first time-point in the recovery phase evaluated for the presence of aggregates, the possibility of an earlier clearance cannot be ruled out. Nevertheless, the presence of aggregates post heat-stress points towards the presence of heat generated proteotoxic stress, thus ideal conditions for studying stress response.

Measuring the impact of heat-stress on viability and proliferation led to cellular level evaluation of heat-stress models. Using the xCELLigence real-time cell analysis (RTCA) system the cell behavior was monitored over a period 72 hours in control cells grown at 37˚C and in cells exposed to heat at 44˚C for 1, 2, and 3 hours in the case of DBMSCs, DPMSCs and 1 and 2 hours in case of pMSCs. The calculated Cell Index (CI) values indicate a decrease in proliferation as can be seen by decreasing percentage difference in Cell Index (CI) (Figure2, Table 1). In the case of DBMSCs and DPMSCs a 3-hour exposure at 44°C can be seen to leads to a statistically significant ($P < 0.05$) reduction in cell-proliferation, which is observable even at 24 hours post heat-stress. This decreased cellular proliferation points towards the persistence of cellular stress, potentially an active heat-shock response. But this is not in complete resonance the protein aggregation state of the cell, for which the cells seem to have cleared a significant portion of heat-stress induced protein aggregates (Figure 1). Therefore, a conclusion here is that mere, clearance of protein aggregates doesn't mean a complete reversal of the cellular stress. There potentially are other heat-induced damages in these cells which do not allow the return of the cells to normal physiological state. This points out the criticality of studying stress-response over multiple time points, which potentially can lead to the detection of stage-specific processes, and consequently the identification of the stage-specific factors relevant to cell survival mechanisms.

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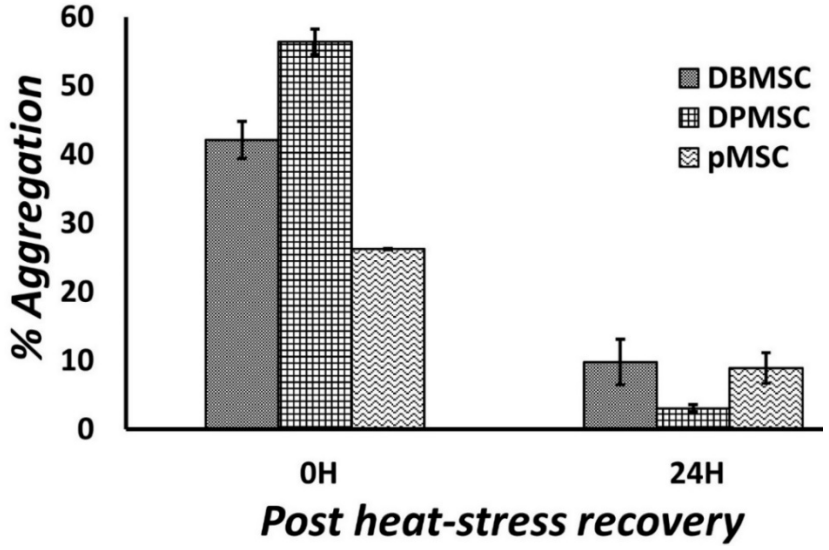

Figure 1. Estimation of protein aggregation in heat-stress models of DBMSCs, DPMSCs and pMSCs. Increase in aggregation was measured as percentage change in fluorescence as compared to total proteome of cells grown at 37°C. All the three cell types exhibited highest presence of aggregates immediately after heat-stress i.e., recovery time 0H confirming existence of proteotoxic stress.

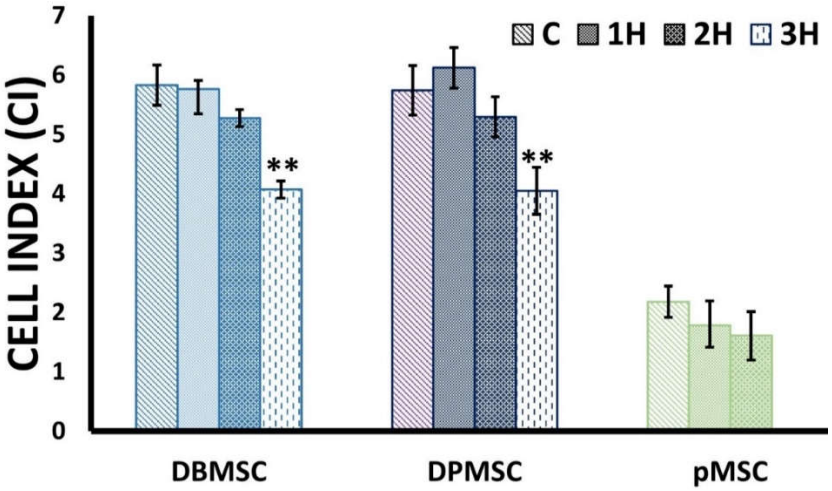

Figure 2. Cell proliferation assay in heat-stress models. Cells grown at 37°C (C) and heat-exposed at 44°C (For 1H, 2H, and 3H) were seeded in E-plate and monitored automatically. Cell Index (CI) values depicted here are 24 hours post heat-stress and show statistically significant ($P < 0.05$) reduction at 3 hours exposure duration [ED] at 44°C in DBMSCs and DPMSCs. In case of pMSCs although CI reduction of around 25% is observed but was not detected to be statistically significant.

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Table 1. Cell proliferation analysis in heat-stress models. Percentage Difference in Cell Index (CI) between heat-stressed cells and control cells (grown at 37°C). These values were calculated over CI values estimated at 24 hours post heat-stress.

Cell Type	Exposure Duration [ED]	% Difference Cell Index (CI)
DBMSCs	1 Hour	1.13
	2 Hours	9.45
	3 Hours	**30.15**
DPMSCs	1 Hour	-6.61
	2 Hours	7.74
	3 Hours	**29.48**
pMSCs	1 Hour	18.28
	2 Hours	**26.40**

3.2. Dose-responsive characteristic of heat-stress and stress response

From the protein aggregation and cell proliferation measurements, we concluded that for DBMSCs and DPMSCs ED: 44˚C, ET: 3 Hours, and for pMSCs ED: 44˚C, ET: 2 Hours were optimal for inducing proteotoxic stress. We intended to validate further these stress models for ability to elicit a tangible and effective stress response. Chaperones in general, have both housekeeping and stress-responsive functions, therefore, act as sensors for stress to help coordinate communication between protein homeostasis and stress signaling processes [21,34].

Gene expression modulation is central to cellular adaptation to stress. Consequently, fold change in gene expression of stress response genes like chaperones is a vital parameter to gauge levels of the cellular stress response. Therefore, we relied on the quantitative measurement of Hsp70 gene expression to evaluate the establishment of heat-stress and heat-shock response/proteotoxic stress response. Hsp70 gene expression was measured immediately after heat-stress exposure and at 1, 6, 9, 12, and 24 hours post heat-stress at 42˚C for 1 and 2 hours and at 44˚C for 1, 2, and 3 Hours. We tested this approach with Decidua Basalis mesenchymal stem cells (DBMSCs) because these cells are from the maternal-fetal interface that exposes them to higher levels of circulating inflammatory factors and reactive oxygen species consequently rendering them with high oxidative stress resistance [8,35]. Therefore, it becomes logical to validate the heat shock response in a cell type which already has an existing stress response because the heat-shock response is also a function of oxidative stress [27]. Figure 3A shows a time-course analysis of Hsp70 gene expression during the recovery phase post-heat-stress at 42˚C for 1 and 2 hours and 44˚C for 1, 2, and 3 Hours. Hsp70 expression differed very marginally in the exposure temperatures of 42˚C and 44˚C for exposure durations of 1 and 2 hours; in fact, there is almost no difference whether the cells are exposed to heat at 42˚C for 1 hour or 2 hours (Figure 3A, Table 2). There is substantial Hsp70 expression at 0H which is immediately after heat-stress exposure and this expression of Hsp70 keeps going up marginally up to 1H post exposure and is statistically significant at both 0H and 1H.

Table 2. Heat-stress model of DBMSC. Hsp70 gene expression fold-change values from five circumstance conditions involving a combination of two exposure temperatures (ET) and three exposure durations (ED).

Exposure Temperature [ET]	Exposure Duration [ED]	Hsp70 Induction
42˚C	1HR	40.67±7.7
	2HR	41.04±6.3
44˚C	1HR	56.07±8.4
	2HR	68.77±2.38
	3HR	108.13±1.54

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The dose-responsive nature of Hsp70 gene expression is evident as the exposure temperature [ET] and exposure duration [ED] increase. In the case of ET: 42˚C, the Hsp70 gene expression at 6H has come down to Log_2 fold change of around 1, while in case of ET: 44˚C, ED: 1-hour, it is 3.5 and increases to more than 5.5 when ED>1H. Although a decrease can be noticed beyond 6H in ET: 44˚C, the Log_2 fold change is still more than 3 (Figure 3B). This difference in gene expression fold change also follows a pattern in p-values with the significant expression time points going from two (0H and 1H) for ET: 42˚C, ED:1-hour to five (0H, 1H, 6H, 9H, and 12H) in case of ET: 44˚C, ED: 3-hours (Figure 3C). Thus, an active stress response is operational up to 1 hour in low magnitude heat-stress (ET: 42˚C, ED:1-hour) as compared to at least up to 12 hours in a high magnitude heat-stress (ET: 44˚C, ED:3-hours). Considering Hsp70 expression as an indicator of heat-shock response, we can conclude that as the magnitude of heat-stress increases in terms of exposure temperature (ET) and exposure duration (ED), the stress response increases proportionately and keeps increasing both in duration and magnitude. It is noteworthy to mention that stress-response pathways are transient in nature and consequently controlled tightly in both magnitude and duration, which is proportional to the severity or dose of the stress itself [36,37]. This tight control of stress response ensures its synchronization with the physiological state of the cell and consequently leads to effective recovery.

The regulation and control of stress response *magnitude* is controlled through activating mechanisms that are down-regulated once cellular homeostasis is reset. For example, Hsp70 gets ubiquitinated by HSF1-induced ubiquitin ligase CHIP for proteasome degradation once the misfolded or unfolded proteins are depleted [38]. The regulation of *duration* of stress-response involves negative feedback loop mediated down-regulation of HSF-1 in heat-shock response (HSR), or through pathway-specific transcription factors like XBP-1 in case of unfolded protein response (UPR) and ATFS-1in mitochondrial UPR (UPRmt) through negative feedback loops [27,37,39]. In addition to regulation by negative feedback loops, increased production of elements like ubiquitin ligases targets the active transcription factors for degradation [37,38]. Thus, there is close coordination between dose-responsive nature of stress-response pathways and their regulation to ensure efficient recovery with judicious utilization of cellular machinery. Therefore, it is imperative to develop comprehensive understanding of these pathways and be studied at different stages of the response, which might be potentially tied to different cell-fate decisions. We believe our time-course-based cellular stress models offer an excellent platform to study these different phases of the stress response as each condition identified potentially represents a significant component of the overall heat-shock response. Keeping this in consideration, for Decidua Basalis mesenchymal stem cells (DBMSCs) the exposure temperature (ET) of 44˚C accompanied by exposure duration (ED) of 3 hours are the appropriate conditions for studying heat stress response. Utilizing similar approaches, ET: 44˚C, ED: 3 hours for Decidua Parietalis mesenchymal stem cells (DPMSCs) and ET: 44˚C, ED: 2 hours for placental MSCs (pMSCs) from chorionic villi are ideal conditions for heat-stress induction (Table 3).

Table 3. Heat-stress conditions in three PDSCs.

Cell Type	Exposure Temperature [ET]	Exposure Duration [ED]
DBMSCs	44˚C	3 Hours
DPMSCs	44˚C	3 Hours
pMSCs	44˚C	2 Hours

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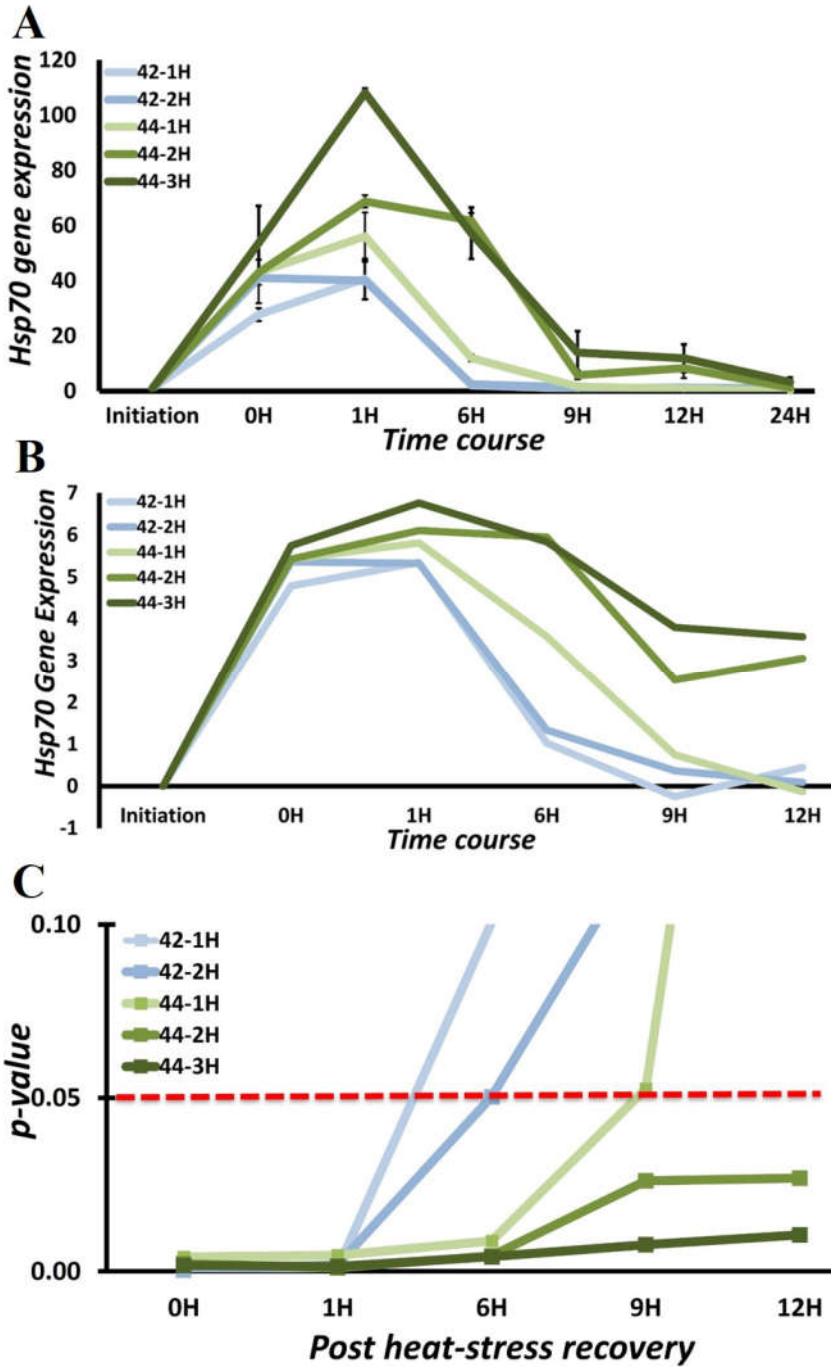

Figure 3. Dose responsive nature of heat-stress in DBMSCs (A) Time-course analysis of Hsp70 gene expression induction at 42°C for 1 and 2 hours and at 44°C for 1, 2, and 3 Hours. (B) Log_2 Fold Change plot for Hsp70 induction. Temperature elicits larger response as can be judged from difference between 44-2H and 44-1H. As the magnitude of heat-stress increases, the heat-shock response increases both in magnitude and duration as seen at 9H and 12H for 44-2H and 44-3H. (C) Dose responsiveness shows pattern in statistical significance as p-value varies decreases with respect to increase in magnitude of heat stress from 42-1H to 44-3H. It is further evident from $P < 0.05$ in case of 44-2H and 44-3H up to 12 hours post heat stress.

3.3. Chaperone gene expression during heat-shock response

We performed a time-dependent analysis of chaperone gene expression in our heat-stress models of DBMSCs, DPMSCs, and pMSCs. Human Heat Shock Proteins & Chaperones RT² Profiler PCR Array (Qiagen) allows for simultaneous expression analysis of 84 genes from the HSP90 Family (81 to 99 kDa), HSP70 Family (65 to 80 kDa), HSP60 Family

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(55 to 64 kDa), HSP40 Family (35 to 54 kDa), small HSPs (<34 kDa), and Chaperone cofactors. The genes covered in this array are significantly involved in the regulation of protein folding and therefore, are part of any proteotoxic stress response pathways like heat-shock response [15]. Therefore, to analyze the time-dependence or stage specificity of these proteins during the stress response, we analyzed their expression at different time points in the recovery, in addition to immediately after heat-stress. Thus, the time-dependent chaperone gene-expression profile involved gene expression at immediately after exposure to heat stress (0H), and at hours 1, 3, 6, and 24 post heat stress when the cells were recovering at 37°C. At 0H, we expect to identify the genes induced near-simultaneously with stress initiation. Those expressed in later stages can have a more prominent role in post-stress recovery. We set the statistically significant ($p<0.05$) Log_2 cut-off fold change for a gene to as a subject for further analyses at 1.5. For comparative analyses this condition should be fulfilled immediately after stress exposure i.e., 0H or at least in one of the analyzed recovery time-point conditions. On this basis, 15 genes (Figure 4A, Table 4) among heat-shock proteins and chaperones in DBMSCs, 12 genes (Figure 4B, Table 4) in DPMSCs, and 08 genes (Figure 4C, Table 4) in pMSCs are the overexpressed genes. In addition to these, the Heat shock 70kDa protein 6 (HSPA6) is overexpressed at 0H (and subsequently studied points) in both DPMSCs and pMSCs as concluded from the low average threshold cycle (C_t~16) in both as compared to C_t value in control samples. The exact fold change cannot be calculated reliably because of the unreliable C_t value (>30) in controls in both cases. 08 genes HSPA1A, HSPA1B, HSPA4L, HSPH1, DNAJB1, DNAJB4, CRYAB, and BAG3 are common to all the three cell types and in fact are the only ones overexpressed in pMSCs other than HSPA6. DNAJA1, HSPB1, and HSPB8 are the common genes overexpressed in DBMSCs and DPMSCs only. In addition, HSPA5 can be detected specifically in DPMSCs, while as HSPA1L, DNAJB9, HSP90AA1, and HSPD1 are overexpressed only in DBMSCs.

Stem cells, in general, are characterized by elevated protein synthesis because of their high proliferative requirement, consequently requiring enhanced protein quality control mechanisms like elevated chaperones. Among the chaperones, heat-shock proteins are abundantly expressed in pluripotent stem cells than in terminally differentiated cells, providing this enhanced competence to these cells. Different stem cells express different heat-shock proteins, and these overexpressed heat-shock proteins perform multiple functions. S*tem-cell self-renewal* is one such process involving complex regulatory networks in which the heat-shock proteins in embryonic stem cells (ESCs) play a prominent role by interacting with the transcription and regulatory factors that have a role in stem-cell renewal [40–42]. For example, in embryonic stem cells (ESCs), higher expression of heat-shock proteins (Hsps) and their subsequent interaction with transcription factors is essential for cell development and functioning [40,42]. Cancer stem cells (CSCs) like other stem cells are also characterized by enhanced expression of Hsp genes, e.g., Hsp70 protein 5 (HspA5) also known as GRP78, displays a significantly increased presence in isolated head and neck cancer stem cells (HN-CSCs), the downregulation of which has been reported to be associated with reduction in self-renewal properties and inhibition of tumorigenicity[43–45]. Like embryonic and cancer stem cells, high HSPA5 expression has been detected in hematopoietic stem cells (HSCs) as well [46]. In characterization of shared and unique chaperone expression profiles in different types of stem cells, viz., embryonic, mesenchymal, and neural stem cells, high expression levels of Hsp70 protein 5 (HspA5), Hsp70 protein 8 (HspA8), and Hop (Stip1) has been reported in all these three cell types [40,47]. ESCs, on the other hand represent a unique chaperone expression signature that is composed of Hsp70 protein 4 (HspA4), Hsp27 (HspB1), and Hsp90β (HspCb) [40,47]. Thus, proteome stability maintenance wise stem cell renewal is a demanding process and consequently is accompanied by high expression of different chaperones and co-chaperones that potentially act as a buffer against different stressors and consequently help these cells maintain their stemness. To our knowledge, there is no information available regarding the baseline expression of heat shock proteins in placenta-derived stem cells nonetheless, the overexpression of different chaperones points towards stress-induced expression as a result of heat-induced proteotoxic stress.

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Summing up, 17 out of 84 genes of the Human Heat Shock Proteins & Chaperones RT² Profiler PCR Array are overexpressed altogether as part of the heat-induced proteotoxic stress response in the three placenta-derived stem cells (PDSCs). On the basis of protein family or group distribution, these 17 genes belong to 08 categories: *Small Heat Shock Protein*, *Mitochondrial 60 kDa heat shock protein*, *Heat shock 70 kDa protein*, *Heat shock protein 90kDa alpha (cytosolic) class A*, *Heat shock protein 105 kDa*, *DnaJ homolog subfamilyA*, *DnaJ homolog subfamily B*, and *BAG family molecular chaperone regulator 3* (Table 5). Within these, 06 of the overexpressed genes belong to Heat shock 70 kDa protein, around 35% of the overexpressed genes, followed by 04 genes (around 25%) belonging to DnaJ homolog subfamilies. That Hsp70 family chaperones are the major ones overexpressed is not surprising because Hsp70 family members like Hsp70 protein 4 (HspA4), Hsp70 protein 5 (HspA5), Hsp70 protein 8 (HspA8), have a significant role in stem-cell self-renewal [40]. Thus, overexpression here correlates with their role in any enhanced chaperone requiring activity. Hsp70 proteins work in tandem with other chaperones and co-chaperones through an intricate network to ensure the regulation of proteostasis.

J-proteins and nucleotide exchange factor (NEF) co-chaperones regulate the ATPase reaction cycle of Hsp70s by working in substrate recruitment [48,49]. Therefore, the next group of highly expressed chaperones, DnaJ homologs is not surprising. These J-proteins are part of a large family of proteins with around 40 members in humans, all of which are characterized by the presence of a J-domain that binds to the N-terminal ATPase domain of Hsp70 [22,50]. Likewise, heat shock protein 105 kDa, also identified as HSPH1 acts a co-chaperone for HSPA1A and HSPA1B by functioning as a nucleotide exchange factor [20,49,51]. Another Hsp70 family interacting protein overexpressed in our system is the BAG family molecular chaperone regulator 3 (BAG3). All BAG proteins are characterized by a conserved BAG domain in their C-terminal region, and BAG3 along with some other members of this family physically interact with Hsp70 potentiating a role for these proteins in Hsp70 targeting factors [52,53]. It can be thus concluded that the proteotoxic stress response in PDSCs involves overexpression of Hsp70 family proteins, and to support and coordinate their activities, their interacting cochaperones and other co-factors overexpress along with.

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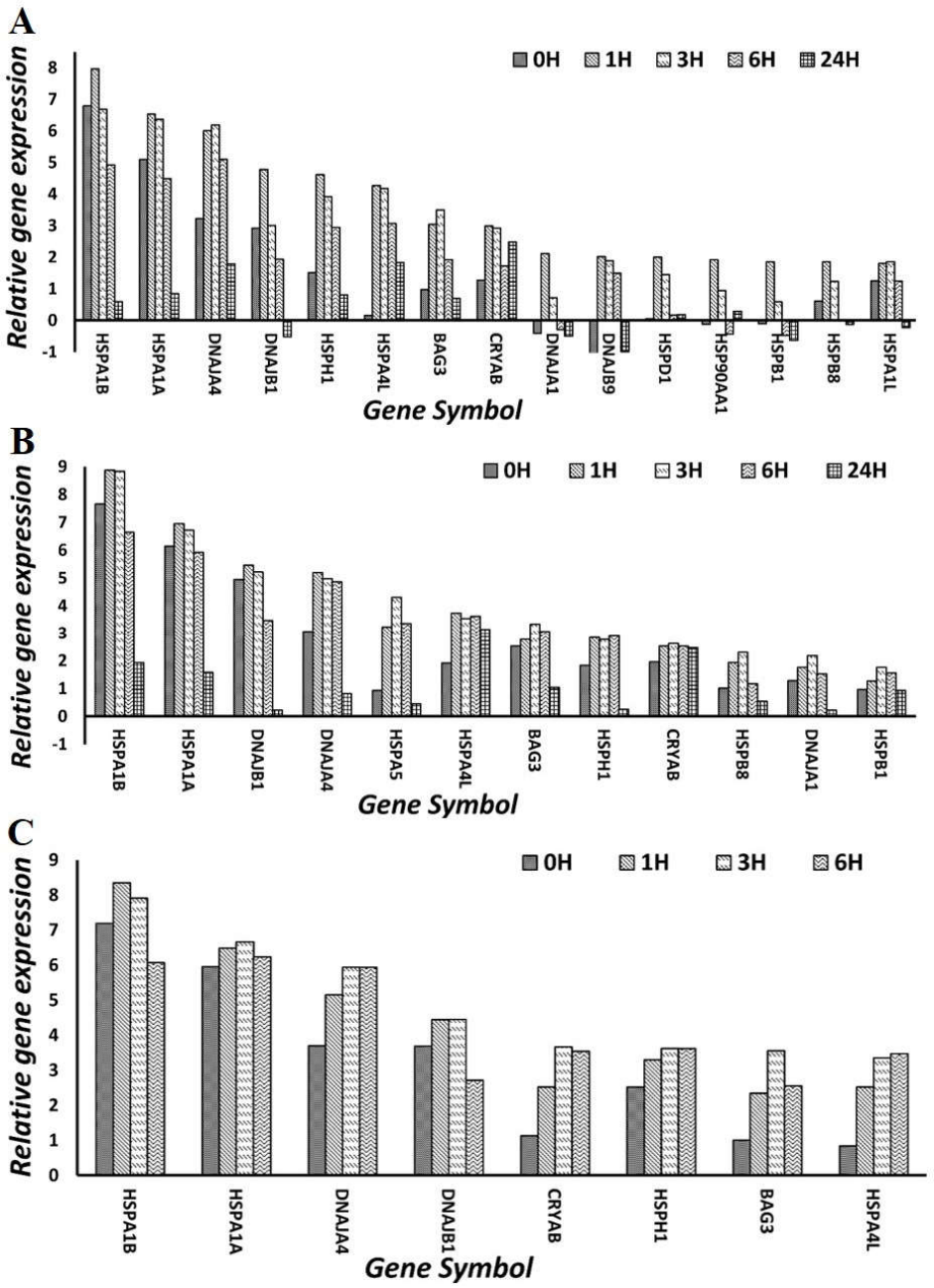

Figure 4. Human Heat Shock Proteins & Chaperones gene expression profile at conclusion of heat-stress (0H) and during recovery at 37°C at 1-hour (1H), 3 hours (3H), 6 hours (6H), and 24 hours (24H). (A)15 genes in DBMSCs, (B)12 genes in DPMSCS, and (C) 8 genes in pMSCs were detected to be overexpressed in a statistically significant ($p<0.05$) manner with Log_2 fold-change of 1.5, in at least at one of the analyzed time-points.

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Table 4. RT² Profiler PCR Array results. Tabulated values fit the criteria of Log_2 fold-change>1.5 and p<0.05 at 0H or.in at least one of the analyzed recovery time-point conditions.

		DBMSC					*DPMSC*					*pMSC*			
FAMILY	*GENE*	*0H*	*1H*	*3H*	*6H*	*24H*	*0H*	*1H*	*3H*	*6H*	*24H*	*0H*	*1H*	*3H*	*6H*
Heat shock 70 kDa protein	*HSPA1B*	6.69*	7.96*	6.69*	4.93*	0.60	7.66*	8.87*	8.84*	6.65*	1.94*	7.19*	8.36*	7.92*	6.08*
	HSPA1A	5.1*	7.53*	6.37*	4.49*	0.85	6.15*	6.95*	6.72*	5.93*	1.58*	5.96*	6.49*	6.67*	6.24*
	HSPA1L	1.26	1.81*	1.86*	1.25	-0.22	No Overexpression					No Overexpression			
	HSPA4L	0.61	4.28*	4.18*	3.07*	1.83	1.92	3.74*	3.54*	3.63*	3.15*	0.85	2.52*	3.36*	3.47*
	HSPA5	No Overexpression					0.93	3.23*	4.31*	3.37*	0.46	No Overexpression			
	HSPA6	No Overexpression					C_t>30					C_t>30			
Small Heat Shock Protein	*HSPB1*	-0.10	1.86*	0.59	-0.47	-0.63	0.97	1.27	1.76*	1.56*	0.94	No Overexpression			
	HSPB8	0.62	1.86*	1.24	-0.02	-0.13	1.02	1.94	2.3*	1.18	0.54	No Overexpression			
	CRYAB	1.28	3*	2.92*	1.73*	2.49*	1.96	2.53*	2.62*	2.52*	2.47*	1.13	2.53	3.67*	3.54*
DnaJ homolog subfamily A	*DNAJA1*	-0.41	2.11*	0.72	-0.29	-0.49	1.28	1.76	2.18*	1.53*	0.23	No Overexpression			
	DNAJA4	3.23*	6.01*	6.19*	5.10*	1.79	3.08*	5.19*	4.98*	4.87*	0.82	3.70*	5.16*	5.94*	5.94*
DnaJ homolog subfamily B	*DNAJB1*	2.92*	4.78*	3.01*	1.93*	-0.51	4.95*	5.46*	5.22*	3.48*	0.23	3.69*	4.44*	4.45*	2.72
	DNAJB9	-1.35	2.03*	1.89*	1.5	-0.98	No Overexpression					No Overexpression			
60 kDa heat shock protein, mitochondrial	*HSPD1*	0.06	2.01*	1.45	0.17	0.19	No Overexpression					No Overexpression			
Heat shock protein 90kDa alpha (cytosolic), class A	*HSP90AA1*	-0.12	1.92*	0.95	-0.43	0.29	No Overexpression					No Overexpression			
Heat shock protein 105 kDa	*HSPH1*	1.52	4.62*	3.91*	2.95*	0.81	1.84	2.85*	2.77*	2.91*	0.26	2.52	3.31	3.62*	3.62*
BAG family molecular chaperone regulator 3	*BAG3*	0.98	3.05*	3.50*	1.93*	0.70	2.54*	2.78*	3.34*	3.07*	1.04	1.01	2.35	3.56*	2.56*

Table 5. Classification of overexpressed genes according to Heat Shock Protein or Chaperone.

Chaperone Family	Overexpressed Genes	Proportion
Small Heat Shock Protein	HSPB1, HSPB8, CRYAB	3/8
60 kDa heat shock protein, mitochondrial	HSPD1	1/1
Heat shock 70 kDa protein	HSPA1A, HSPA1B, HSPA1L, HSPA4L, HSPA5, HSPA6	6/11
Heat shock protein 90kDa alpha (cytosolic), class A	HSP90AA1	1/1
Heat shock protein 105 kDa	HSPH1	1/1
DnaJ homolog subfamilyA	DNAJA1, DNAJA4	2/4
DnaJ homolog subfamily B	DNAJB1, DNAJB9	2/11
BAG family molecular chaperone regulator 3	BAG3	1/1

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3.4. Temporal dynamics of chaperone gene expression

The fundamental pieces of information derived from time-course gene expression analysis include identification of (a) *maximal gene-expression time point*, i.e., time-point post-heat-stress at which the highest number of genes overexpress, and *genes overexpressed significantly and substantially* (b) immediately after heat-stress, i.e., at 0H (c) at different stages of recovery at 37°C, over the course of 24 hours.

We discussed in the previous section that 15 genes in DBMSCs, 12 in DPMSCs and 8 in pMSCs overexpress during heat-stress and course of recovery over 24 hours at 37°C. Figure 5A depicts the time-series distribution of these genes in the three PDSCs. Analyzing the time-series expression, the *maximal gene-expression time point* occurs between 1-6 hours into the stress response and varies between the three cell types (Figure 5B). At 1H post-heat-stress DBMSCs hit saturation in the number of overexpressed genes, with almost all the 15 overexpressed genes showing significant and substantial overexpression (Figure 6A). Thus, the maximal gene-expression time point in DBMSCs is at 1H. The number of overexpressed genes then goes down to 10 at 3H and 8 at 6H, thus reaffirming 1H post-heat-stress or time before that as the transcriptionally crucial time point in heat-induced proteotoxic stress response. DPMSCs in comparison, do not have a single point high expression point, instead shows a relatively stable gene expression between 1-6 hours, with the maximal gene expression at 3-6 hours where a maximum of 12 genes are overexpressed (Figure 6B). On the contrary, pMSCs show a relatively different expression pattern with the maximal gene-expression time point at 3H post-heat-stress where eight genes are expressed significantly and substantially (Figures 6C). Chaperones are among the first and predominant class of proteins synthesized as part of the heat-shock response, and this is globally applicable to all living systems [27,54]. This very well correlates with their demand in protein refolding and proteostasis restoration. The maximal expression time point between 1-6 hours correlates well with the expression patterns reported from the other systems like Drosophila [54], *C. elegans* [55], cells like HeLa [56], and mouse embryonic fibroblasts [57], and humans [58]. As far as the inter-cellular differences among PDSCs are concerned, these are due to the different endogenous stress resistance properties in these cells because of the different niches they occupy within the placenta [7–9,35].

An interesting observation that can be made from analyzing the temporal distribution of gene expression is that the stage at *completion of heat-stress exposure i.e.,0H* is characterized by minimum variation in number of the overexpressed genes. Only 4-5 genes overexpress in the three PDSCs: 4 in DBMSCs and pMSCs, and 6 in case of DPMSCs (Figures 5A). Incidentally, four of the genes, HSPA1A, HSPA1B, DNAJA4, and DNAJB1 are common to all the three cell types and are expressed significantly and predominantly at 0H (Table 4, Figure 4). In addition, the Heat shock 70kDa protein 6 (HSPA6) is overexpressed at 0H in both DPMSCs and pMSCs as concluded from the low average threshold cycle (C_t~16), but fold change and statistical significance calculation is not possible because of the high average threshold cycle (C_t >30) in control samples. BAG3 is only significantly overexpressed in DPMSC at 0H. Thus, in all the three placenta-derived stem cells the very early stages of the transcriptional response to proteotoxic stress are primarily mediated through two Hsp70 isoforms, HSPA1A and HSPA1B. HSPA1A and HSPA1B coexpress with their ATPase activity stimulating partner DNAJB1, a member of DnaJ Heat Shock Protein Family (Hsp40) along with another member of the same family DNAJA4[50].

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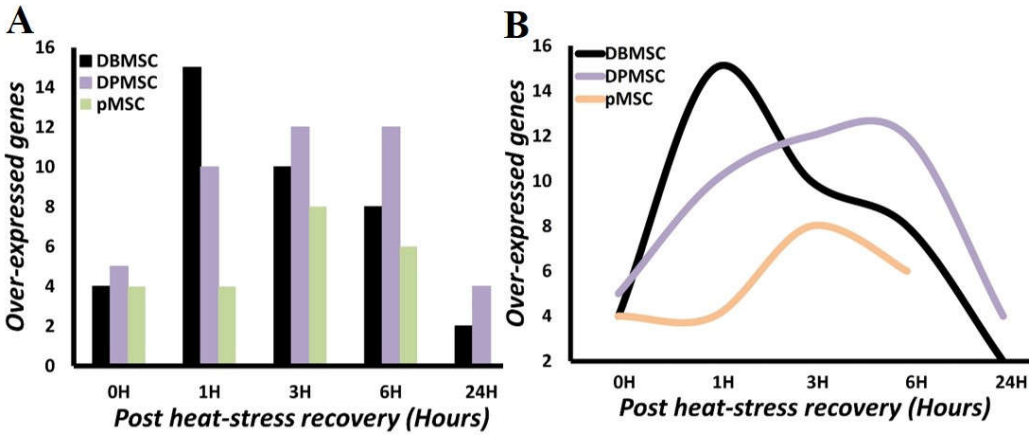

Figure 5. Number of over-expressed genes. Number of genes overexpressed from Human Heat Shock Proteins & Chaperones RT² Profiler PCR Array immediately at conclusion of heat-stress (0H) and during recovery at 37°C at 1-hour (1H), 3 hours (3H), 6 hours (6H), and 24 hours (24H). Minimum variation in number of overexpressed genes is seen at 0H with 4-5 genes overexpressed across the three stem cell types.

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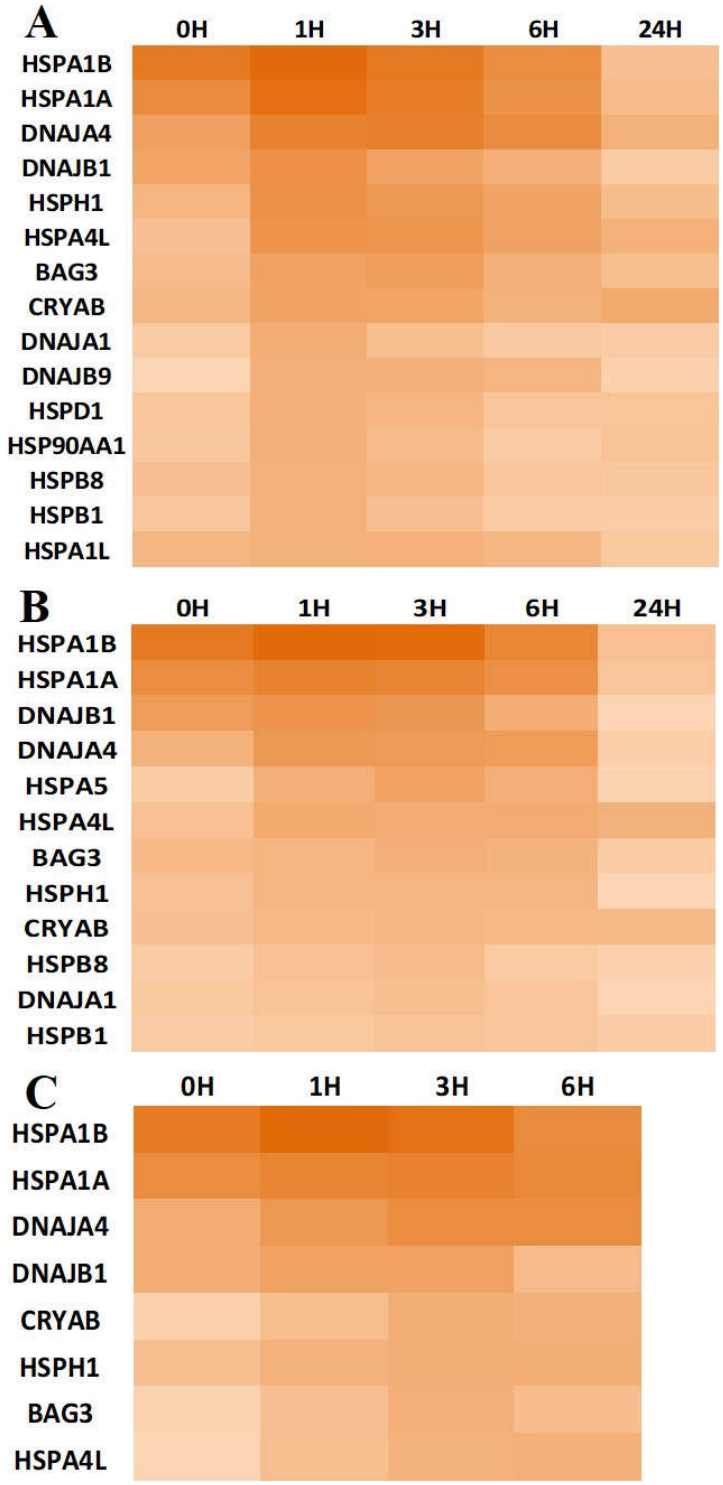

Figure 6. Heat maps showing temporal expression of significantly expressed genes. Maximal gene-expression time point at 1H in DBMSCs (A), 3-6H in DPMSCs (B), and 3H in pMSCs (C). Additionally in all the three cell types, significantly high expression of HSPA1A, HSPA1B, DNAJA4, and DNAJB1at 0H is visble.

3.5. HSPA1A and HSPA1B are primary determinants of heat induced proteotoxic stress response

Primary determinants of stress response are the genes that are substantially and significantly overexpressed during stress exposure and can therefore be measured immediately after stress-exposure. This significant overexpression immediately on the onset of stress indicates their potential primary influence in the stress response. Therefore, the four

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overexpressed genes HSPA1A, HSPA1B, DNAJB1, and DNAJA4 detected to be highly expressed at 0H (Section 3.4) in all the three PDSCs fit this definition.

An additional criterion to define the criticality of the genes in stress response can be the level of expression during stress compared to expression during the recovery phase; small or insignificant fold change differences will mean extremely high importance for stress response. The stress response is a dose-related process and, consequently, gene-expression calibration has to happen in proportion to the stress stimulation. We detected the maximum protein aggregation levels at 0H (Figure 1), representing the peak proteotoxic stress level. Burst in stress response gene expression ensures the time-bound cooperation of different constituents of gene expression machinery to ensure proportionate stress response [59,60]. Chaperones have a primary role in ensuring maintenance of protein folding states, so their gene-expression cannot be expected to vary significantly post the stress exposure stage. We, therefore, calculated the '*recovery gene-expression fold-change*' by normalizing the calculated gene expression fold-change to 0H fold-change. This sets a value of 1 for gene-expression fold-change at 0H, and recovery phase gene-expression fold-change is analyzed in comparison to this (Figure 7A). We set a threshold of <3 for 'recovery phase gene-expression fold-change' for a gene to qualify as a primary determinant of stress response. Although an arbitrary number, this is set keeping in consideration that active transcription in the recovery phase is a hallmark of stress response and is expected to persist even hours after stress exposure [56]; therefore continued expression at 1H and beyond is not surprising, even though the expression is at low levels.

Only two genes, HSPA1A and HSPA1B, from among the four early phase overexpressed genes fit this criterion of criticality with HSPA1A 1H/0H fold-change between 1.4-2.7 while in the case of HSPA1B it is between 2.2-2.3 (Figure 7A). In pMSCs, the HSPA1A expression keeps going up to 6H, but that increase is very insignificant. An important point to consider while postulating the criticality of HSPA1A and HSPA1B is their position in the order of over-expressed genes and the fold change in gene expression at 0H, i.e., immediately after heat-stress exposure. HSPA1B and HSPA1A are the two topmost overexpressed genes among the genes in Human Heat Shock Proteins & Chaperones RT^2 Profiler PCR Array (Figure 4, Table 4). Additionally, the gene expression fold-change values for these genes are very high; Log-2 value between 6.7-7.2 (Table 4). No other genes for which the fold change values could be calculated come close to those for HSPA1A and HSPA1B, further augmenting the argument in favor of their critical importance to heat induced proteotoxic stress response.

We further analyzed the HSPA1A and HSPA1B gene expression temporal characteristics and also identified the transcriptionally active period in the stress response phase. As alluded to above, the proteotoxic stress response is characterized by a rapid increase in HSPA1A and HSPA1B gene expression with a very highly significant measurable gene expression immediately after heat-stress exposure: 5-7 log fold increase (HSPA1A:5-6.2, HSPA1B: 6.7-7.2) (Figure 7B). This immediate and high expression of these Hsp70 isoform genes is along the expected lines considering that they are fundamentally part of heat-shock response, which is a rapid gene expression program characterized by the instant overexpression of stress response genes [27] (REF). There is an observable upward trend in gene expression at 1H with a very stable around two-fold change (2.2-2.3) in HSPA1B. In HSPA1A there is variation in the fold-expression with 2.7 in DBMSCs and <2 in DPMSCs and pMSCs. Nonetheless, this 1H is the 'maximal gene expression point' of these genes. The gene expression doesn't reduce substantially even up to 3H, around only 0.1-0.2-fold reduction in expression is observed in most cases. There are two outliers to this range, with DBMSC-HSPA1B accompanied by a reduction of 0.6-fold and pMSC-HSPA1A increasing by 1.13-fold. At 6-hours post-heat-stress exposure a steady decline in gene-expression can be identified in all the analyzed conditions, albeit with different decline rates. Irrespective of the differences in decline rates, there is a similar directionality in HSPA1A and HSPA1B gene-expression patterns in these three placenta-derived stem cells. Thus, a conclusion is drawn that there is an active transcription of HSPA1A and HSPA1B up to six hours post-heat-stress exposure pointing toward their critical role in the heat-induced

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proteotoxic stress response in the three placenta-derived stem cells. We discussed previously that the overall maximal expression time point in all the over-expressed genes lies between 1-6 hours post-stress exposure (Section 3.4, and the same holds for HSPA1A and HSPA1B. The predominantly high expression of these proteins in the very early stages of the stress response is in resonance with their function in stoichiometric amounts in proportion to the aberrantly folded proteins.

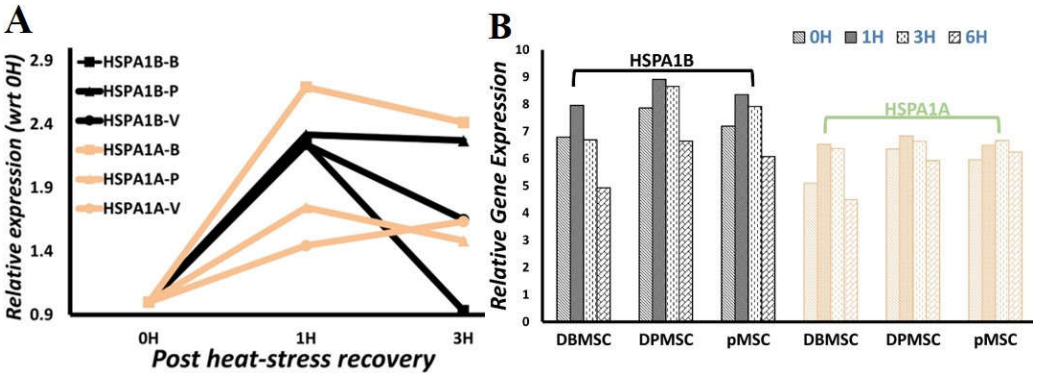

Figure 7. (A) Variation of HSPA1A and HSPA1B gene expression in 'post heat-stress recovery phase'. Less that 3-fold change in gene expression with respect to expression after heat-stress exposure i.e., 0H (B) Gene expression for HSPA1B and HSPA1A. Log_2 fold-change values at completion of heat-stress (0H) and during recovery at 37°C at 1-hour (1H), 3-hours (3H), and 6-hours (6H) point toward active transcription up to six hours post heat-stress.

3.6. HSPA1A and HSPA1B protein expression follows a temporal pattern

After identifying the critical importance of HSPA1A and HSPA1B transcription in proteotoxic stress response in placenta-derived stem cells (PDSCs), we assessed their protein expression using the same time-series-based approach. Initially, we evaluated the protein expression immediately after heat-stress exposure, i.e., at 0H and at 1H, 3H, 6H, 9H, 12H, and 24H during recovery at 37°C (Data not shown). For further analyses we selected the time points that appeared to be relevant and significant in the protein expression process; (i) *0H*, immediately after cells are relieved of stress, (ii) *1H*, first hour after recovery and important from gene-expression point of view, (iii) *6H*, where gene expression can be seen to be receding, and (iv) *24H*, where gene-expression almost hits the pre-induction states.

In non-stressed control cells i.e., cells continuously growing at 37°C HSPA1A protein expression is more remarkable than HSPA1B. However overall, there is a similar trend in the expression of both proteins, although with different magnitudes (Figure 8). In the case of HSPA1A the significant fold-change varies between 2 and 4.5, while for HSPA1B it ranges from 11 to 20. In terms of temporal distribution, in all the three cell types, both HSPA1A and HSPAB1A are characterized by protein expression in the first recorded sample, i.e., immediately after heat-stress exposure (0H). Although the protein levels at this point (0H) are not present at statistically significant levels, their presence points to an immediate requirement for these proteins in their functional form. At 1H, i.e., after cells have recovered at 37°C for one-hour, significant overexpression for both HSPA1A and HSPA1B can be seen in DBMSCs and DPMSCs, while in the case of pMSCs, the protein expression levels are still low at this stage. 6H post-recovery at 37°C is the critical time point from protein-expression point of view in all the three cell types and for both proteins. Thus 6H is the 'maximal protein-expression' point where statistically significant maximal protein expression is observed in all cases. The fold-change in protein expression ranges between 3.1-4.2 and 16-21 for HSPA1A and HSPA1B, respectively. We can firmly say that six hours after the cells are relieved of heat stress, the cells attain the maximal expression of HSPA1A and HSPA1B proteins and considering proteins as functional molecular entities, potentially representing an essential stage in the proteotoxic stress response pathway. The

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protein expression starts receding after this point but not significantly, and even at 24-hours post-heat-stress protein expression fold-change ranges between 2.8-3.5 and 8-17 for HSPA1A and HSPA1B, respectively. Thus, the heat induced proteotoxic stress response as represented by HSPA1A and HSPA1B expression is significantly, and substantially active even 24 hours post-exposure of heat stress. Pertinent to note here is that the reduction in expression from 6H to 24H is more prominent in HSPA1B which is reduced approximately by 50% except for pMSC while as for HSPA1A the reduction in expression is between 10-20%. Considering all the points from protein expression, it is clear that both HSPA1A and HSPA1B have a significant role in heat-induced proteotoxic stress response in placenta-derived stem cells, with marked differences among the two proteins in terms of levels of over-expression and temporal variation. We discuss in detail in the following sections comparative dynamics of HSPA1A and HSPA1B gene and protein expression and the dominance of HSPA1B over HSPA1A.

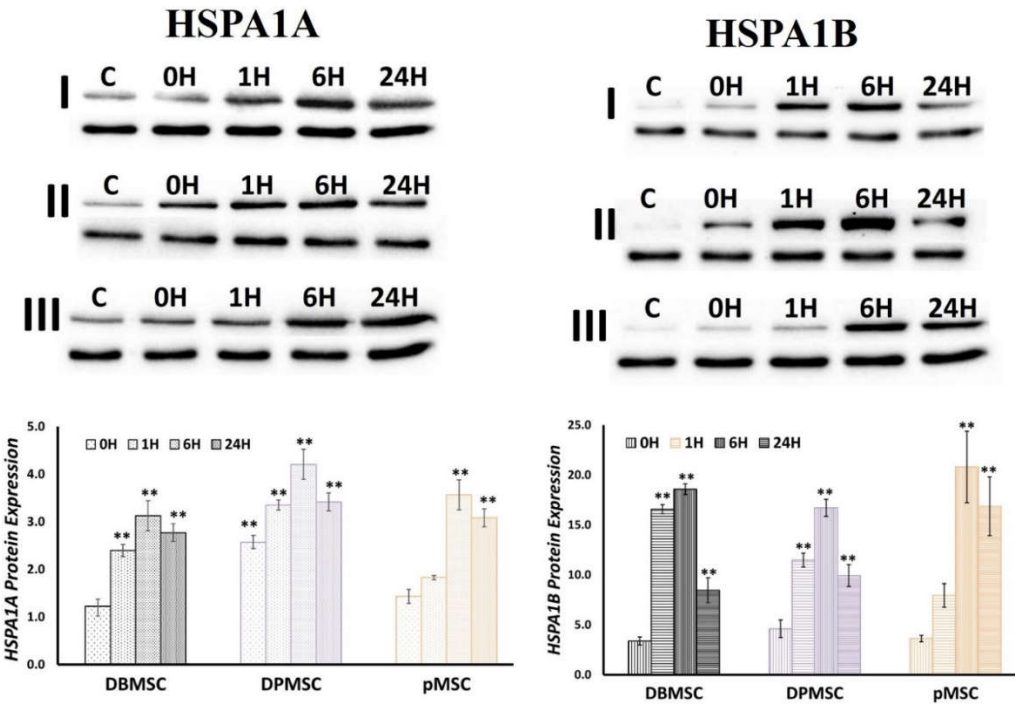

Figure 8. HSPA1A and HSPA1B protein expression in (I) DBMSCs, (II) DPMSCs, and (III) pMSCs. Protein expression in heat-stressed cells, immediately at conclusion of heat-stress (0H) and during recovery at 37°C at 1-hour (1H), 6-hours (6H), and 24-hours (24H). More HSPA1A (Left panel) in control samples as compared to HSPA1B (Right panel). Protein expression is reported to be statistically significant (**) at p-values <0.05.

3.7. Transcription-translation Dynamics: HSPA1A and HSPA1B gene and protein expression kinetics

The gene and protein expression data for HSPA1A and HSPA1B and interpretation of that data in the above sections from three PDSCs in a time-course manner make us believe that further integrative analysis to arrive at a modular interpretation of proteotoxic stress response is possible. To achieve this unified model, we clustered the respective data for gene- and protein- expression for the three cell types. This data clustering is possible because there are no large-scale differences between the expression fold-changes at the analyzed time points (Figures 9A and 9B). This simplistic methodological adaptation is possible because we are primarily relying on relative changes in gene- and protein- expression, and there is a strong co-directionality [61] in expression patterns in all three cell types. Although the subject of expression studies is relative changes, one needs to be cognizant that the changes measured are in the quantities/concentrations of respective molecular entities, mRNA, and protein. Thus, the choice of method holds the key to any

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empirical estimations [62]. Nevertheless, all methods have limitations, and any interpretations should consider these factors. We have employed RT-PCR [63,64] and immunoblotting [65–67], classical and indirect measurement methods but reliably quantitative. In this way, we aim to analyze the transcriptional-translational dynamics and related aspects of proteotoxic stress response in PDSCs in a unified manner.

The aspects of stress response and dynamics of transcription-translation are interpreted at two levels: on transitioning of cells from *(i)* steady-state to expression induction, i.e., expression at 37°C vs. expression under stress, *(ii)* stress-phase to recovery-phase, i.e., expression at 0H vs. expression post stress recovery at 37°C. The first scenario involves utilizing the actual Log_2 expression fold change values, which are the values relative to normal cells growing at 37°C, thus making these healthy control cells as the reference point. For the second scenario, we use the 0H normalized Log_2 fold values, thus making 0H as the reference point, which means evaluating cells recovering at 37°C with reference to stressed cells. At some point, this second stage should lead to its steady state after the cells have fully recovered. But we will be analyzing the stress-related events based on the assumption that the active protein expression even at 24-hours post-stress exposure points toward an active operational stress response (Section 3.6, Figure 9B). Previously (Sections 3.5 and 3.6) it was discussed that for HSPA1A and HSPA1B 1H and 6H respectively, are the 'maximal expression point' for gene- and protein- expression and an active transcription is detected in case of both proteins even at 6H post stress exposure (Figure 9A). Therefore, we are drawing inferences about transcription-translation dynamics for the above stated two cellular states and try to interpret the events for the post-stress transcriptionally and translationally active 6-hour phase (Figures 9C,9D) and the duration of the 24-hours recovery (Figures 9E,9F).

That there is no lag between gene and protein expression can be gauged from the fact that the protein-expression can be measured immediately, albeit at different levels, even immediately after heat-stress i.e., 0H (Figures 8,9B). However, there is a vast difference in the amounts of mRNA and protein at this point, with very high significantly transcribing mRNA while protein expression is almost insignificant in most of the cases (Figure 9C). As a matter of principle, it is not expected that induction of transcription can lead to an immediate increase in protein levels, because RNA processing, like maturation and export, requires some time along with the translation, therefore the poor mRNA-protein correlations at this stage [68–70]. Although in the case of stress-responsive genes mechanisms, like bypassing the mRNA quality control are in place that ensures minimal lag between the processes of transcription and translation [71]. Additionally, stress response pathways are antagonistic to growth-related programs and have distinct transcriptional mechanisms. Consequently, they do not essentially follow the similar regulatory pathways as in housekeeping or even growth-related programs. Therefore, the apparent quantitative discordance between mRNA and protein in the very early phase of the stress response has its origin in the differential regulatory mechanism. Stress response gene expression has a characteristic 'burst' pattern which ensures a proportionate stress response in magnitude and duration [59,72]. This 'transcriptional bursting' represents a hyper-activated state of the transcription machinery in response to the physiological requirement. It is coordinated through cooperation of different contributors of gene expression like chromatin remodeler action at promoter site, recruitment dynamics of transcription factors, and PolII functional dynamics [60,73]. This initial spiking in the mRNA has been observed in other stress proteins like Trafd1 in LPS-stimulated mouse dendritic cells [74], in heat shock proteins in case of rapamycin challenge to yeast [75], and in HSPA5/GRP78 in the ER stress model in HeLa cells [56]. This points toward some unified transcriptional regulatory process in the initial phase of the stress response. Pertinent to the Hsp70s, Hsp70 proteins have a self mRNA stabilization ability [76,77] and in Trypanosoma has been observed during heat-shock[76] mRNA8. At this point, it is too early to pinpoint which of the mechanisms directs the presence of higher levels of mRNA; nevertheless, HSPA1A and HSPA1B gene expression in PDSCs during proteotoxic stress is characterized by behavior seen in stress response in other systems.

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Both mRNA and protein keep increasing in a homodirectional manner at a comparatively rapid rate in the first hour, but with visibly marked difference between HSPA1A and HSPA1B (Figures 9C, 9E). At 1H, gene expression attains the maximal point, while as protein expression at this point attains a substantial jump from 0H. A clearer view of the transcription dynamics during recovery phase are made when making comparisons of recovery states with that attained at completion of stress exposure (Scenario ii proposed above). While the period up to 0H appears to be a transcriptionally active phase, but the recovery phase gene expression seems to be relatively static. At 1H, post-stress translation also starts picking at a comparative rate and, in-fact dominates the transcription in case of HSPA1B (Figure 9D, Table 6). This firmly confirms that the stress exposure is accompanied by a very high and rapid transcription of proteotoxic stress response genes HSPA1A and HSPA1B while the cells are experiencing stress and keep increasing till 1H, but at a very low rate as compared to during stress exposure. Thus, transcription is primarily a function of stress exposure and transcription rates start stabilizing to a new steady state within one hour of stress exposure. This correlates well with the concept that the regulation of gene expression during heat stress occurs at the level of transcription [57,73].

Post 1H, a steady decline in gene expression sets in while as protein expression continues to go up, but the gene expression is still above the stress phase expression rates. At 6H, the gene expression value compared to stress phase expression is ≤1, meaning transcription has attained a steady state. It is important to mention that decrease in measurable mRNA is also an outcome of its translation and subsequent downstream processing. An increase in protein expression can be observed both for HSPA1A and HSPA1B, with protein:mRNA ratio at 6H being around 2 and 12, respectively, while at 1H, it is around 1(Figure 9D). Despite this translational predominance at six hours post-heat-stress, the continued gene-expression at substantial levels up to six hours points towards significant consonance between gene- and protein- expression. Thus, up to six hours post stress-exposure can be considered both transcriptionally and translationally active for HSPA1A and HSPA1B in the proteotoxic stress response in human PDSCs.

Post 6H, the trend observed in transcription and translation is the same as before, with the observed differences becoming stable and more visible. This higher protein expression persists even at 24 hours (with differences in HSPA1A and HSPA1B discussed in the next section) while measurable mRNA levels are close to pre-stress phase period. In the DTT induced ER stress model in HeLa cells GRP78, an important ER stress response factor the down-regulation of mRNA expression and upregulation of protein expression has been reported to set in at 16-hour time point; a relatively high expression of both gene and protein being ascribed to its stress phase importance [56]. Irrespective of the underlying mechanistic factors, the continued overexpression of both HSPA1A and HSPA1B over prolonged period points towards their high relevance to proteotoxic stress response in PDSCs and apparently involves an intricate regulatory network to maintain the fine balance between these two proteins.

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Table 6. The gene- and protein expression fold change values in comparison to 0H i.e., expression.

	HSPA1B							
	GENE EXPRESSION				PROTEIN EXPRESSION			
	DBMSC	DPMSC	pMSC	Average	DBMSC	DPMSC	pMSC	Average
1H	2.25	2.32	2.24	2.3	4.91	2.5	2.2	3.20
3H	0.93	2.27	1.65	1.61				
6H	0.28	0.05	0.46	0.4	5.5	3.62	5.73	4.95
24H	0.02	0.02	0.03	0.02	2.5	2.15	4.65	3.10
	HSPA1A							
	GENE EXPRESSION				PROTEIN EXPRESSION			
	DBMSC	DPMSC	pMSC	Average	DBMSC	DPMSC	pMSC	Average
1H	2.7	1.74	1.44	1.96	1.96	1.31	1.28	1.51
3H	2.41	1.48	1.63	1.84				
6H	0.86	0.86	1.50	1.01	2.55	1.64	2.49	2.23
24H	0.04	0.04	0.05	0.01	2.26	1.33	2.16	1.92

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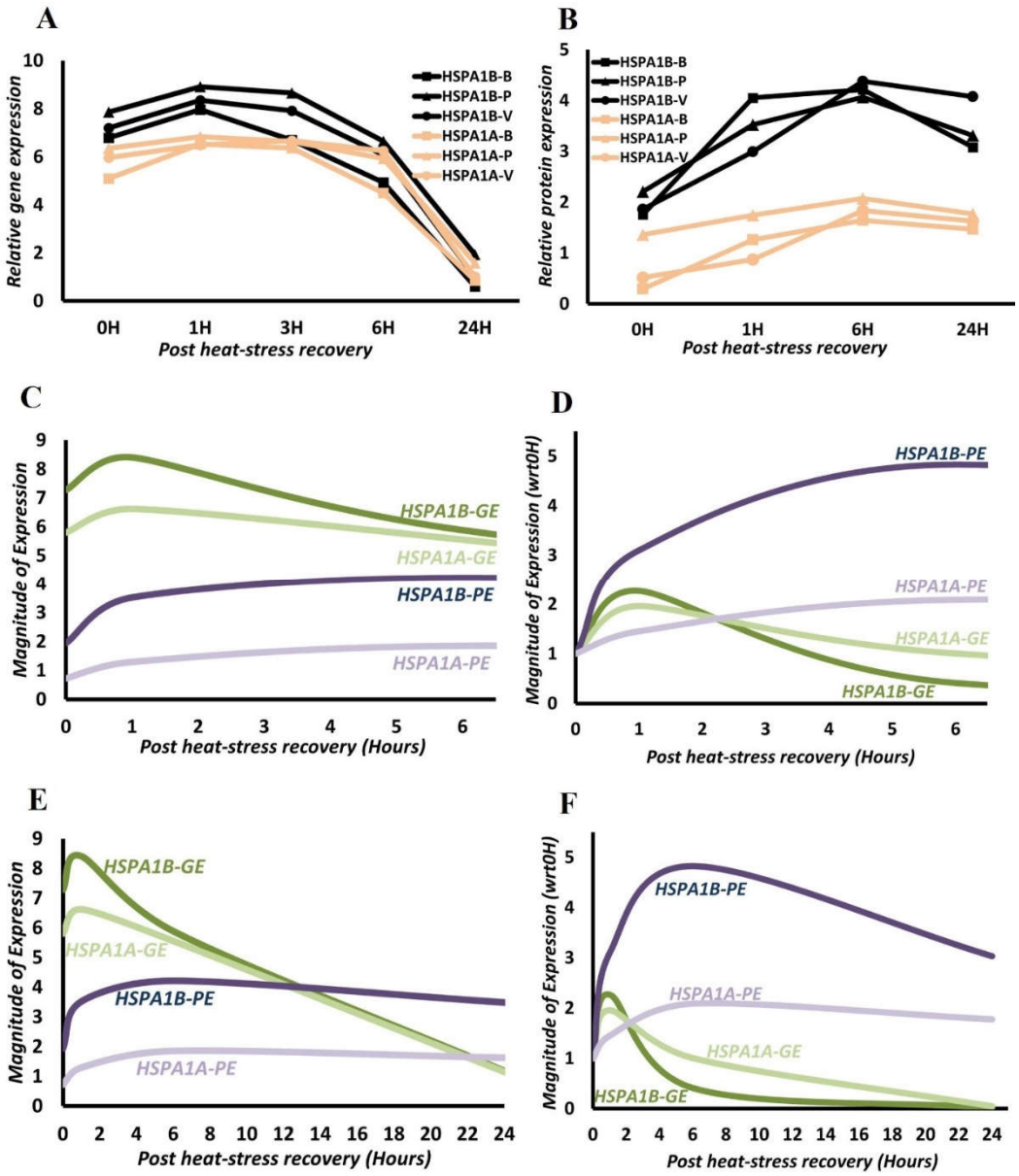

Figure 9. HSPA1A and HSPA1B transcription-translation dynamics. (GE: Gene-expression. PE: Protein-expression) (A) Time-course of gene-expression. (B) Time-course of protein-expression. For both Log_2 fold-change values as function of time are plotted over 24 hours (C, E) Smoothened scatterplot of the averaged Log_2 fold-change fold change in expression with respect to expression in control cells (D, F) Smoothened scatterplot of the averaged fold change in expression with respect to expression at 0H.

3.8. HSPA1B expression dominates HSPA1A expression

A significant fact evident from understanding transcription-translation dynamics is the relative difference between HSPA1A and HSPA1B protein expression. HSPA1B protein expression compared to HSPA1A is 2-fold all along, while the gene-expression ratio fluctuates between different time points (Figure 9D). The difference is noticeable in the mRNA consumption/decay rate for HSPA1B. The slope for HSPA1A and HSPA1B expression with respect to control starts overlapping at around 6H (Figure 9E), which means the presence of similar levels of mRNA. At 1H, the HSPA1B:HSPA1A is around 3.5, reaching to 1.25 at 6H, clearly pointing towards a higher rate of HSPA1B mRNA utilization. The faster HSPA1B mRNA consumption rate post heat-stress is clearly visible in the different slopes of the HSPA1B and HSPA1A gene expression curves during stress-phase gene expression (Figure 9F). Despite being at a higher magnitude of gene expression after stress-

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exposure, just after two hours into recovery, HSPA1B falls below HSPA1A. HSPA1A, on the contrary, follows a steadier rate of mRNA consumption. The difference in mRNA/gene expression levels very well correlates with the highly differential protein expression rates and yields of two proteins, with HSPA1B protein being present in very high measurable levels as compared to HSPA1A.

Taking this into consideration, there is a clear-cut dominance of HSPA1B expression at both levels and is observed across the three cell types (Figure 10). The differences between the expressions of these Hsp70 isoforms can be further analyzed at two levels; (i) time point in the recovery phase and (ii) at molecular level whether it is gene or protein expression. We analyzed these differences by calculating the HSPA1B/HSPA1A fold-change ratios for different studied conditions. As mentioned previously (Section 3.4), the maximal gene expression stays up to six hours, so comparisons within this time frame can assess differences in gene expression correctly. Within this 6-hour time period, in almost all the conditions (cell type and Hsp70 isoform combination), the maximum expression is at 1H (Figure 10A). At this time point, the HSPA1B/HSPA1A for gene-expression is 2.7-3.8 (Figure 10C), lowest in the case of DBMSCs and almost similar for DPMSCs and pMSCs. This difference is visible at the protein level (Figure 10B) as well, with HSPA1B/HSPA1A of 3.4 and 4.3 in DPMSCs and pMSCs, respectively, while as this ratio is around 6.9 in DBMSCs (Figure 10C).

Ignoring the cell-based differences, it is evident that HSPA1B is produced at least 3-fold more than HSPA1A, as early as1H after cells recover at 37°C. A close to 3-fold difference in both gene and protein expression is a definitive indicator of the differential significance of HSPA1B in the proteotoxic stress response pathway. This difference in protein expression is visible even at 6H, which is the maximal protein expression time point, and the HSPA1B/HSPA1A, at this point, varies between 3.9-5.9, a proportional increase from 1H ratio both in the case of DPMSCs and pMSCs, but a slight relative decrease for DBMSCs (Figures 10C and 10D). Irrespective of these differences in cell types, there is clearly a way higher expression of HSPA1B as compared to HSPA1A. This trend is seen even 24-hours after stress with HSPA1B/HSPA1A around 3 in DBMSCs and DPMSCs, while in pMSCs it is still around 6H levels. While at 24H, the measurable levels of gene expression have effectively reached pre-stress induction levels; these palpable differences in protein expression levels firmly point towards the significant role of HSPA1B compared to HSPA1A in proteotoxic stress response among placenta-derived stem cells. With somewhat similar transcription magnitudes but substantial differences in translation, it becomes plausible to conclude that an apparent higher requirement for HSPA1B during proteotoxic stress works through utilization of different regulatory mechanisms during HSPA1A-HSPA1B transcription and translation. We discussed in Section 3.3 the diversity of Hsp70 family members and other chaperones in stem-cell renewal and related processes (Also reviewed in [40]) and this interplay between HSPA1A and HSPA1B can probably be specific to PDSCs.

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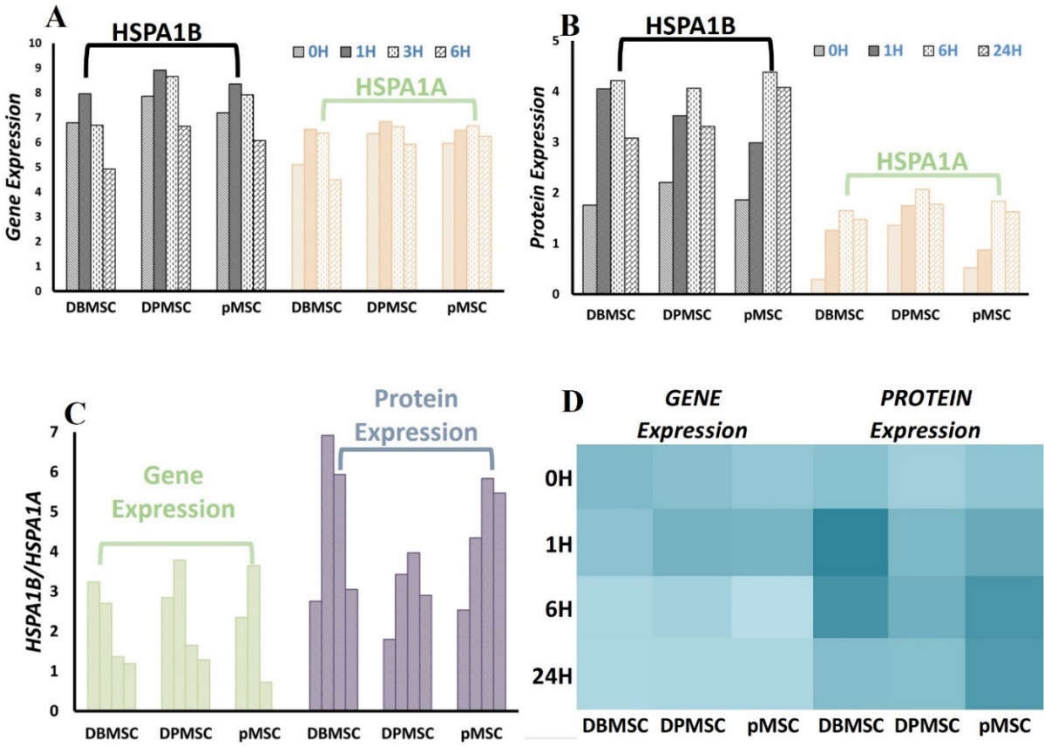

Figure 10. HSPA1B Expression Dominates HSPA1A Expression. (A) Gene- and (B) Protein-expression plots for HSPA1A and HSPA1B. Log_2 fold-change values plotted for visualization of relative differences in HSPA1A and HSPA1B expression. From A and B, it is obvious that overall, the gene-expression fold change is more as compared to protein-expression fold change and second this change in both cases is prominent in HSPA1B. (C) Ratio of HSAP1B expression to HSPA1A expression. Ratios are calculated using the original fold-change values and not Log_2 values. (D) Heat-map representation of the HSPA1B/HSPA1A ratios. HSPA1B dominance is more in protein expression and is very well observed even 24-hours post stress exposure.

4. Conclusions

Therapeutic applications of stem cells can be exploited further by enhancing the properties that contribute to their stemness or help them overcome the challenges they face post differentiation. Heat-shock pre-conditioning as a modulation tool in cell stabilization is one such tool already in practice with multiple applications. Numerous studies report better outcomes in transplanted cells exposed to heat shock before therapeutic use [78–82]. The origin of heat shock as a manipulation tool lies in its ability at milder levels to elicit a stress response that helps cells withstand severe forms of stress. Despite the benefits that heat-shock pre-conditioning affords, there still is potential to impact stem cell characteristics in adverse ways [82,83]. Therefore, it is more prudent to develop an in-depth understanding of various pathways that contribute to stem cell behavior with the potential to enhance the viability and behavior of transplanted stem cells.

Stress response pathways are very highly regulated in their magnitude and duration, and any manipulation of these pathways will require prior knowledge of different actors of these pathways. Our approach to the development of proteotoxic stress models lays a platform for in-depth analysis of stress response. Identification of the two Hsp70 isoforms is the first step in the understanding of the proteotoxic stress response in PDSCs. However, their functioning is not static and fluctuates in response to the cell's physiological state. Hsp70 function modulation happens through their interactions with other proteins, therefore a complete understanding of stress response should involve knowledge of the interaction partners, regulatory networks, and their role in regulating different cell-physiological states. This precise knowledge can be exploited in a more targeted approach to stem-cell enhancement through application of highly advanced gene-editing technologies like CRISPR-Cas9. An advantage of chaperone-based systems is developing peptide-

based entities with chaperone-like activities applied in therapeutics [84–88]; similar strategies can help develop design peptides to enhance stem cell survival. Therefore, comprehensive knowledge of stress-response pathways will go a long way in developing the understanding of mechanisms underlying stem-cell behavior and direct in framing effective and safe strategies in manipulating these pathways.

Author Contributions: Conceptualization, S.S.M., B.M.A., S.M., A.B., and T.S.K.; Methodology, S.S.M., B.M.A., A.B., A.Z., R.A.; Data Analysis, S.S.M., and B.M.A.; Writing—original draft preparation, S.S.M.; Writing—review and editing, S.S.M., S.M., B.M.A., A.B., and T.S.K.

Funding: This is King Abdullah International Medical Research Center, MNGHA, Riyadh project number RC17/060/R

Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Review Board (Reference # IRBC/1320/18) at King Abdullah International Medical Research Centre, Ministry of National Guard Health Affairs, Riyadh, Saudi Arabia.

Informed Consent Statement: All placentae were obtained after seeking informed consent from the volunteers.

Acknowledgments: We would like to thanks Ms.Aileen David for all her administrative support that helped in the successful completion of this work.

Conflicts of Interest: The authors declare no conflict of interest.

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