

Short heat shock has a long-term effect on mesenchymal stem cells' transcriptome

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Abstract

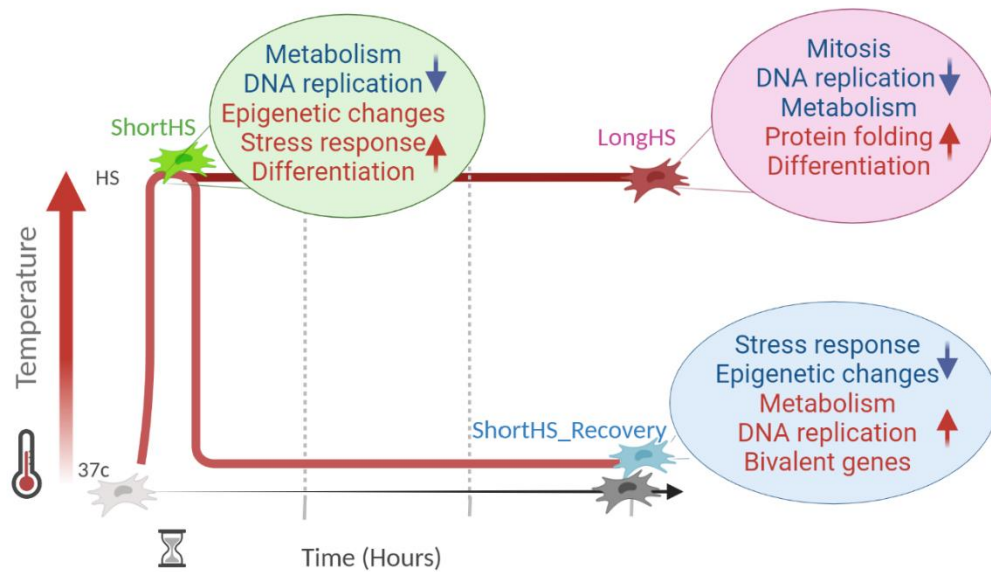
Background: Mesenchymal stem cells (MSCs) are multipotent stromal, non-hematopoietic cells with self-renewal and differentiation properties and are therefore a preferred source for cellular therapies. However, a better understanding of culture techniques is required to harness their full potential. Here we aim to compare the effects of short and long heat shock (HS) on the transcriptomic landscape of MSCs.

Methods: MSCs were extracted from the umbilical cord of a bovine fetus, cultured, and validated as MSCs. Early passage cells were exposed to 40.5°C for six hours or three days. RNA sequencing and bioinformatics analysis were performed to systematically examine the transcriptional changes following each treatment and to identify specific biological features and processes.

Results: The data indicates that while long heat stress influences many cell processes, such as immune response, cell cycle, and differentiation, the short HS mostly upregulates the cellular stress response. Once normothermia is resumed the long-term effects of the short HS can be revealed: although most genes revert to their original expression levels, a subgroup of epigenetically marked genes termed bivalent genes, maintains high expression levels. These genes are known to support cell lineage specification and are carefully regulated by a group of chromatin modifiers. One family of those chromatin modifiers, called MLL genes, is highly over-represented in the cluster of genes that are transiently upregulated following six hours of HS. Therefore, our data provide a mechanistic explanation for the long-term phenotype of short HS on development-related genes and could be used to predict the long-term effect of HS on cell identity.

Conclusions: Understanding the influence of culture conditions on morphology, phenotype, proliferative capacity, and fate decision of MSCs is needed to optimize culture conditions suitable for clinical or commercial use. Here, we suggest that simple and short stress can alter the cell's proliferation and differentiation capacities and therefore, following future optimizations, be used to shift the cells toward a more desirable functionality.

Graphical abstract



Keywords

Heat shock, bovine, mesenchymal stem cells, transcriptome, stress, bivalent genes, umbilical cord.

Background

Mesenchymal stromal cells (also called mesenchymal stem cells, MSCs) are a heterogeneous group of non-hematopoietic multipotent stem cells that assist in the preservation of homeostasis in many organs and tissues [1]. Additionally, physiological MSC stores are essential for the regeneration of many tissues in the body, acting through common signaling pathways such as Wnt, BMP and Notch [2]. MSCs in vitro are most frequently derived from adult tissue sources such as bone marrow and adipose tissue or birth associated tissue such as placental tissue, amniotic membranes and umbilical cord [3,4]. When grown in culture, MSCs can self-renew, differentiate into several tissues, and modulate the immune response in their surroundings [5–7]. When activated, MSCs secrete biologically active compounds and generate exosomes that modify the function of their cellular microenvironment [8–10]. Due to these characteristics, MSCs are commonly suggested as

candidates for cell-based therapy or as the cell source for tissue engineering and cultured meat products. However, despite their promise, it is still unclear how the properties and key biological functions of MSCs *in vitro* are influenced by parameters such as cell source, culture conditions, and varied pretreatments or enrichment protocols.

MSCs have been investigated and applied in cell-based therapy as immunomodulatory agents or as the cell source for tissue regeneration. To enhance their therapeutic efficacy, numerous chemical and physical agents have been assessed for their ability to speed cell expansion or enhance differentiation to the required cell types. These pretreatments, also termed priming, of MSCs with cytokines, drugs, or environmental stress conditions, were proven highly beneficial in many clinical settings [11]. For example, MSCs cultured under hypoxic conditions usually maintain their ‘stemness’ better, as illustrated by their increased proliferation capacity, better survival rates, and increased glucose consumption [12]. Therefore, the extent to which MSCs fulfill their immunomodulatory or regenerative potential can be altered by changing their extracellular environment. Hence, understanding the mechanisms through which the external conditions of the cells direct their biological functions is necessary. Nevertheless, the characterization and elucidation of these distinct effects remain challenging.

Lately, research has focused on the response of MSCs to sublethal exposure to cellular stressors such as hypoxia, heat, and nutrient depletion. These stressors resemble either ischemic or inflammatory microenvironments and are considered the major challenges to cell survival *in vivo* [13]. These preconditioning strategies induce hormesis - a phenomenon in which low doses of a harmful stressor produce a cascade of beneficial biological effects - and have been shown to improve migration, regenerative and proangiogenic potential, and stemness of MSCs [14,15]. Specifically, priming MSCs by heat shock (HS) is an effective way to protect cells undergoing transplantation, as

it enhances the survival rate and reduces the apoptotic rate of transplanted MSCs [16–19] On the 74
cellular level, thermal stress is associated with oxidative stress, endoplasmic reticulum (ER) stress, 75
and neurochemical stress, and impairs protein folding, cell cycle, and mitochondrial function 76
[16,20–22]. 77

On the transcriptional level, cells exposed to HS generally activate a cascade of heat shock proteins 78
and factors which in turn regulate downstream pathways that protect the stressed cells. The effect 79
of external temperature has been examined in various animals and tissues such as bovine PBMCs 80
[23], mammary tissues [24,25] and granulosa cells [26]. Another study exposed rats to heat stress 81
and examined the transcriptional changes in 3 different tissues [27]. Although many key genes and 82
pathways that respond to acute thermal stress were identified in these studies, only a few genes 83
were common across tissues, suggesting a high level of tissue specificity in the cellular response to 84
thermal stress. Additionally, although short preconditioning with temperatures above the standard 85
has been shown to prevent or reverse age related impairment and significantly improve the 86
regenerative potential and therapeutic potential of human MSCs [16,28–32], other studies have 87
shown reduced proliferative and regenerative abilities [22,33,34]. This inconsistency is possibly due 88
to the different treatment protocols (namely, different temperatures and durations of heat stress) 89
and the origin of MSCs used but could also be attributed to the intrinsic heterogeneity of MSCs. 90
Studies using single-cell RNA-seq have shown that MSCs are a heterogeneous population containing 91
pre-MSCs and lineage committed MSCs [35–37]. Hence, the effect of environmental factors, 92
including heat, on MSCs transcriptome and function is not yet fully understood. Furthermore, the 93
long-term epigenetic effects of variations in culture conditions are only beginning to be uncovered 94
[38,39]. An example of such an effect is hypoxia-preconditioning, which was shown to significantly 95

reduce global 5hmC in swine MSCs but had no effects on H3K4me3, H3K9me3, or H3K27me3 [39]. 96

Overall, the long-term effects of stress-preconditioning on MSCs are barely characterized. 97

Here, we set out to evaluate the immediate and long-term effects of HS treatment on the bovine 98
 umbilical cord (bUC)-derived MSCs transcriptome. In our previous study, we demonstrated that HS 99
 treatment changed the proliferation, differentiation, and immunomodulatory potential of bUC- 100
 MSCs [22]. Several HS protocols were used and shown to have a common effect of reducing 101
 proliferation and inducing oxidative stress and premature senescence. However, different HS 102
 protocols showed profound variation in gene expression and immunomodulatory potential. 103
 Surprisingly, even one hour of treatment at 42°C had a long-term effect on bUC-MSCs function and 104
 transcriptional pattern 3 days after the HS, which partially persisted even after eleven passages 105
 (about 40 days) in culture [22]. However, at temperatures higher than 41°C, the survival rate of 106
 mammalian cells decreases with increasing exposure time; to avoid this bias we performed our HS 107
 at 40.5°C [40,41]. Here, to gain an unbiased view of the cell-intrinsic response to heat shock, we 108
 performed gene expression profiling using RNA sequencing (RNA-Seq) following exposure of the 109
 bUC-MSCs to moderate HS at different times. We hypothesize that transcriptional response to heat 110
 stress has two manifestations: a major immediate reaction of stress response related genes, and a 111
 minor response of permanently altered transcripts. This work will advance our understanding of 112
 the cellular response to thermal stress and the long-term effects on the functionality of MSCs and 113
 the enduring impact of short and mild HS event on the organismal stem cell pool. 114

Methods 115

Cell Culture 116

Bovine mesenchymal stem cells were isolated, cultured and characterized based on generally 117
 accepted criteria [6,42] as we have previously reported [22,43]. Briefly, umbilical cords of Holstein 118

dairy cows were obtained from abattoirs located in the north of Israel. In the lab, the umbilical cords 119
were digested and plated as previously described in [44], followed by expansion in low glucose 120
Dulbecco's Modified Eagle's Medium (Gibco, 31885-023 or Biological Industries (BI), 04-001-1A), 121
containing 10% Fetal Bovine Serum (FBS) (BI, 04-001-1A), penicillin-streptomycin solution 1% (BI, 122
03-031-1B), Glutamine 1% (BI, #03-020-1B) and cryopreserved at different passages using FBS with 123
10% DMSO (Sigma-Aldrich, W387520). The media was changed every 2-3 days and all cells were 124
cultured in a humidified incubator with a controlled environment of 5% CO₂ and a temperature of 125
37°C, unless mentioned otherwise. 126

Cell Characterization 127

Three bovine MSCs lines were used in this study. Two were previously examined and verified 128
[22,43] and the third was examined and verified in this study (Supplementary Figure 1 (S1A-S1C)) 129
before subsequent heat shock experiments. One line was used for the RNA-seq experiment, while all 130
three lines were used for RNA-seq validation and further examinations. All the experiments 131
described were done on cells in passages 2-4, and at least 3 biological replicas were used unless 132
specified otherwise. 133

Cell Death Quantification Using Propidium Iodide (PI) 134

For quantification of cell death in culture, cells were harvested by trypsinization, washed with PBS, 135
and re-suspended in 10 g/mL PI for 5 min on ice. The percentage of live/dead cells was determined 136
within 1 hour of staining by flow cytometry (CytoFLEX, Beckman Coulter, Indianapolis, IN, USA). 137
10,000 events were collected per sample. At least three biological repeats were used for each 138
treatment. Data analysis was performed using Flow-Jo software. 139

Heat-shock Induction 140

MSCs at early passages (P2–P4) were seeded in 6-well plates (Costar, 3516) at different 141

concentrations to allow similar confluence at the end of the experiment, as follows: 142

Time of treatment	Temperature	# of cells seeded	abbreviation
six hours	37°C	250,000	ShortNT
	40.5°C	250,000	ShortHS
Three days	37°C	42,000	LongNT
	Six hours at 40.5°C, three days recovery at 37°C	72,000	ShortHS_Recovery
	40.5°C	72,000	LongHS

The cells were incubated for 24 hours in normal conditions before treatment onset (time 0). 143

Cell Counting 144

Following the HS, the cells were washed with Dulbecco's Phosphate Buffered Saline (BI, 02-021-IA) 145

and trypsinized (BI, 03-053-1A). 6μL of trypsinized cells were mixed with 6μL of trypan-blue (Sigma, 146

T8154), loaded into an appropriate slide, and counted using an automated cell counter (TC20, Bio- 147

Rad Laboratories Hercules, CA, USA). Three to five biological repeats were performed. 148

RNA-sequencing 149

Total RNA from the heat shocked cells was extracted via PureLink RNA Minikit (Invitrogen, 150

12183018A). RNA concentrations were measured using a NanoDrop ND-1000 spectrophotometer 151

(NanoDrop Technologies) and the quality of extracted RNA was confirmed (RNA integrity number 152

(RIN) > 9) using an Agilent 2100Bioanalyzer before further processing. Library preparation was 153

done using the INCPM mRNA library of the Weizmann Institute, Rehovot. The quality of cDNA 154

libraries was determined using a tape-station. Sequencing was done using Nextseq Illumina in the 155

INCPM of Weizmann Institute, with single-end reads of 75bp. Sequencing was done on 14 samples 156

from the same MSC line as in [22], and after initial quality analysis one sample was removed and
we were left with 13 samples to perform the in-depth analysis: 3 biological replicas for ShortNT,
ShortHS and LongHS treatments, and 2 biological replicas for LongNT and ShortHS_Recovery
treatments.

Data Analysis

Following sequencing, reads were trimmed using Cutadapt, mapped to genome (Bos_taurus.ARS-
UCD1.2) and counted using STAR and HTSeq. Further quality control of alignment sequencing data
was done using the Qualimap. Normalization of the counts and differential expression analysis were
performed using DESeq2. Hierarchical and K-means clustering as well as data visualizations were
performed using R packages. Gene names were taken from the Ensemble BioMart ARS-UCD1.2
dataset. Gene Ontology (GO), KEGG and Reactome pathways enrichment analyses were conducted
using both ranked gene list analyses using Gene Set Enrichment Analysis (GSEA) software version
4.1.0 and DEGs list analysis using g:Profiler version e103_eg50_p15_eadfi41 and ClueGO v2.5.7
(Cytoscape plug-in) with default parameters. KEGG pathway maps were downloaded from KEGG
pathway database. String datasets were used for functional protein association networks. Epigenetic
complexes analysis was performed using the EpiFactors Database [45] as well GO annotations of the
MGI database (Mouse Genome Informatics). The cytokine pathway list was taken from PathCards
v5.7.551.0. Bivalent enrichment was done by comparing our list to “3,868 bonafide bivalent genes”
in supplementary table 1 [46]. For detailed information see Supplementary Materials and Methods.

RNA extraction, Reverse transcription, and Real-time PCR

RNA extraction from cells was carried out using GenElute™ Total RNA Purification Kit (Sigma,
RNB100-50RXN). RNA was then reverse-transcribed into cDNA using qScript™ cDNA Synthesis
Kit (Quanta-bio, 95047-100). Real-Time PCR (RT-qPCR) reactions were performed using Fast SYBR

Green Master Mix (Applied Biosystem, 4385614) in an ABI Step-One Plus Real-Time PCR system. All
primers were verified by standard curve evaluation and are shown in Supplementary Table S1.
Primers for expression analysis were designed on exon-exon junctions and a -RT control was
performed each time. Relative mRNA fold change was calculated with the $\Delta\Delta CT$ method, using 1-2
control genes (PSMB2 and RPS9) as reference.

Statistical Analysis

GraphPad Prism (La Jolla, CA, United States) was used for statistical analysis and visualization of
RT-qPCR. $p < 0.05$ was considered statistically significant. For enrichment analysis hypergeometric
p-value was calculated using The Graeber Lab online calculator 2009 ©
(<https://systems.crump.ucla.edu/hypergeometric/>). See Supplementary Materials and Methods for
more detailed information.

Results

RNA-seq analysis of heat shock treated and control cells.

To examine the effects of thermal stress on the cell's transcriptome, we evaluated the transcriptional
response of MSCs to *in vitro* heat shock (HS). MSCs were extracted from the UC of a preterm fetus
as previously described [22]. Prior to the HS treatment, MSCs marker expression (Supplementary
Figure S1A), proliferation (Supplementary Figure S1B), and differentiation capacities
(Supplementary Figure S1C) were examined and validated as in [22,43].

HS treatment protocols were designed to examine the short- or long-term effects of exposure to mild
HS conditions (Figure 1A). One day after seeding MSCs from early passages (P2-P4), were moved
from 37°C to 40.5°C for six-hour (ShortHS) or three days (LongHS) or a six hours HS followed by
three days recovery back at 37°C (ShortHS_Recovery). Each HS treatment had a matching 37°C
normothermic (NT) control (ShortNT and LongNT). The two time points were selected to capture

the immediate transcriptional effects of HS vs. the stable and delayed ones. Immediately after the completion of the six hours or three days treatments, the cells were harvested, viability was checked and pellets were stored at -80° for later matched RNA extraction and sequencing. The viability of the cells and their MSC marker expression levels remained high following the various treatment protocols (Supplementary Figure S1D, S1E). Two or three biological repeats were done for each treatment (Supplementary Table S2). All RNA samples were run together on Nextseq Illumina and showed a high percentage and quality of mapping to exons and low levels of rRNA and mitochondrial DNA contaminations (Supplementary Figure S2A, S2B). Principal component analysis shows that the HS treatment determined the transcriptome profile as samples from the same treatment clustered together and apart from the other treatment groups (Figure 1B). Hierarchical clustering uncovered a secondary factor affecting the transcriptome which could be attributed to the time passed from seeding (Supplementary Figure S2C) or the population doubling (Supplementary Table S3). Several genes were also evaluated by RT-qPCR to validate the sequencing results (Supplementary Figure S2D).

We found that the various HS treatments significantly changed the expression levels of a total of 3667 genes ($p_{adj} \leq 0.05$ and $\log_2(FC) \geq 1$) (Figure 1C). After six hours of HS, 712 differentially expressed genes (DEGs) were found, whereas after three days of HS 1,371 DEGs were found, most of them downregulated (Supplementary Figure S2E). In the cells that were allowed to recover at 37°C for three days after the short HS, 2,037 genes were changed versus the short HS. However, only a slight difference is found when compared to the long NT sample. This suggests that after a short HS there is a major recovery from the stress, at least on the transcriptional level. The results indicate that short thermal shock affects MSCs' gene expression rapidly and widely but mostly transiently.

To discern the dominant systematic changes following the different HS treatments, we performed k-means clustering on all 3,667 DEGs (Figure 1D and Supplementary Figure S3). The resulting clusters revealed three major modes of change. Clusters 2 and 4 show the common effect of both long and short HS on the transcriptional pattern. Conversely, in clusters 3 and 7 the change is only observed after the long HS, portraying that more genes were induced after three days, possibly due to a slower transcriptional response rate (Figure 1C). Clusters 1 and 6 show the effect of culturing time on the transcriptional pattern.

While changes in expression patterns are evident from the k-means clustering analysis, we wanted to systematically examine the transcriptional changes following each treatment alone and identify specific biological features and processes.

Cell cycle and immune response are downregulated as developmental pathways are activated following a long heat shock

To get a comprehensive understanding of all the changes that occurred following the HS, we delved into the analysis of each treatment using the gene set enrichment analysis (GSEA) and gene ontology (GO) tools (as described in the supplementary materials and methods). First, we focused on the changes observed in cells exposed to long HS vs. long NT control (Figure 2A). On the morphological level, cells appeared flattened, with expanded cytoplasm and detectable stress granules (Figure 2B, stress granules marked with arrows). On the transcriptional level, 1,192 DEGs ($p_{adj} \leq 0.05$ & $\log_2(FC) \geq 1$) were found (Figure 1C). GSEA analysis show downregulation of cell proliferation and metabolism; namely cell cycle and DNA repair, glycolysis and oxidative phosphorylation (Figure 2C and Supplementary Figure 4A) and upregulated response to stress (Figure 2C). Interestingly, many differentiation and cell-fate related pathways (Figure 2D, Supplementary Figure 4B, C) were also upregulated, as previously suggested [47]. For example, general terms of cell growth and

morphogenesis were enriched, as well as more specific terms like connective tissue, chondrocytes, 249
osteoblasts, kidney, and neuronal system are upregulated. In addition, there is apparent 250
upregulation of cell adhesion molecules, which represents cell-cell interactions and the interaction 251
between the cell and the extracellular matrix (ECM). These molecules promote a broad spectrum of 252
cell signaling that directly or indirectly modulates stem cell proliferation, self-renewal property, 253
adhesion, and multilineage differentiation [48,49]. There is downregulation of gene sets related to 254
inflammation and the immune system, as well as DNA damage and ROS response. This could be 255
explained by the long duration of the HS, which might have required the cells to attenuate their 256
stress response. What is more, these changes were accompanied by the upregulation of post- 257
translational modifiers, specifically histone demethylases and Polycomb group (PcG) complex 258
members (Figure 2E). Additionally, KEGG analysis showed enrichment and upregulation of 259
pathways such as MAPK, PI3K-Akt, RAS and RAP1 (Figure 2F). In general, those pathways are known 260
to regulate cell size, survival, differentiation, adaptation to growth conditions, and stress responses 261
[33]. PI3K-Akt activates the mTOR signaling pathway [33,50], which is also elevated following heat 262
stress in bovine granulosa cells [47]. Activation of PI3K-Akt and mTOR pathways plays a central role 263
in cellular senescence and organismal aging and thus acts as a driver of stem cell depletion and 264
reduced tissue regenerative capacity [51–53]. 265

In summary, our data demonstrate that three days of HS treatment are not detrimental to cell 266
viability but have a major effect on cell fate and aging. Although the impact of long HS on 267
differentiation might prove useful as a pretreatment protocol for MSC transplantations, this duration 268
of HS is scarcely experienced in physiological settings. Hence, we wished to examine the effect of 269
short (six hours) HS on the MSCs. 270

Stress responses are induced while cellular functions decline following a short heat shock. 271

Initially, we focused on the immediate effects of short HS (Figure 3A) using GSEA analysis. Five out 272
of the ten most upregulated genes in this treatment (HSPA1A, HSPA6, HSPA4L, HSPB1, HSPH1, 273
Figure 3B) were heat stress genes, along with genes that regulate redox maintenance (SLC27A5), 274
insulin levels (MAFA) and mitotic progression (MISP). The most downregulated genes following 275
short HS are involved in RNA interference (RNAi) process (TSNAX), post-splicing multiprotein 276
complex (UPF3A), cell polarity (DAAM2), metabolism regulation (DHRS12) and chromatin and 277
transcription process (H1-2). These genes are mostly included in the Hallmark gene sets that are 278
enriched for up- and down- regulated processes following six hours of HS (Figure 3C and 279
Supplementary Figure 5A, 5B). Short heat stress induced stress response and differentiation 280
pathways while halting oxidative phosphorylation and cell cycle. Upregulated biological processes 281
enriched in GSEA were stress response, autophagy, development, and mRNA transcription (Figure 282
3D, Supplementary Table S4), while downregulated processes were all related to DNA replication 283
(Figure 3E and Supplementary Figure 5B). In addition to GSEA, we performed the analysis in 284
g:Profiler, KEGG and Reactome which also suggests that stress response and ER activity were 285
induced whereas DNA replication, with an emphasis on initiation, was decreased (Figure 3F). After 286
discovering dramatic short-term changes, we proceeded to check how many of these changes 287
remained stable. To elucidate these stable changes, the treated cells were allowed to recover in 288
normothermia for three days before the transcriptional analysis. 289

Long term effects of short heat stress. 290

To identify the transcriptional changes which occurred after short HS but remained stable even after 291
3 days of recovery, we compared the three different time points: control (as the zero-time point), six 292
hour HS and six hour HS followed by three days of recovery (ShortNT vs. ShortHS vs. 293

ShortHS_Recovery, Figure 4A). Thus, we re-analyzed the data (see Supplementary materials and 294
methods) using K-means clustering to visualize transcriptomic differences (Figure 4B). While after 295
recovery most genes regained similar expression levels to that of the control sample, two clusters in 296
which the differential expression persisted were identified. Cluster 2 genes were lowly expressed in 297
the control sample but were then upregulated after the short HS and remained active after recovery. 298
GO annotations and KEGG pathways enriched in this cluster are mostly related to stress and protein 299
degradation (Figure 4C). This implies that some elements of the stress response to acute stress last 300
for more than three days. Cluster 3 is the mirror image of cluster 2, presenting stably downregulated 301
genes related to proliferation and metabolism but also senescence and DNA damage (Figure 4D). 302
This might suggest that following short HS, the cells enter a state of quiescence to maintain their 303
stemness. This data is in agreement with our population doubling time analysis, which shows slower 304
proliferation after both the long and the short HS (Figure 4E). 305
Similar analysis done on the other two clusters, 1 and 4, raised an interesting point. Of the most 25 306
significantly enriched GO terms in cluster 4, only 3 are not related to transcriptional regulation, 307
mostly epigenetic modification (Figure 4F). This raised the intriguing hypothesis that the epigenetic 308
landscape is altered following the short HS, possibly with long term consequences. To examine how 309
a short period of stress could have that kind of persistent effect, we used the EpiFactors Database 310
[45] and related MGI annotations to identify enriched epigenetic modifiers in the data. Several 311
epigenetic complexes were found highly enriched in Cluster 4, i.e., upregulated after six hour HS 312
and then back to low expression after recovery (Figure 4G). Interestingly, those complexes are all 313
related to the mixed lineage leukemia (MLL) proteins which catalyze the trimethylation of histone 314
H3 Lys 4 (H3K4me3), a mark associated with active or poised transcription and found in the 315
promoters of most active or poised genes [54]. Bivalent genes are lineage-specification genes that 316

carry both H3K4me3 and H3K27me3 histone marks and are regulated by the balance between the two histone marks. This balance is mediated by PcG members and the MLL coactivator complex [55]. We searched for known targets of the MLL complex in our list and found cluster 2 (i.e. upregulated in both short- and long-term) to be significantly enriched for bivalent genes [46] crucial to cell fate regulation: several FGF family members, FZD3, JUN, FOXF1, VEGFA and KDM6B, to state a few (Supplementary Figure S6). This was in contrast to the other clusters, where these and other bivalent genes were significantly underrepresented (Figure 4H). Overall, this data sheds light on the long-term effects of short HS and suggests that a short stress event can modulate the epigenetic regulation of key cell fate genes.

Next, we wished to uncover the transcriptional changes shared by all HS-treated cells.

Key characteristics of MSC vary with heat stress duration.

So far, we have shown considerable effects of short and long HS *in vitro*. To examine to what extent the DEGs are shared between the timepoints, we had to eliminate the effect of culturing time and the circadian clock and focus solely on the effect of HS. To that end, we removed all DEGs that changed between the short and long NT controls (see Supplementary materials and methods for the analysis) and examined which DEGs were commonly upregulated (Figure 5A) or downregulated (Figure 5B) in all HS. A total of 102 upregulated and 93 downregulated DEGs were shared by short and long HS treatments, a highly significant enrichment over what could be expected by chance. While the commonly upregulated genes were mostly related to stress response, the commonly downregulated genes were not significantly enriched to any specific pathway or process. Hence, we examined if the different DEGs are annotated to shared biological processes and pathways which are therefore affected by both short and long HS. Important cellular functions like oxidative phosphorylation and cell cycle were found to be impaired in all HS; immune system was

downregulated, and the hedgehog signaling pathway was significantly upregulated following HS, 340
even after the recovery period (Figure 5C, Supplementary Figures S7A-D). 341

To understand the effect of HS on the biological processes related to the key capacities of MSCs, 342
namely self-renewal, differentiation and immunomodulation, we compared the relevant biological 343
processes and functions between different treatments. For cell cycle we saw that while in all three 344
treatments the downregulated DEGs were enriched for the general term ‘cell cycle’, only the short 345
HS is downregulating specifically DNA replication pre-initiation (e.g., genes like MCM2 and 7, CDC6 346
and E2F1). The three days samples showed downregulation of genes required for the G2/M phase 347
(namely cyclin A and B and CDC25), suggesting a block before mitosis (Figure 5D). Interestingly, the 348
cells that got the short HS but were let to recover for three days in normothermia show both the 349
G1/S and the G2/M effects although to a lesser degree, suggesting that even short thermal stress 350
might disrupt cell cycle regulation and proliferation. These results are supported by the population 351
doubling we saw in the culture following the experiments (Figure 4E) as well as in our previously 352
described cell cycle analysis [22]. 353

As for differentiation, it appears that several developmental pathways were activated, depending on 354
the heat shock treatment (Figure 5E, Supplementary Figure S7E-F). The long HS induced various 355
differentiation pathways; the most enriched were the differentiation to mesoderm/mesenchymal 356
fate. Notably, both canonical and non-canonical WNT pathways were upregulated after long HS, 357
suggesting a general shift in cell identity. The short HS upregulates the canonical WNT but 358
downregulates the non-canonical (WNT5a related) pathway (Figure 5E, Supplementary Figures S7E, 359
F). In line with the fact that KEGG definitions like “canonical WNT pathway” includes both activators 360
and suppressors, this and other pathways are enriched in both the down and upregulated DEGs. 361
Interestingly, HOX genes related to skeletal morphogenesis and several epigenetic factors (e.g., 362

Setd2, Kdm6a, Jarid1) were upregulated following both long and short HS; suggesting that heat shock treatment may slow proliferation while promoting MSC's commitment and differentiation. Immunomodulatory properties of MSCs have previously been shown to decline following stress [22,56]. Major downregulation of immune related pathways was detected after HS (Figure 5F). GO analysis in GSEA revealed considerable effects on the production of interleukins (ILs), TNFs and INF- γ . In addition, we observed the downregulation of antigen processing, acute inflammatory response, response to chemokines, and the migration and chemotaxis of macrophages, neutrophils, and granulocytes (Supplementary Table S5). The list of 760 genes in the "Cytokine Signaling in Immune system SuperPath" (<https://pathcards.genecards.org>) was analyzed and 38 of them (for example IL1Rs, IL7 and IL18) were to be downregulated after long HS while 17 (like FGF2 and FGFR2 which are related to differentiation [57,58]), are upregulated (Figure 5G). Overall, genes related to IL production are significantly downregulated (hypergeometric p-value = 2.49e-04, Figure 5G).

Discussion

Here, we show that the transcriptional changes following heat shock *in vitro* are broad and can alter every functional aspect of MSC identity. We analyzed RNA-seq from bUC-MSCs in normothermia vs. short or long exposures to HS, to investigate the subsequent changes in the transcriptional landscape that might lead to a shift in the cell's identity and fate. We were particularly interested in identifying the long-term effect of thermal stress on cell proliferation, differentiation, and immunomodulation capacities; as those key MSC features were amongst the most abundant GO term groups. To examine the relevance of our data to physiological heat stress, we compared our DEGs to a list of 55 genes defined as thermal stress responsive in a bovine study done *in vivo* [60]: 36 of the 55 genes were found to be differentially expressed following short, long or both HS groups. The genes EIF2A, HSPA1A, HSP90AA1, and HSF1 were considered by the authors key genes that

responded to thermal stress of Holstein dairy cattle. From our experiments, HSPA1A, and HSP90AA1 386
were indeed upregulated DEGs for short and long HS groups, and EIF2A for shortHS group only. 387
HSF1 was not differentially expressed in our treatments, maybe due to our use of 40.5°C which is 388
mild relative to the 42°C used by the authors. In addition, Fang et al. bioinformatics analyses 389
pinpointed biological processes and pathways associated with thermal stress that are very similar to 390
those we have identified, e.g., protein folding, transcription factor binding, immune effector process, 391
negative regulation of cell proliferation, PI3K- Akt signaling pathway, and MAPK signaling pathway. 392
The immediate transcriptional changes found following short HS include the onset of several cellular 393
stress responses and cell cycle arrest in G1/S. This checkpoint arrest is a known adaptive cellular 394
response to heat stress, in which cells slowdown proliferation rate while rapidly upregulate the 395
transcription and translation of HSPs to maintain cell homeostasis and retain their cellular functions 396
[60]. Our previous results, which showed cell cycle arrest at G1/S after short HS [22] also support 397
this finding. Two possible interpretations arise, each with a different implication for stemness and 398
differentiation: in one, following the short HS the cells enter a quiescent state; this is in alignment 399
with the observed metabolic changes (reduced amino acid metabolism, reduced oxidative 400
phosphorylation, increased glycolysis, and mitophagy induction of mitochondrial renewal, etc., as 401
reviewed in [61]. This suggest that short HS treatment encourages the retention of MSC stemness. 402
In the other interpretation, a long G1 may allow for the accumulation of the epigenetic changes 403
needed for the initiation of fate decisions [62]. This interpretation might explain the transient 404
upregulation of many epigenetic factors and the stable changes in many lineage commitment genes 405
observed after three days recovery. To mechanistically challenge those two interpretations and to 406
determine whether HS promotes stemness or differentiation. Further study, preferably on the single 407
cell level, is required to mechanistically challenge these two interpretations. 408

Evident changes were observed in the morphology and proliferation rate of the cells three days after 409
the Short or long HS treatment. However, on the transcriptional level, the majority of ShortHS DEGs 410
reverted to normal expression levels after three days recovery, and only a subset of the DEGs 411
remained. In this subset of stable DEGs we see the upregulation of several developmental genes 412
known as "bivalent" for their dual histone lysine tri-methylation marking in both K4 (active mark) 413
and K27 (repressive mark). Those genes are known targets of the MLL complex, which carries out 414
the methylation of histone H3 lysine 4 and was transiently overexpressed after the initial HS event. 415
So, a sequence of events can be hypothesized. First, the short HS upregulates the MLL complex 416
members, thus shifting the balance between the active and repressive histone marks. Consequently, 417
the chromatin of the previously poised (and silent) bivalent genes opens to enable transcription of 418
developmental genes, priming the cells toward cell fate determination. Evaluation of this hypothesis 419
will require follow-up studies examining changes in epigenetic marking following HS. 420
The idea that cells after HS pretreatment are more prone to differentiation is tempting. If indeed 421
priming MSCs by specific stress can help us direct their fate, it could provide us with another tool in 422
the cellular therapy toolbox. Moreover, as the idea of cellular agriculture, or cultured meat, gains 423
increasing attention, more cost effective and clean ways to differentiate the source cells toward the 424
intended fate (usually muscle or fat) are needed. Current differentiation protocols require large 425
amounts of expensive and unstable growth factors, which raise costs and hinder commercialization 426
of cultured meat. Every pretreatment protocol that is easy, cheap, and reduces the time and 427
resources necessary for differentiation, will have huge economic and environmental consequences. 428
On the clinical level it is interesting to note the negative effect of long HS on MSC immune activity, 429
possibly compromising the cells' immunomodulatory functions. Indeed, reduced 430
immunomodulation was previously observed when we co-cultured MSCs subjected to HS with 431

macrophages [22]. The reduced immunomodulation capacity of the stressed MSCs might account 432
for the increased production of inflammatory cytokines found in mammary epithelial cells following 433
HS [63]. Hence, the increased rates of inflammatory diseases in dairy cows during the summer 434
months could be partially attributed to the failure of the malfunctioning MSCs to modulate the 435
inflammatory response of the surrounding cells to HS. Overall, identifying the long-term effect of 436
HS on MSC capacities suggest an explanation for the seeming contradiction between the in vitro 437
experiments which show beneficial effects to HS priming and the in vivo data that demonstrate 438
harmful physiological consequences to thermal stress (reviewed by [64]). 439

Although we have demonstrated that HS affects MSCs' transcriptome, our study has some 440
limitations. Population heterogeneity, circadian clock, culturing time, and culture conditions all have 441
effects on RNA-seq results that need to be controlled for (Supplementary Figure S8). To counter this 442
drawback we (1) made sure that at the end of the experiment (either six hours or 3 days) all the 443
treatment groups had similar culture density (Supplementary Table S2) (2) sequenced two or three 444
independent biological repeats from each treatment group (Supplementary Table S3) (3) to reduce 445
heterogeneity that might originate in genetic background, the same MSC line was used in all three 446
repeats. To counter the effects of culturing time, we used untreated (NT) controls, one for the short 447
HS, and the other for the long HS. When we compared gene expression at the short and long time 448
points, all DEGs that were apparent between the NT control time points (and were assumed to be 449
the result of culturing effect) were ignored. Interestingly, two transcription factors regulating the 450
circadian clock, BHLHE40 and TIMELESS, were found among those ignored DEGs. These two 451
transcription factors together regulate the expression of 202 other DEGs in this group 452
(Supplementary Figure S8B). These oscillating genes may partly explain the relatively high number 453
of DEGs due to culturing effect since the ShortHS and ShortNT samples were taken at noon (24h 454

after seeding + six hours treatment) while the three day samples (LongHS, LongNT and ShortHS_Recovery) were collected in the morning (exactly 96h after seeding).

Conclusions

The use of MSCs for cell therapy or cultured meat has several advantages, such as high availability, low production price, and fast and easy differentiation. Nonetheless, our limited understanding of their heterogeneity, fate determination, and immunomodulation holds back further MSC applications. It is therefore essential to improve the consistency and efficacy of MSCs. Such enhancement can be achieved using in-vitro preconditioning treatments such as HS [14]. Here we show that the correct application of HS allows for the enhancement of desired MSC characteristics and induction of a wide range of stem cell fates and differentiation pathways.

List of abbreviations

MSCs:	467
Mesenchymal stem cells	468
bUC-MSCs:	469
Bovine umbilical cord-derived mesenchymal stem cells	470
ER :	471
endoplasmic reticulum	472
FBS:	473
Fetal Bovine Serum	474
RNA-seq:	475
RNA sequencing	476
RT-qPCR:	477
Reverse transcriptase- quantitative polymerase chain reaction	478
HS:	479
Heat shock	480
NT:	481

Normothermic	482
DEGs:	483
Differentially expressed genes	484
GSEA:	485
Gene set enrichment analysis	486
GO:	487
Gene ontology	488
ECM:	489
Extracellular matrix	490
PcG:	491
Polycomb group	492
MLL:	493
Mixed lineage leukemia	494
H3K4me3:	495
The trimethylation of histone H3 Lys 4	496
ILs:	497
Interleukins	498
Ethics approval and consent to participate	499
The procedures of isolated UC-MSCs were conducted abiding by the Institutional Animal Care and Use Committee of the Israel Ministry of Agriculture and Rural Development, permit #11380.	500 501
Consent for publication	502
Not applicable.	503
Availability of data and materials	504
The datasets supporting the conclusions of this article are available in the GEO repository, GSE214467 study at: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE214467	505 506
Some datasets supporting the conclusions of this article are included within the article additional files.	507 508
Competing interests	509
The authors declare that they have no competing interests.	510 511

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Authors contributions	516
IRH: conception and design, designing and performed the experiments, bioinformatics analysis	517
and data interpretation, and manuscript writing. GS: validation of MSC lines by flow and RT-	518
qPCR. CS: support with MSC extraction and culturing. SS: conception and design, assembly of data	519
and data analysis, manuscript writing. The authors read and approved the final manuscript.	520
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Additional information 707 708

Additional file 1.pdf. A detailed list of additional files, Supplementary Figure 1-8, Supplementary figure legends 1-8, Supplementary Materials and Methods, and Supplementary Table 1-3. 709 710

Additional file 2. xlsx. Supplementary Table 4 711

Additional file 3.xlsx. Supplementary Table 5 712

Additional file 4.xlsx. Supplementary Table 6 713

Additional file 5.xlsx. Supplementary Table 7 714

Figure legends: 715 716

Figure 1: Transcriptome changes in response to heat shock. (A) A schematic timeline showing the HS treatments from which RNA-Seq libraries were prepared. MSCs were plated and 24h later exposed to heat shock (HS, 40.5°C) for: 6h (ShortHS), 6h + 3 days recovery at 37°C (ShortHS_Recovery), 72h (LongHS), followed by collection for RNA extraction. Control cells were parallelly plated and maintained in normal temperature (NT, 37°C). Each treatment has colour code which will be used throughout the article: ShortNT (red), ShortHS (green), LongNT (olive), ShortHS_Recovery (blue), LongHS (purple). (B) Scatter plot for principal component analysis of global gene expression (RNA-seq) demonstrates clear separation of gene transcripts between groups. (C) Number of differentially expressed genes (DEGs) with $\text{padj} \leq 0.05$ and $\log_2(\text{FC}) \geq 1$ that change between the treatment groups. (D) K-means clustering of 3667 DEGs. 717 718 719 720 721 722 723 724 725 726

Figure 2: Downregulation of cell cycle and immune response genes while developmental pathways genes are induced following long heat shock. (A) Illustration of long heat shock treatment. (B) Cells after LongHS get enlarged and flattened. Bright field x4 (up) and x10 (down). Arrows point to stress granules. (C) Results of GSEA Hallmark analysis showing enriched gene 727 728 729 730

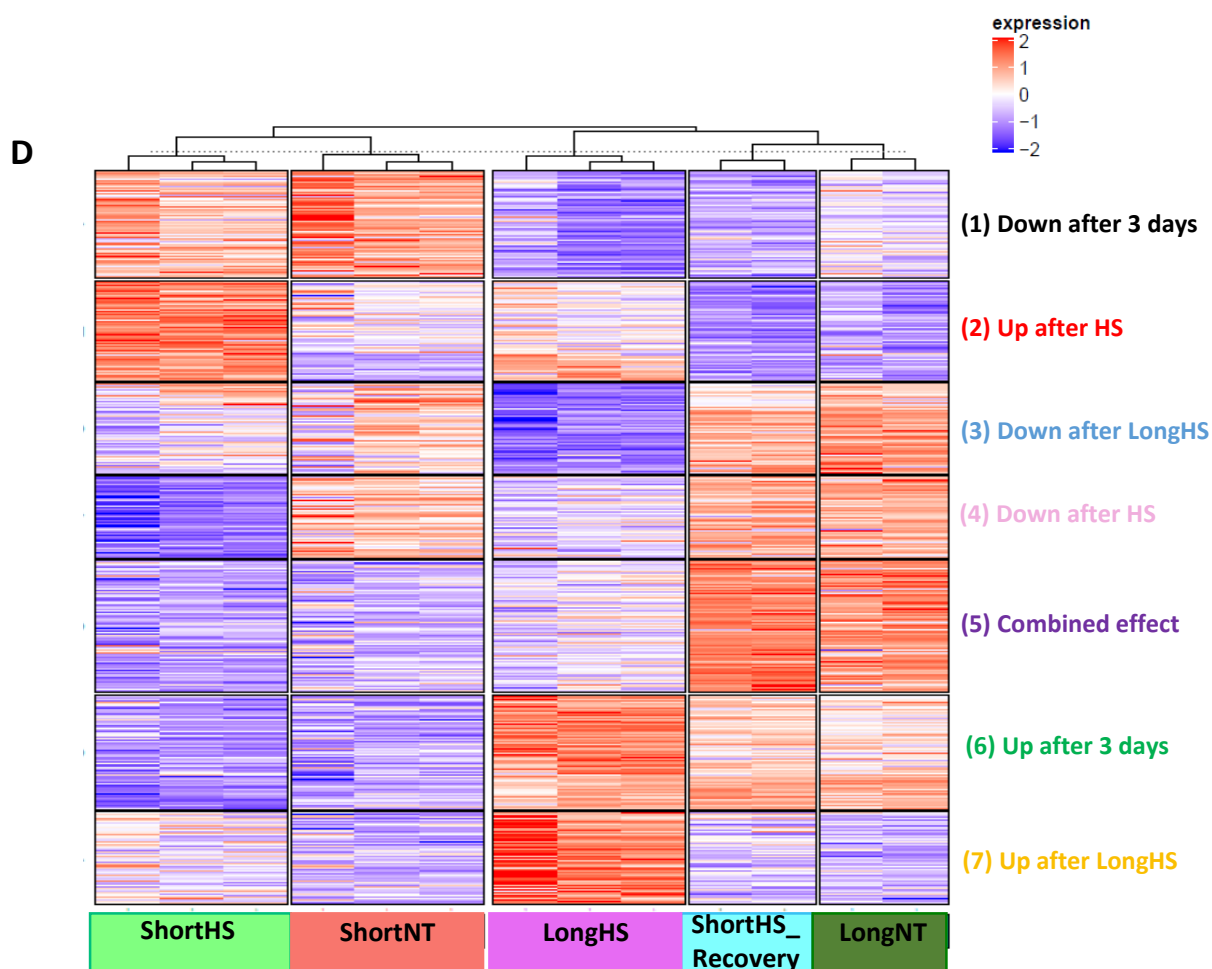
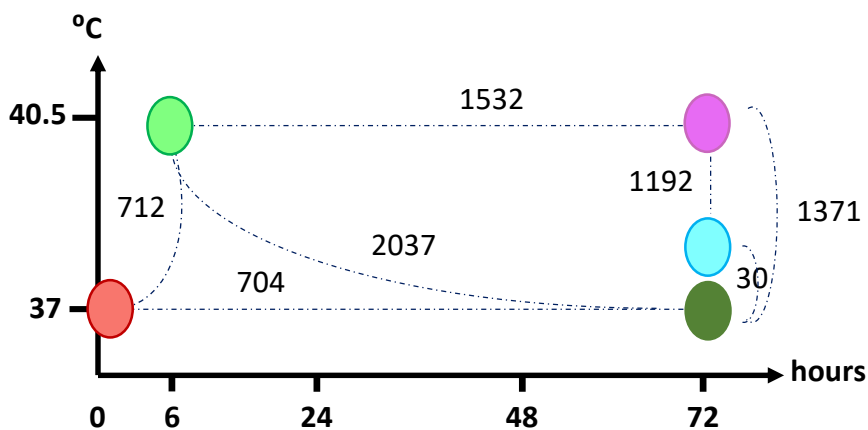
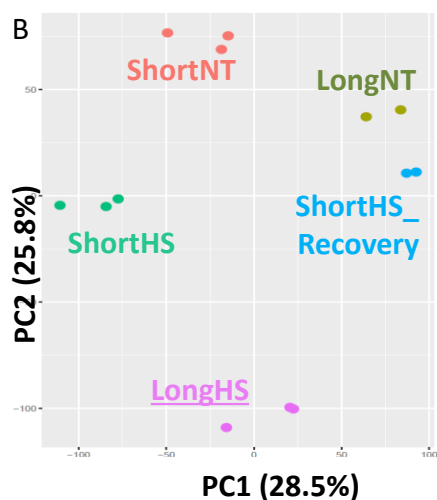
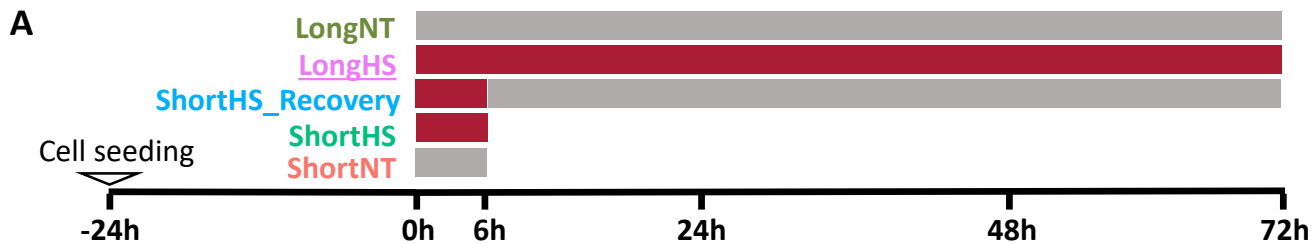
sets (FDR q-val < 25% and p-val < 0.05). A positive normalized enrichment score (NES) values, mark in red, indicates upregulation in the LongHS phenotype. (D) Differentiation pathways significantly upregulated in LongHS vs. LongNT as per g:Profiler analysis. Plot was generated using GOpot R package. (E) Upregulated histone modifications and PcG complex following LongHS as per GO analysis with GSEA. (F) KEGGs significantly upregulated in differentially expressed genes of LongHS vs LongNT using ClueGo. Colors represented p-value: light red 0.05, red 0.01, brown 0.001. The node size represents the term enrichment significance.

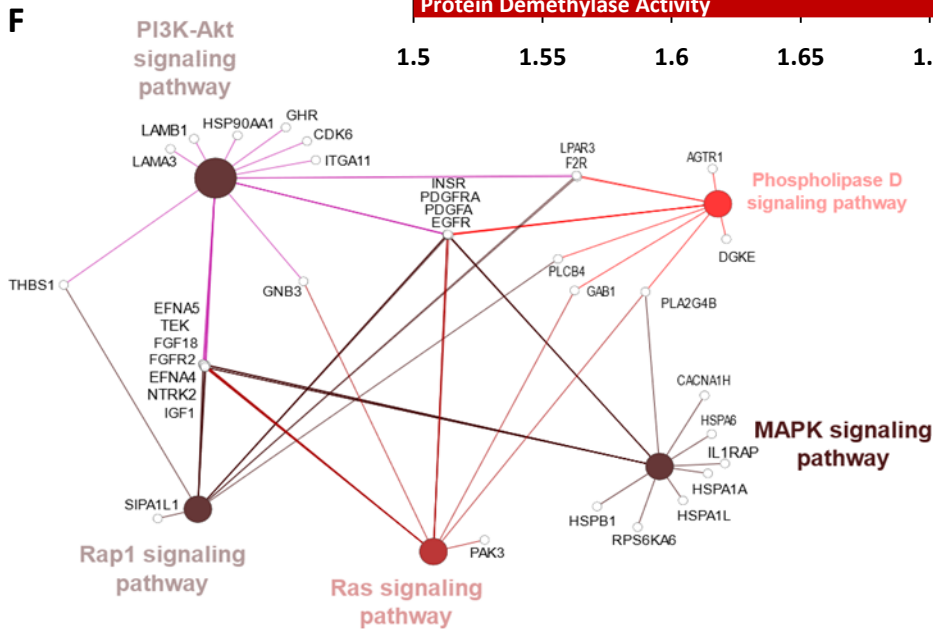
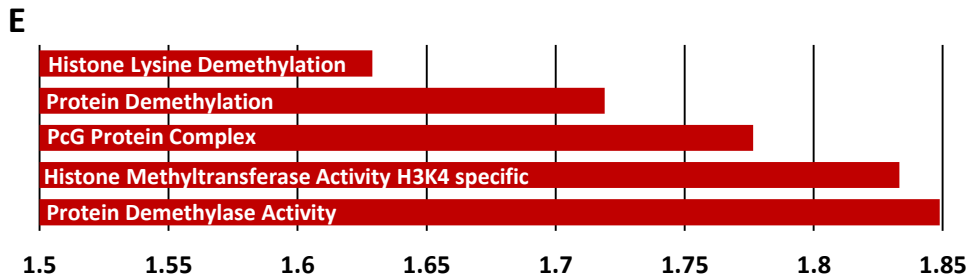
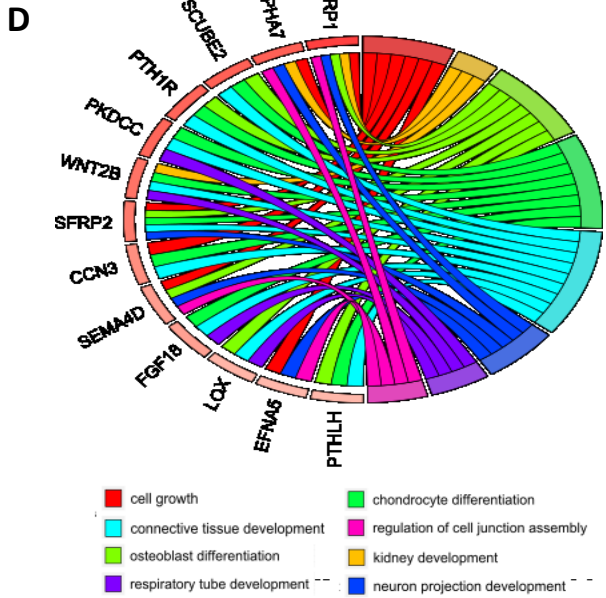
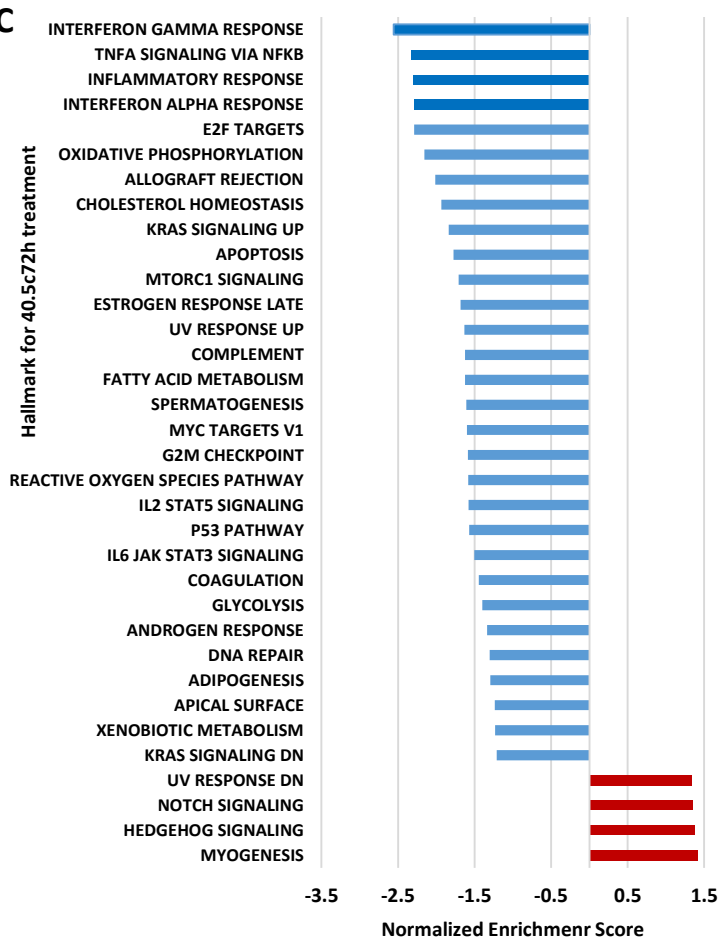
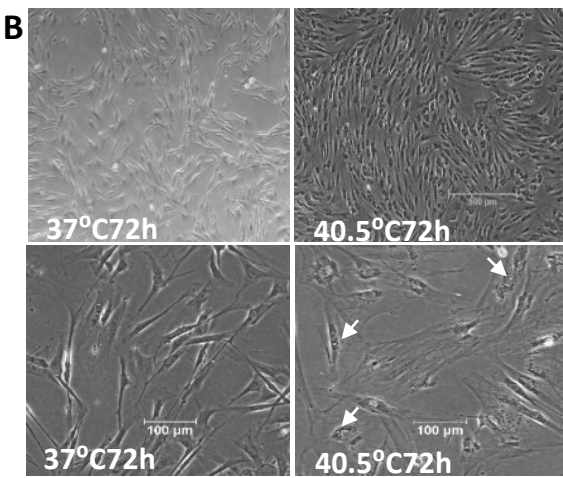
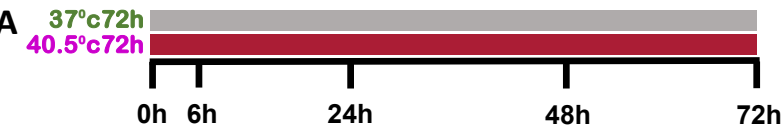
Figure 3: Induced stress response and reduced cellular functions following short heat shock. (A) Illustration of short heat shock treatment. (B-E) Gene set enrichment analysis (GSEA) results of 40.5c6h vs. control comparison. RNA-Seq was performed on samples collected after 6 hours at 40.5c compared to cells cultured in 37c (control). (B) Heat map of the top 30 marker genes upregulated and downregulated in short heat stress. Expression values are represented as colors and range from red (high expression), pink (moderate), light blue (low) to dark blue (lowest expression). (C) Results of GSEA Hallmark analysis showing enriched gene sets (FDR q-val < 25% and p-val < 0.05). A positive normalized enrichment score (NES) values, marked in red, indicate enrichment in the 40.5c6h phenotype. (D) Upregulated GSEA biological processes significantly enriched (FDR q-val < 0.25 and p-val < 0.01) in the comparison of ShortHS vs. ShortNT, ordered by the increasing NES (dark color is the most enriched). Due to high number of GOs, the GOs with similar process were combined under one general phrase, which is represented by one part of the pie chart (Supplementary Table S4). The number defines average normalized enrichment score. (E) Downregulated GSEA biological processes significantly decreased (FDR q-val < 0.25, p-val < 0.01) in the comparison of ShortHS vs. ShortNT, ordered by the increasing NES. (F) Functional enrichment analysis of the upregulated (red) and downregulated (blue) differentially expressed genes (DEGs) between ShortHS and ShortNT cells using g:Profiler.

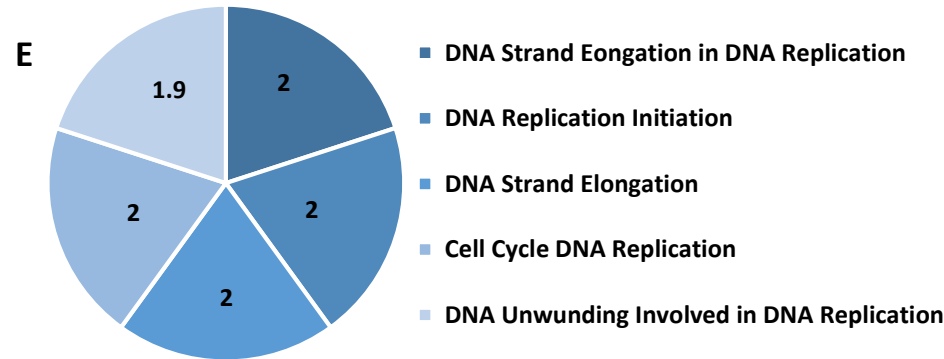
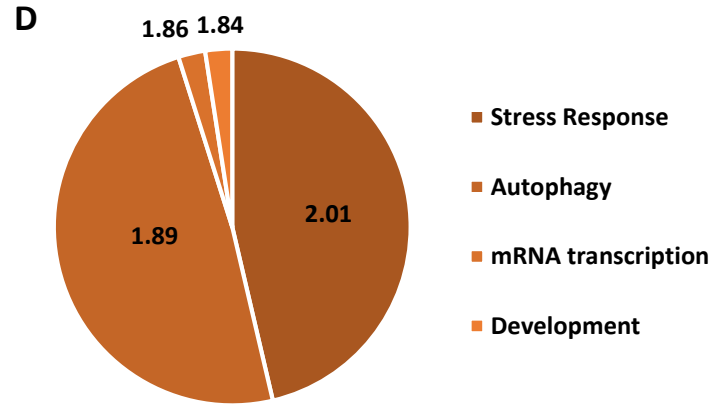
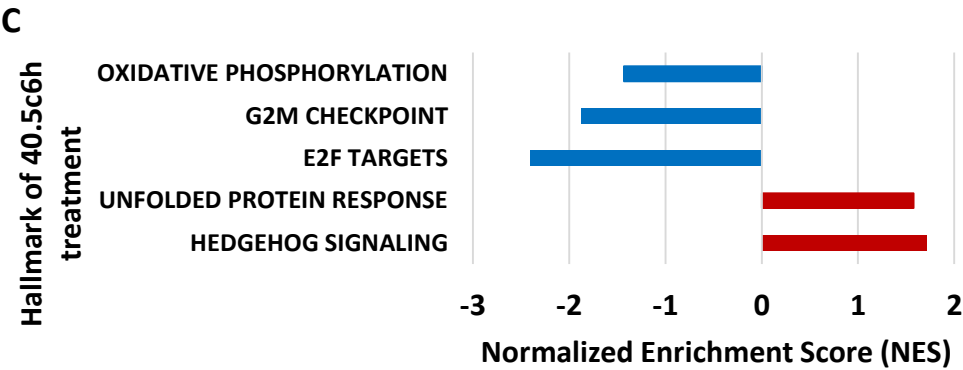
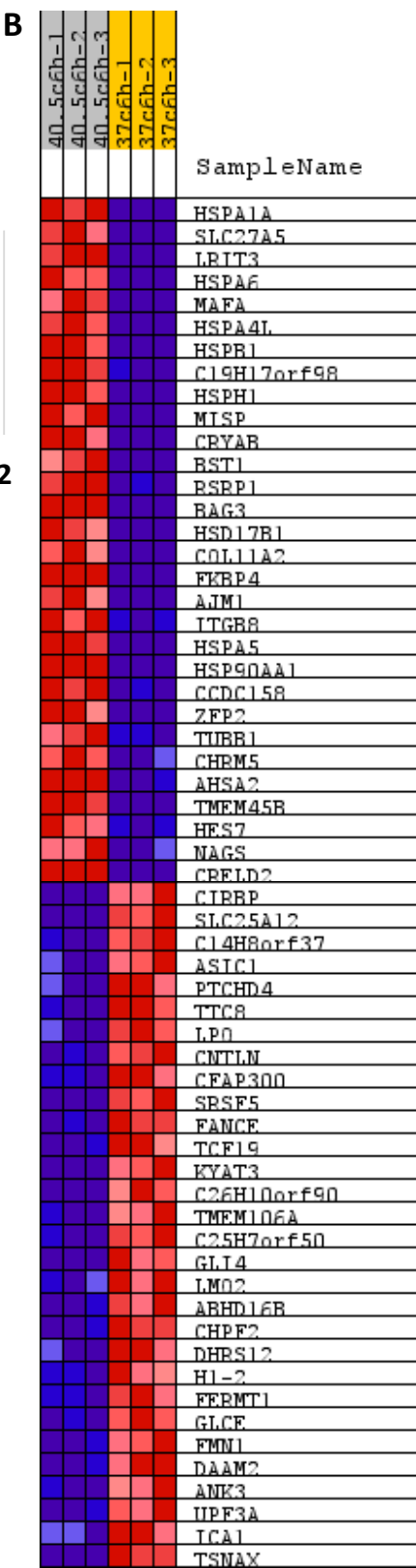
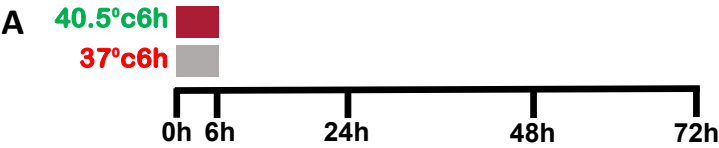
Figure 4: Transient upregulation of chromatin modifiers after short heat shock and stable activation of lineage commitment genes, maintained even after three days recovery. (A) Illustration of ShortHS_Recovery vs ShortHS treatment. (B) Heat map of genes differentially expressed (padj < 0.05) between ShortHS and ShortNT. (C) Biological processes enriched in cluster 2 (pink) (D) Biological processes enriched in cluster 3 (blue) (E) Population doubling time for prolonged treated groups. (F) Biological processes enriched in cluster 4 (purple) (G) MLL (GO:0044665, GO:0044666), HAT (GO:0000123) and HMT (GO:0035097) are upregulated following ShortHS but return to normal/downregulated during recovery period (H) Bivalent genes are upregulated following ShortHS and remain in this state even after recovery period.

Figure 5: Cell cycle and metabolism are commonly reduced following all heat shock protocols, while other processes related to key characteristics of MSC are differently regulated. (A) Venn diagram of upregulated genes (padj < 0.05) between the treatment groups. (B) Venn diagram of downregulated genes (padj < 0.05) between the treatment groups. (C) Heat shock common hallmarks and their enrichment score. Above hyphenated line are hallmarks common to all 3 HS groups. (D) Cell cycle related biological processes, effected by HS, and their enrichment score. No0455: CDC25-Cell cycle G2/M (KEGG pathway), GO:0007049: Cell cycle (MGI database), R-BTA-69002 – DNA Replication Pre-Initiation (Reactome). (E) Enrichment of

biological processes related to development effected by HS. GO:0060070: Canonical WNT 772
 signalling, GO:0048863: Stem cell differentiation, GO:0048468: Cell development, GO:0009888: 773
 Tissue development, GO:0007492: Endoderm development. GO:0007498: Mesoderm 774
 development, GO:0048762: mesenchymal cell differentiation. (F) Enrichment of biological 775
 processes related to immune system effected by HS. GO:0002526: Acute Inflammatory response, 776
 GO:1905517: Macrophage migration, GO:0032635: IL-6 production, Cytokine pathway list was 777
 taken from PathCards v5.7.551.0. (G) Cytokine pathways genes that are differentially expressed in 778
 LongHS vs. LongNT. 779

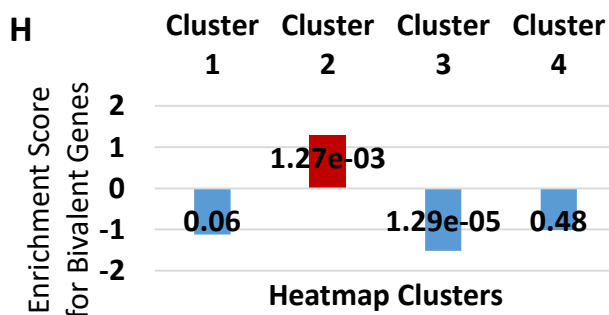
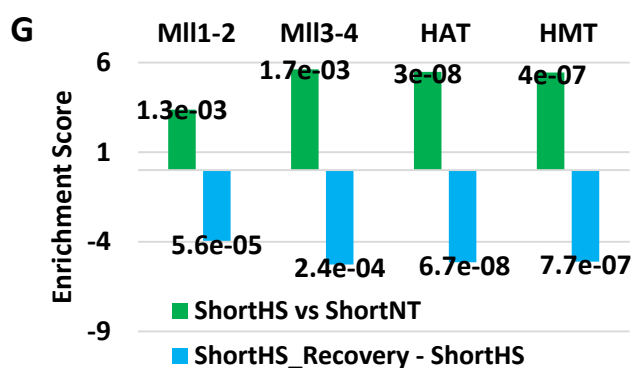
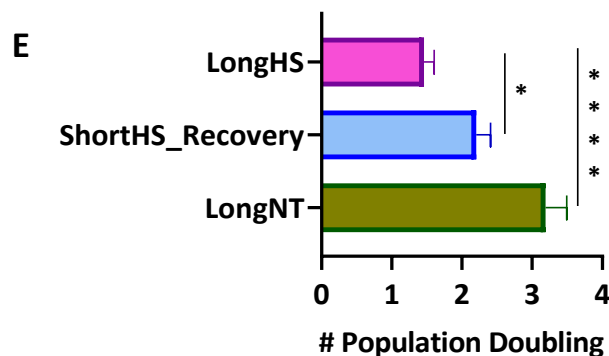
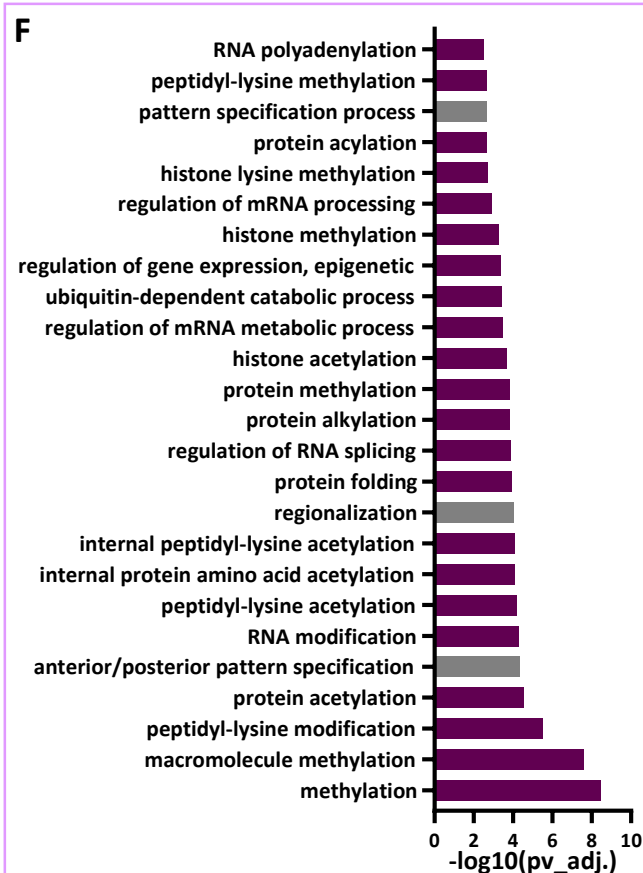
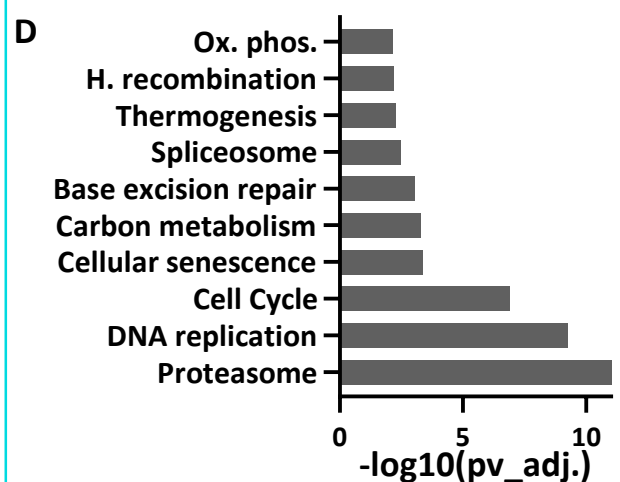
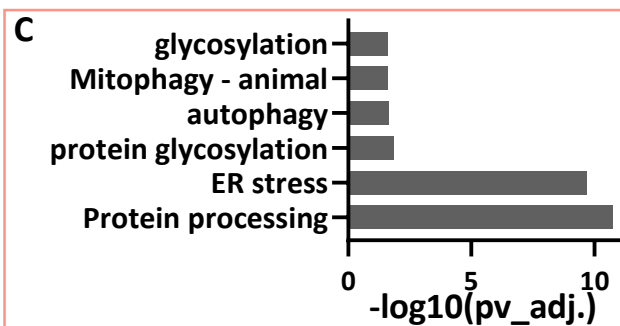
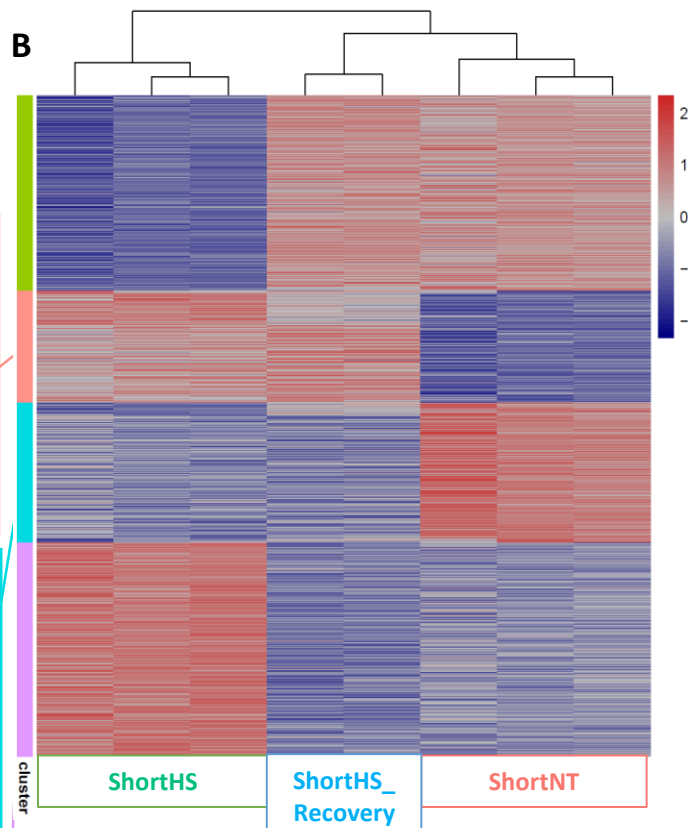
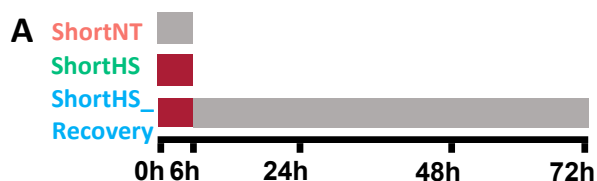




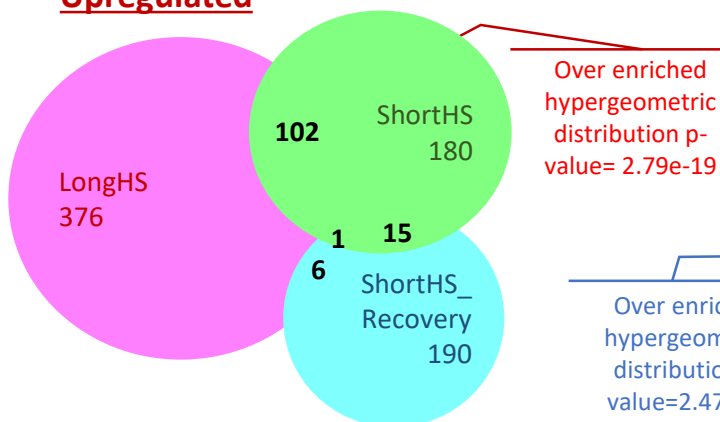


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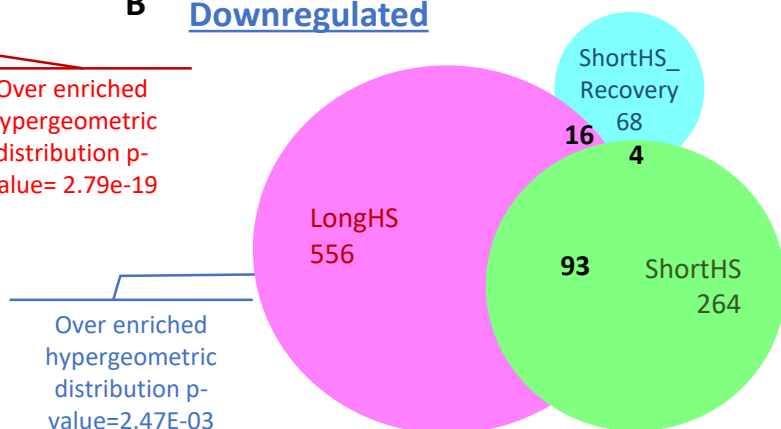
Term name	Term ID	Padj
Protein processing in endoplasmic reticulum	KEGG:04141	1.720×10^{-9}
DNA-binding transcription activator activity	GO:0001216	1.222×10^{-2}
HSP90 chaperone cycle for steroid hormone receptors (SHR)	REAC:R-BTA-337...	2.349×10^{-4}
Regulation of HSF1-mediated heat shock response	REAC:R-BTA-337...	3.540×10^{-4}
Cellular response to heat stress	REAC:R-BTA-337...	3.540×10^{-4}
DNA replication initiation	GO:0006270	2.933×10^{-5}
organic acid catabolic process	GO:0016054	3.993×10^{-3}
carboxylic acid catabolic process	GO:0046395	1.330×10^{-2}
DNA-dependent DNA replication	GO:0006261	1.708×10^{-2}
small molecule catabolic process	GO:0044282	2.304×10^{-2}
DNA replication	KEGG:03030	1.031×10^{-3}
Cell cycle	KEGG:04110	1.126×10^{-3}
Tyrosine metabolism	KEGG:00350	2.121×10^{-2}
Glycine, serine and threonine metabolism	KEGG:00260	4.270×10^{-2}
DNA Replication Pre-Initiation	REAC:R-BTA-690...	2.363×10^{-3}
Metabolism of amino acids and derivatives	REAC:R-BTA-712...	3.758×10^{-3}
DNA Replication	REAC:R-BTA-693...	1.445×10^{-2}
Phenylalanine and tyrosine metabolism	REAC:R-BTA-896...	2.615×10^{-2}
Assembly of the pre-replicative complex	REAC:R-BTA-688...	2.615×10^{-2}



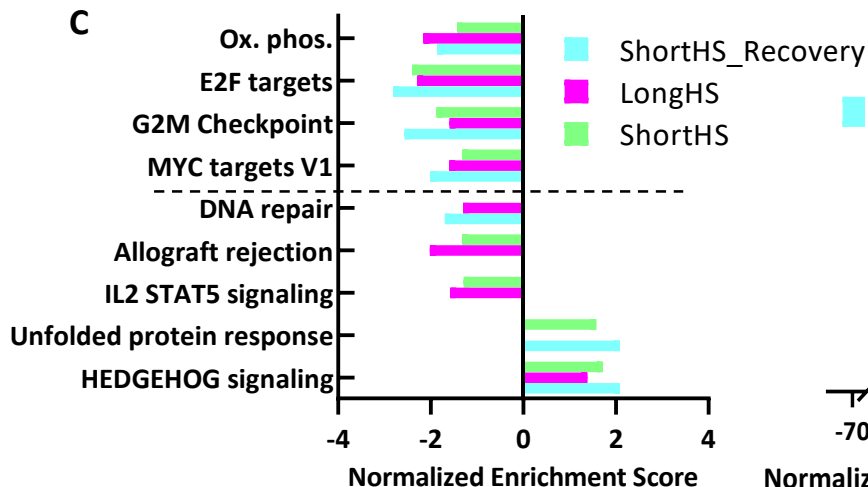
A Upregulated



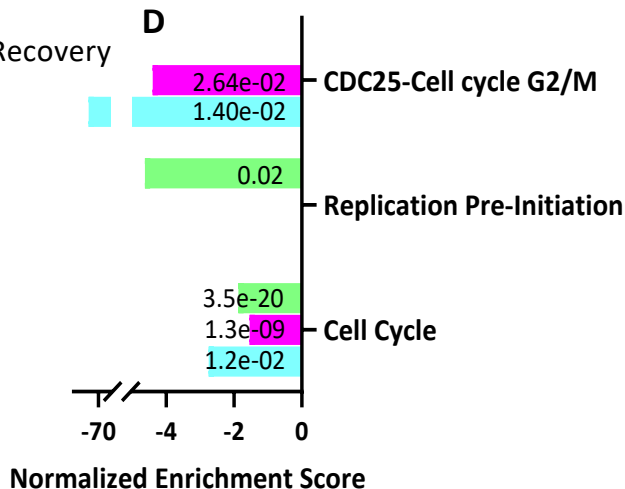
B Downregulated



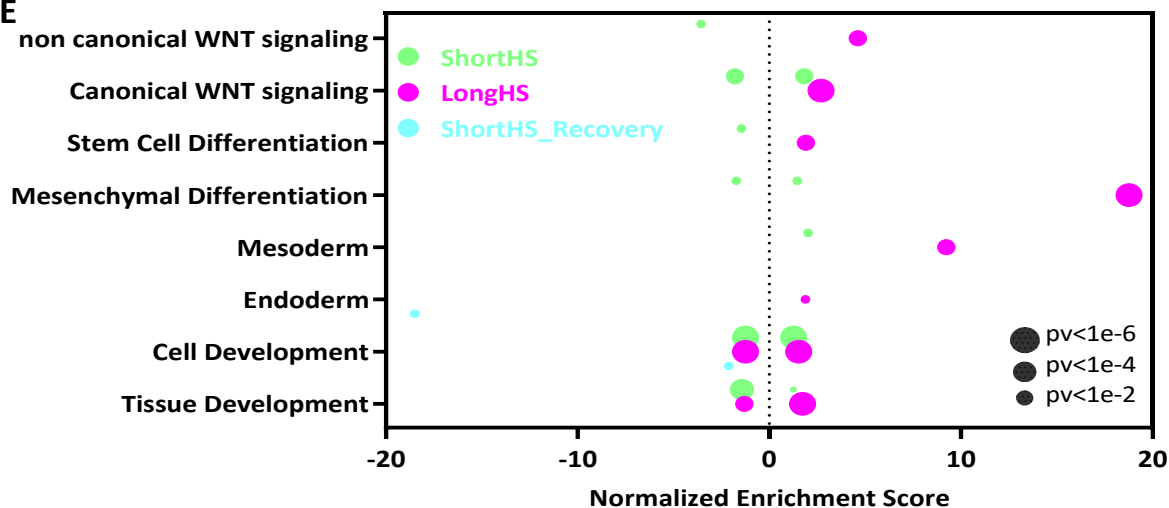
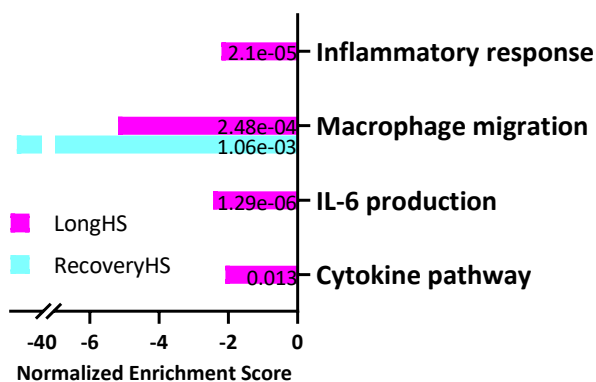
C



D



E

**F**

G

