Hyperosmolar stress induces global mRNA responses in placental trophoblast stem cells that emulate early post-implantation differentiation

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Abstract

Hyperosmolar stress acts in two ways on the implanting embryo and its major constituent, placental trophoblast stem cells (TSC). Stress causes homeostasis that slows development with lesser cell accumulation, increased cell cycle arrest, and apoptosis. Stress may also cause placental differentiation at implantation. To test for the homeostatic and differentiation-inducing consequences of stress, TSC were exposed to hyperosmolar stress for 24hr and tested using whole mouse genome arrays and Real-time quantitative (Q)PCR. At 0.5hr, all 31 highly changing mRNA (>1.5-fold compared with unstressed TSC) decreased, but by 24hr 158/288 genes were upregulated. Many genes upregulated at 24hr were near baseline levels in unstressed TSC, suggesting new transcription. Thus few genes change during the early stress response, but by 24hr TSC have adapted to start new transcription with large gene sets. Types of genes upregulated at 24hr included homeostatic genes regulating growth and DNA damage induced (GADD45β/γ), activator protein (AP)-1 (junB/junC/ATF3/4), heat shock proteins (HSP22/68), and cyclin-dependent kinase inhibitor [CDKI; p15, p21]. But, stress also induced transcription factors that mediate TSC differentiation to trophoblast giant cells (TGC)(Stra13, HES1, GATA-binding2),

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placental hormones [proliferin, placental lactogen (PL)1, prolactin-like peptide (PLP)M], and extracellular matrix genes (CCN1/2). Transcription factors for later placental cell lineages, spongiotrophoblast (MASH2, TPBPα) and syncytiotrophoblast (GCM1, TEF5) and placental hormones (PLPA, PLII), were not induced by 24hr stress. Thus stress induced the temporal and spatial placental differentiation normal after implantation. Although differentiation was induced, markers of TSC stemness such as inhibitor of differentiation (ID)2 remained at 100% of levels of unstressed TSC, suggesting that retained mRNA might mediate dedifferentiation were stress to subside.

Introduction

Peri-implantation stress leads to slower embryo development [1; 2], with accumulation of fewer stem cells through mechanisms of apoptosis and cell cycle arrest in embryos and TSC [3; 4]. In addition, models such as delayed implantation may also be an example of a balanced stress which can cause embryonic stasis and cell cycle arrest [5]. However, shear stress on the hatching blastocyst [6] or cytokine-to-receptor signaling from the uterus to placental cells [7] may initiate placental differentiation [8]. The predominant cell type in the implanting blastocyst is the TSC and a subpopulation of this cell type must differentiate soon after implantation to produce the first placental hormones that mediate the antiluteolytic response needed for survival of the conceptus [9]. Proliferation and differentiation defects in cytotrophoblasts early in the first trimester can lead to diseases of placental insufficiency such as preeclampsia [10].

Hyperosmolar stress can induce yeast undergoing somatic growth to differentiate and produce α-mating type factor that induces development through induction of conjugation structures and sporulation [8]. After hyperosmolar stress was used to clone stress enzymes in yeast, hyperosmolar stress and inflammatory factors and cytokines such as lipopolysaccharides and tumor necrosis factor, respectively, were used to clone stress enzymes in mammalian cells. Hyperosmolar stimulation and inflammation are inducers of stress signaling and can be used to probe prioritized developmental decision-making. Hyperosmolar stress can induce multiple homeostatic changes in the implanting embryo and its constituent TSC [3; 4], but it is not known if hyperosmolar stress modulates developmental programs.

Peri-implantation development is the most susceptible period of mammalian development to embryo loss [11] and embryos in this period are susceptible to sublethal *in vitro* and *in vivo* stress effects leading to post-natal consequences such as hypertension and learning anomalies [12; 13; 14]. During assisted reproductive technology (ART), embryo culture [1; 15] and handling techniques such as pipetting activate stress activated protein kinase (SAPK)[16] and if sustained leads to loss of cell accumulation at lower stress levels and apoptosis at higher levels. In addition, the stress of embryo culture during IVF can lead to changes in placental hormone production in the first trimester [17]. Understanding stress effects on normal and pathophysiologic peri-implantation development is needed to understand how stress may have short-term lethal and long-term sublethal placental consequences.

We report here that hyperosmolar stress induces a time-dependent program of developmental mRNA in TSC consistent with the hypothesis that stress can regulate normal or pathophysiologic development.

Materials and Methods

Reagents

Sorbitol, FGF4, and heparin were from Sigma Chemical Co. (St. Louis, MO). KSOM and KSOM+ amino acids (KSOM+AA) were from Specialty Media (Phillipsburg, NJ). DMEM/F-12, fetal bovine serum, and RPMI1640 were from Gibco (Grand Island, NY). The primary antibody for total JunB (SC73) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and the antibody for actin (CS4967) was purchased from Cell Signaling Technology (Beverly, MA). CCN2 antibodies were described previously [18].

Collection and culture conditions for mouse embryos, culture of TSC

Standard techniques were used for obtaining mouse embryos [19]. Female MF1 mice (4–5 weeks old, Harlan Sprague Dawley, Indianapolis, IN) were super-ovulated, and their embryos were obtained as described previously [1; 6; 15]. Animal use protocols were approved by the Wayne State University Animal Investigation Committee (AIC). In all studies, embryos were equilibrated for 2 hour in KSOMaa and stressed with the 400mM sorbitol for the time period indicated. KSOMaa had ranges of 250–270mOsmol that increased 2.82-fold to 674mOsmol with 400 mM sorbitol. TSC were from Dr. Rossant (Lunenfeld Research Institute, Ontario, Canada). TSC were cultured as described previously [20]. TSC media range from 265–300mOsmol. The change in osmolality caused by sorbitol in all media were measured as described previously [3; 4]. For TSC basal media produced 298mOsmol and the addition of 400 mM sorbitol produced 713mOsmol. This was a 2.4-fold increase.

Microarray experiments

Total RNA was isolated from TSC stressed with 400mM sorbitol for each time point during 24hr in 2–3 independent biological experiments. All amplifications started with 2ug total RNA. For each replicate, 10ug aRNA was fragmented and hybridized to Murine Genome 430.2 gene chips following Affymetrix instructions. Analysis of the raw data was performed with dCHIP, a model-based method for expression analysis. Repeat biological and technical experiments were performed at Stanford and Wayne State (WSU). In comparing the replicates between Stanford and WSU, there was an anticipated time-dependent correlation. Unstressed TSC have biological variability, putatively due to changing fractions of default differentiation, and had the lowest correlation. Correlation increased to the highest level at 24hr of hyperosmolar stress, when biological and molecular responses became the most focused to achieve cellular (homeostatic) and 'organismal' (developmental) survival.

Analysis of microarray data

The microarray data were analyzed using several software packages. D-Chip (2005 Version) (www.dchip.org) was used to normalized the data set [21]. The median intensity from all 11 un-normalized array chips varies between 91 to 122 with P call between 48.1% ~57.1%. The original signals were scaled towards the median intensity array (the default is the one with median overall intensity of 95) in the group at the probe intensity level. Normalization was done by determining the normalization curve with invariant probe sets. The resulting signals, or model-based expression indexes, were the weighted average of PM/MM differences (PM/MM model) of selected probes estimated using a multiplicative model [21]. In our normalization process, those low values were truncated to 100. GeneSpring 7.0

(Silicon Genetics, Redwood City, CA) was used to identify differentially expressed genes and hierarchical clustering. Samples were grouped according to different time points. ANOVA method was used to analysis the difference. In this ANOVA analysis, we used parametric test, variances were assumed equal, and p-value cutoff was 0.05. Since there are 45116 probes in Affymatrix Chips, Benjamini and Hochberg method was used for multiple testing corrections for false discovery rate (FDR). All the significant genes between two groups were filtered with expression level based on 1.2 and 1.5 fold changes.

Gene Ontologies (GO) classification

Differentially expressed genes among different embryo culture conditions, identified by microarray gene expression analysis, were classified into gene ontology groups using GO Tree software [22]. Gene Ontology Tree Machine (GOTM) interprets biological, molecular, and cellular functions of from lists of genes identified in response to TSC stress. GOTM performs a statistical analysis of the GO categories for the input gene list and outputs potential biological areas for further investigation. The differentially expressed genes are classified by their corresponding GO categories, and the observed number of genes in each of these GO categories is recorded. Genes represented on the Affymetrix 430 2.0 chip comprise the reference gene list. The expected number of genes in each GO category corresponds to the number of genes falling into that GO category in the reference gene list. A given GO category is considered enriched when the observed number of genes in that category is greater than the expected number. The GO classifications for stress vs. unstressed conditions were derived, taking into account only non-redundant and specific GO classes for each condition.

Indirect immunocytochemistry and Western blot analysis conditions

Indirect immunocytochemistry was performed as described previously [3; 4; 6]. Photomicrography was done with a Leica DM IRE2 automated epifluorescence microscope (Wetzlar, Germany) controlled by SimplePCI AI software (Compix Inc., Imaging Systems, Cranberry Township, PA). Photomicrographs were formatted using Adobe Photoshop 6.0 (San Jose, CA). FITC intensity measurement and comparison were done with SimplePCI DNN software. SDS-PAGE and Western blots were done as described previously [3; 4].

Real-Time QPCR

TSC were stimulated as for microarray experiments during three independent biological experiments. Primer sequences for were used from PrimerBank Harvard (Wang and Seed, 2003)(Supplemental Table 1). Real-time quantitative (Q)PCR was performed using a SmartCycler Thermal Cycler (Cepheid, Sunnyvale, CA, USA) and carried out with QuantiTect SYBR Green PCR Master Mix (Qiagen), which contained HotStarTaq DNA Polymerase, QuantiTect SyBR Green PCR Buffer, and SYBR Green I. The QPCR reaction mixture contained 1X QuantiTect SYBR Green PCR Master Mix, 0.3uM primer pairs, and 1ul cDNA in a total volume of of 25ul. Melting curves were done by incremental temperature increases of 1.0°C from 60°C to 90°C. These were repeated to ensure that primer-dimers and other nonspecific products had been eliminated. All QPCR measurements were performed in triplicate, with similar results.

Results

Immediate response to hyperosmolar stress at 0.5hr is to irreversibly downregulate a small set of highly changing mRNA transcripts

The kinetics of the TSC hyperosmolar stress response was analyzed by whole mouse genome Affymetrix arrays (430MOE v2). Two or three 400 mM sorbitol stimulation periods

replicated with high correlation, 0.86–0.94 for time points from 0 to 24hr (Supplemental Table 2). Sorbitol at 400mM was chosen because it induces peak SAPK activation in TSC, and activates p38MAPK and AMPK in TSC and embryos at high stimulation indices [[3; 4], unpublished data]. Biological replicates within a single stimulation period had a 0.95 correlation from array series one at 0.5hr and a 0.99 correlation from array series two at 6hr.

Microarray data repeated in array series one and two were normalized for intensity using positive and negative control probes in the array. DCHIP software was used to calculate the significance of changes in intensity for paired time points by two-way ANOVA. The following analyses featured significantly changing genes using 1.5-fold change from unstimulated as a threshold.

By 0.5hr, 100% of significantly changing genes (31 genes) decreased in magnitude (Figure 1A, Supplemental Table 3). The early stress response of TSC is only to downregulate highly-changing mRNA. This is a specific response as >1.5 fold downregulated genes are only 31/17,411 sequences expressed in the unstressed TSC (from a total of 45,101 probes). In contrast to 0.5hr, at 24hr 158/288 highly-changing sequences increased in magnitude (Figure 1B) and 130/288 decreased.

What was the fate at 24hr of the genes downregulated at 0.5hr? The 31 downregulated genes are significantly decreased (p<0.001) at 33.6%, 18.9%, 21.3%, and 52.8% of unstressed levels (mean intensity ratios of 128/381, 72/381, 81/381, and 201/381) at 0.5hr, 2hr, 6hr, and 24hr, respectively (Figure 2, Supplemental Table 3). Although there is a significant drift upwards from 18.9% to 52.8% between 2hr and 24hr (p<.05), the initial downregulation of the 31 genes remains significant through 24hr.

In contrast upregulation occurs slowly with a small, insignificant increase through 6hr (123.5%, mean intensity ratio=270/219, p=0.25), and a high significant increase (303%, mean intensity ratio=663/219, p<0.001) occurring at 24hr. By 6hr, 84% of the 158 genes upregulated at 24hr (Supplemental Table 3) are still expressed at low levels.

The upregulated response at 24hr is homeostatic

What was the quality of the new mRNA synthesis occurring at 24hr? We analyzed the upregulated and downregulated genes at 24hr and the downregulated genes at 0.5hr for the types of functions they might perform. This was done for general cell biological, putatively homeostatic, functions using GOTM software.

The hyperosmolar stress response included many categories of function in the set of the 158 genes upregulated at 24hr. These included regulation of cell cycle, apoptosis, cell signaling pathways, and nuclear function including transcription regulation (Table 1 shows significant categories, Supplemental table 4 shows individual genes). The functional categories in the set of 130 genes downregulated at 24hr include decreased cell cycle commitment, mediators of mitosis, and several changes in macromolecular synthesis (Table 1, Supplemental Table 5). The functions of the set of 31 genes downregulated at 0.5hr included RNA polymerases, processing, and binding proteins and cellular metabolism (Table 1, Supplemental Table 6).

Subsets of genes typical of hyperosmolar stress responses were upregulated in homeostatic gene groups such as adaptor protein (AP)1, growth arrest and DNA damage (GADD)45 and DNA damage inducible transcripts (DDIT), heat shock protein (HSP), and cyclin-dependent kinase inhibitors (CDKI)(Table 2). The function of these genes is to resist stress by stopping the cell cycle and mediating survival.

The upregulated response at 24hr mediates TS cell differentiation

A homeostatic response had been anticipated (Table 2), but an unexpectedly large fraction of the genes upregulated at 24hr were specific to TS cell differentiation (Table 3). Since some other markers for primary and secondary TGC were not significantly changed despite replicate high stimulation indices, we also tested 16 of them by QPCR, rather than repeating a third array time course (Table 3). Transcription factors and endocrine hormones associated with differentiation to primary TGC, such as Stra13, HES1, and PL1 were induced by hyperosmolar stress, as were some secondary TGC genes such as GATA2, proliferin, and PLP-M. Extracellular matrix molecules important in placental differentiation after implantation, CCN1/CCN2, were also induced by hyperosmolar stress. Other secondary TGC markers such as PLPA and PLII were not expressed. The QPCR results confirmed the results from arrays and all 16 genes studied had the same changes with both techniques, although there was some variation in magnitude using the two techniques.

Interestingly, although many TGC markers were induced, many genes specific to TSC were still expressed at nearly the same level and did not change significantly through 24hr of hyperosmolar stress (Tables 4). Extra embryonic development (Eed)/polycombs, Inhibitor of DNA binding (Id)1/2, AP2γ, and cyclin D3 are expressed after 24hr of stress at 81–117% of unstressed cells. Since these genes did not change between 0 and 24hr, the differences were not significant (p>0.05). Typically markers of spongiotrophoblasts and syncytiotrophoblasts arise later during induction of differentiation of TSC *in vitro* and *in vivo*, and markers for these cell types were not induced significantly. Specifically, for spongiotrophoblasts, MASH2 and TPBPa/4311 weren't induced and for syncytiotrophoblasts, GCM1 and TEF5 weren't induced.

A remarkable effect of the 400mM sorbitol is the high amount (76.6+/−6%) of cell death that it causes after 24hr. This is similar to the level of cell death seen in somatic cells under similar sorbitol doses and durations [23]. However, there were very few TGC during the unstressed state (Supplemental figure 1A) and 400mM sorbitol stress suppresses further DNA synthesis in TSC and embryos [3; 4], so no generation of TGC occurred during the 24hr of hyperosmolar stress.

Upregulated mRNA produced upregulated proteins in placental cells and embryos

To follow up the studies on global mRNA expression several genes were studied for protein expression (Figure 3). CTGF/CCN2, GADDβ/γ and p21 were upregulated by hyperosmolar stress in TSC. JunB protein was upregulated by hyperosmolar stress in TSC and embryos. These data suggest that stress-induced mRNA are translated into proteins.

Discussion

Hyperosmolar stress causes differentiation of TSC

Hyperosmolar stress induces the first spatial and temporal differentiation events after implantation. Previous reports of TSC differentiation have used a variety of developmentally relevant stimuli *in vitro* and *in vivo*. These include leukemia inhibitory factor [LIF, [24]], retinoic acid [RA, [25]], diethylstilbestrol [DES, [26]], and the FGF4 removal [27] emulating migration away or loss of contact with FGF4 sources *in vivo* [28]. A surprising finding is how similar stress-mediated differentiation is to these normal stimuli.

Primary TGC arise from the abembryonic pole of the implanting blastocyst from cells that have migrated away from the inner cell mass and the source of FGF4 that maintains TSC in the undifferentiated state [27; 29; 30]. Primary TGC are induced by 24hr of hyperosmolar stress, characterized by the induction of transcription factors Stra13 and HES1 and the

placental hormone PL1. Stra13 is sufficient to override FGF4 and cause differentiation of TSC into primary TGC [31]. Similarly, stress is dominant over FGF4 for inducing PL1 mRNA (shown here) and protein (Zhong et al, submitted).

Hyperosmolar stress induces a subset of markers of early secondary TGC, characterized by the transcription factor GATA2 and the placental hormones proliferin, PLPM, and PLPE. Secondary TGC are those that arise later from a second center of TSC proliferation, the ectoplacental cone. However, stress did not induce the placental hormones PLII or PLPA of secondary TGC [32]. Other later placental lineages including spongiotrophoblasts (MASH2 and TPBPa/4311) and syncytiotrophoblasts (GCM1 and TEF5)[33]. These lineages are the final ones developing at E11.5, when the placenta becomes essential for mouse fetal life [34]. These markers for these three placental lineages are not induced by 24hr of stress or by FGF4 removal [25; 31; 35]. Therefore, hyperosmolar stress does not accelerate differentiation to produce later placental lineages. GCM1 mRNA can be induced within 24hr of FGF4 removal from TSC [31], but is not expressed after sorbitol addition to media containing FGF4. Thus stress may suppress later differentiation events.

In contrast, expression of one marker of differentiation is speeded up during stressed differentiation. PL1 mRNA arises 5 days after FGF4 removal [31], but PL1 mRNA (and protein) is induced by 24hr after sorbitol addition. Thus, stress actively modulates differentiation.

TSC potency under stress appears to be preserved, although signs of differentiation occur. Although, TSC have differentiated into primary and secondary TGC, they maintain levels of Id2 mRNA that are unchanged after 24hr of hyperosmolar stress. Of nine genes investigated five remained at nearly the same level of expression as in unstressed TSC (Eed/107%, Id2/92%, AP2Gamma/94%, CyclinE1/92% and CyclinD3/81%). An additional four genes had decreased to around half the level of expression of unstressed TSC (Cdx2/60%, Eomesodermin/53%, ErrBeta/47%, and CyclinA2/60%). Although, stressed TSC express transcription factors that mediate differentiation to TGC, they still express substantial levels of mRNA transcripts mediating the undifferentiated state. If stress subsided, proteins maintaining reestablishing TSC potency could be translated without new transcription in energy-depleted cells.

The homeostatic response to hyperosmolar stress

The homeostatic genes include ontologic groups that mediate suppression of apoptosis (GADD45β/γ), cell cycle arrest (p15, p21), and heat shock proteins (HSP1a/1b/2/8), and AP1 transcription factors (JunB/C, ATF3/4). The downregulated genes include RNA processing factors that decrease immediately at 0.5hr and remain low through 24hr. Thus the stressed TSC stops growth and prepares for survival.

The stress response is highly dynamic with differences between the rapid changes at 0.5hr and the maturing response at 24hr

A very small specific number of 31 mRNA change highly at 0.5hr and the change is all downregulation. By 24hr, over half of the highly changing mRNA are upregulated. These genes are developmental and homeostatic.

Hyperosmolar 400mM sorbitol causes an 80% decrease in new translation in somatic cells after 0.5hr [36]. Although translation was not measured here, there were no new highly upregulated mRNA at 0.5hr in TSC stressed by 400mM sorbitol. Of the 158 mRNA highly upregulated at 24hr, these were still not significantly upregulated at 6hr. Thus, stressed TSC respond to stress for many hours in a post-transcriptional, and probably post-translational mode.

Stress enzymes and their substrates are modified to stress-induced function by phosphorylation and change in cell location [23]. For example, SAPK is phosphorylated and localized to the nucleus in mouse preimplantation embryos and TSC, and human first trimester placental cell lines [1; 3; 4; 15]. This suggests that SAPK may be responsible for new transcription of homeostatic and developmental transcripts.

Approximately 3/4th of the cells died during 24hr of 400mM sorbitol and the function of the persistent cells needs clarification. The die off at 24hr is seen in adult somatic cells exposed to similar levels of sorbitol [37; 38]. The initial die off is followed by establishment of a persistent cell population that can actually increase in cell number from 48–72hr. We have also seen that 22.4+/−6.1% of TSC persist at 48hr of 400mM sorbitol after 23.8+/−4.4% persisted at 24hr (Zhong et al, submitted). Also, nearly 100% of TSC stressed for 24hr express PL1 protein, suggesting nearly total differentiation. Differentiation in persistent cells could result for two reasons. One is that the differentiated state allows a more efficient response to hyperosmolar stress during culture. Alternately, differentiation may emulate the organismal survival response by TSC as a model for the stressed implanting embryo. This second hypothesis is supported by observations that the stress enzymes maintain HAND1 protein and decrease ID2, and the two stress enzymes are required to upregulate maternal-produced placental lactogen (PL)1 (Zhong, manuscript submitted). PL1 and proliferin are not known to have effects on the placenta or TSC themselves and would not be part of survival program for the TSC. Thus for stress that kills most TSC, surviving stressed TSC differentiate as part of an organismal survival strategy.

The high cell loss after 24hr at 400mM sorbitol also suggests the possibility that differentiation could be an artifact of survival of stress by a fraction of differentiated TGC existing before stress. Several lines of evidence argue against this. First, no increase in TGC was seen in micrographs of stressed TSC prior to mRNA preparation. Second, many mRNA mediating differentiation were at background levels in unstressed cells. Third, 400mM causes 100% of TSC to express PL1 protein (Zhong et al., submitted). Thus, hyperosmolar stress causes differentiation rather than selecting for a differentiated subpopulation.

Placental hormones mediating maternal signaling are important during stress. For example, PLPA is essential for embryonic survival if the gestational female is placed under transient hypobaric hypoxia [39]. Thus placental hormones induced by hypoxic stress mediate a survival strategy.

Sorbitol was used here because it activates stress and stress enzyme responses in all cell types and embryos, because stress enzymes were cloned using hyperosmolar stress, and this continues as the standard stressor for enzymologists [8]. However, sorbitol and other polyols may play a significant normal role during first trimester placentation under hypoxic conditions [40]. But, the level of sorbitol used as a stressor here is about three orders of magnitude higher than the levels detected during human placental development. suggesting that stress and stress enzyme function are the primary consequences of sorbitol. Using lower stress doses and other stressors like TNFα [that activate SAPK similarly to sorbitol [7]] is an important future area of research.

The studies here did not test for dependency of the induction of developmental and homeostatic genes on stress enzymes. Stress enzymes such as SAPK and AMPK have overlapping and distinct functions during the stress response of mouse embryos and the stem cells derived from them [8; 23]. It will be important to test whether stress enzymes regulate homeostatic and developmental genes. It will also be important to test for the induction of differentiation of TSC under lower hyperosmolar stresses that do not produce apoptosis, but

may reflect lower levels of stress caused by IVF, malnutrition, or exposure to toxins such as cigarette smoke.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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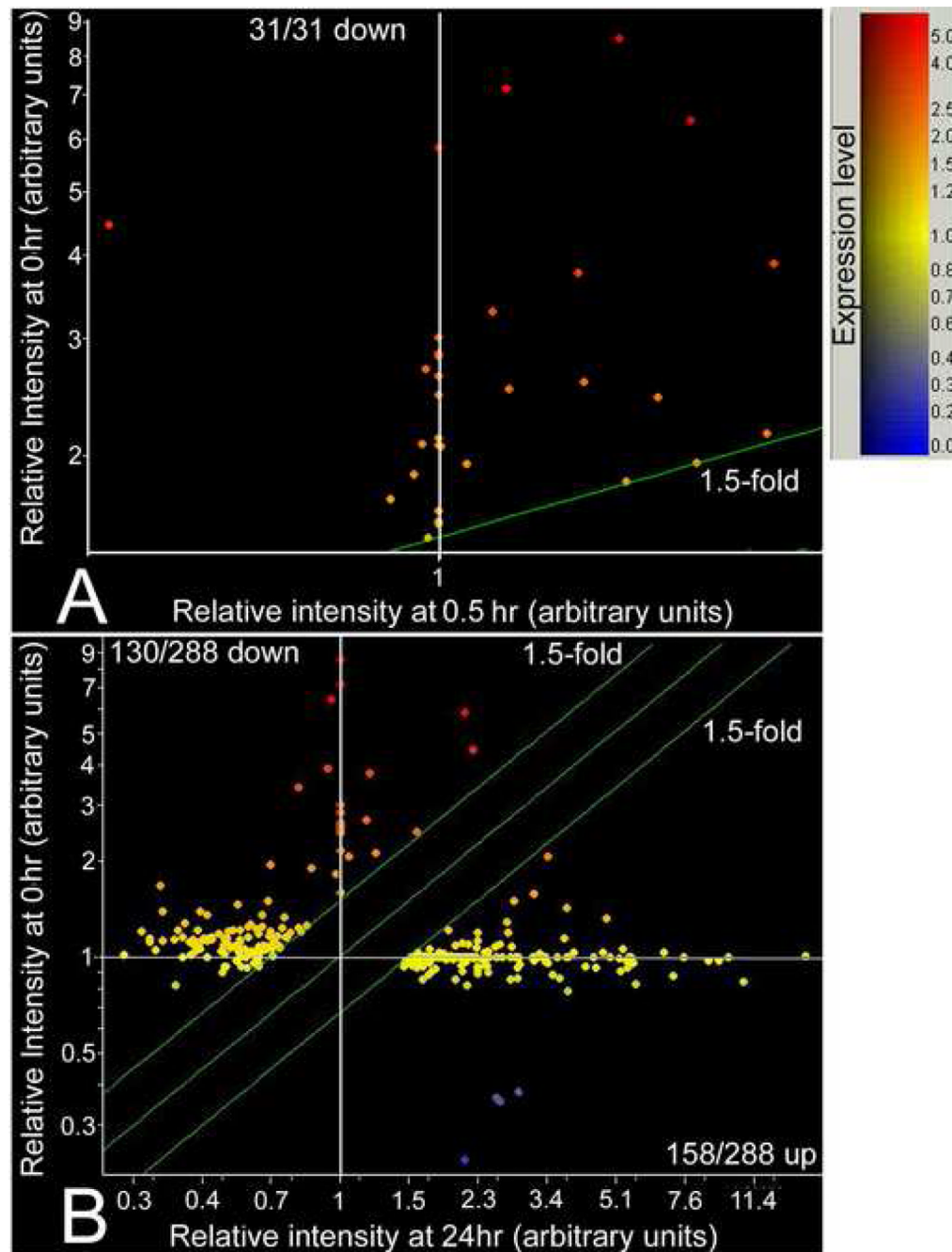
Figure 1.
Scatter plots of highly downregulated genes at 0.5hr and a mix of downregulated and upregulated genes at 24hr. Scatter plots of 45,101 genes on Affymetrix 430 MOE v2. Microarray comparing gene expression in unstressed and stress TSC. Microarray data were analyzed by ANOVA statistics. The combined results of two series of hybridizations identified 31 downregulated genes at 0.5hr (>1.5fold change comparing stressed with unstressed TSC, $p<0.05$) and 130 downregulated and 158 upregulated genes at 24hr of 400 mM sorbitol.
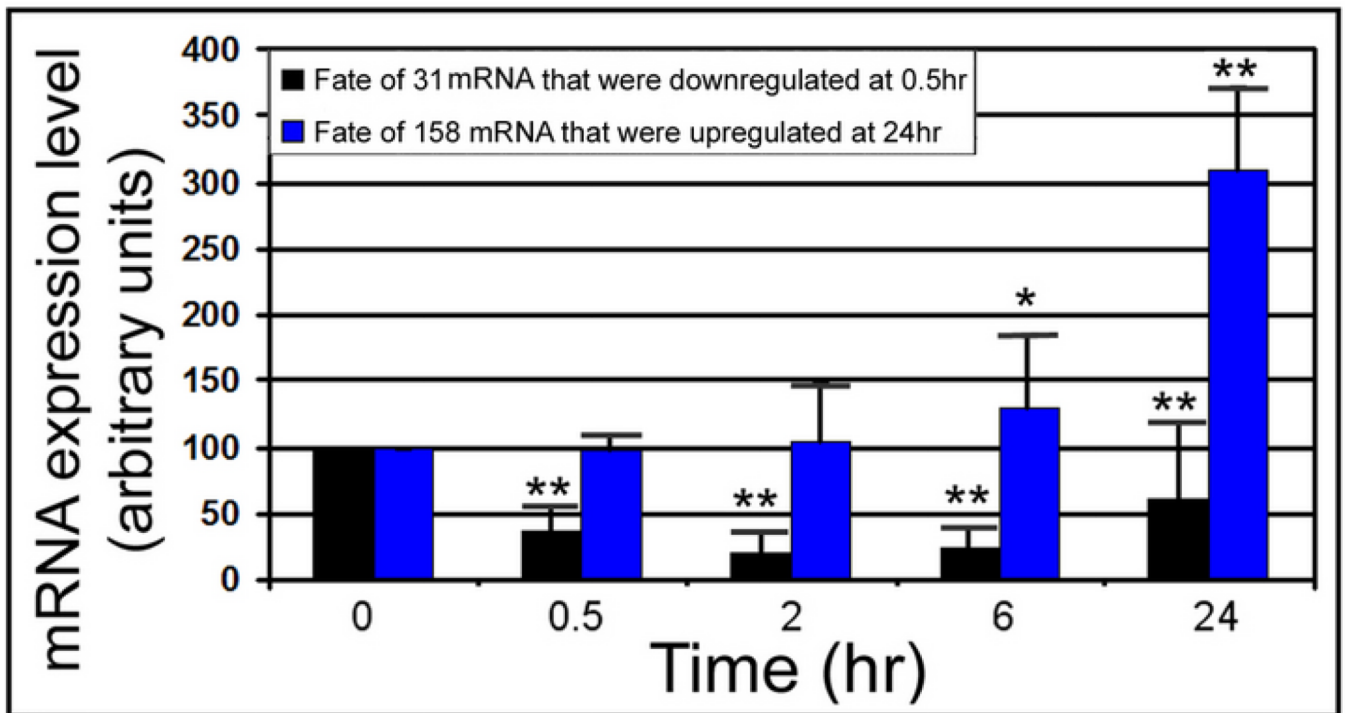
Figure 2.
Of highly changing genes, downregulated genes decrease rapidly and remain significantly lower than unstressed and upregulated genes increase slowly, becoming significantly higher by 24hr. Time courses of the 31 genes downregulated at 0.5hr and 158 genes upregulated at 24hr are displayed in a histogram series. ** Signifies highly significant change (p<0.001), and * signifies significance (p<0.05). Y error flags show standard errors of average intensity of the group.
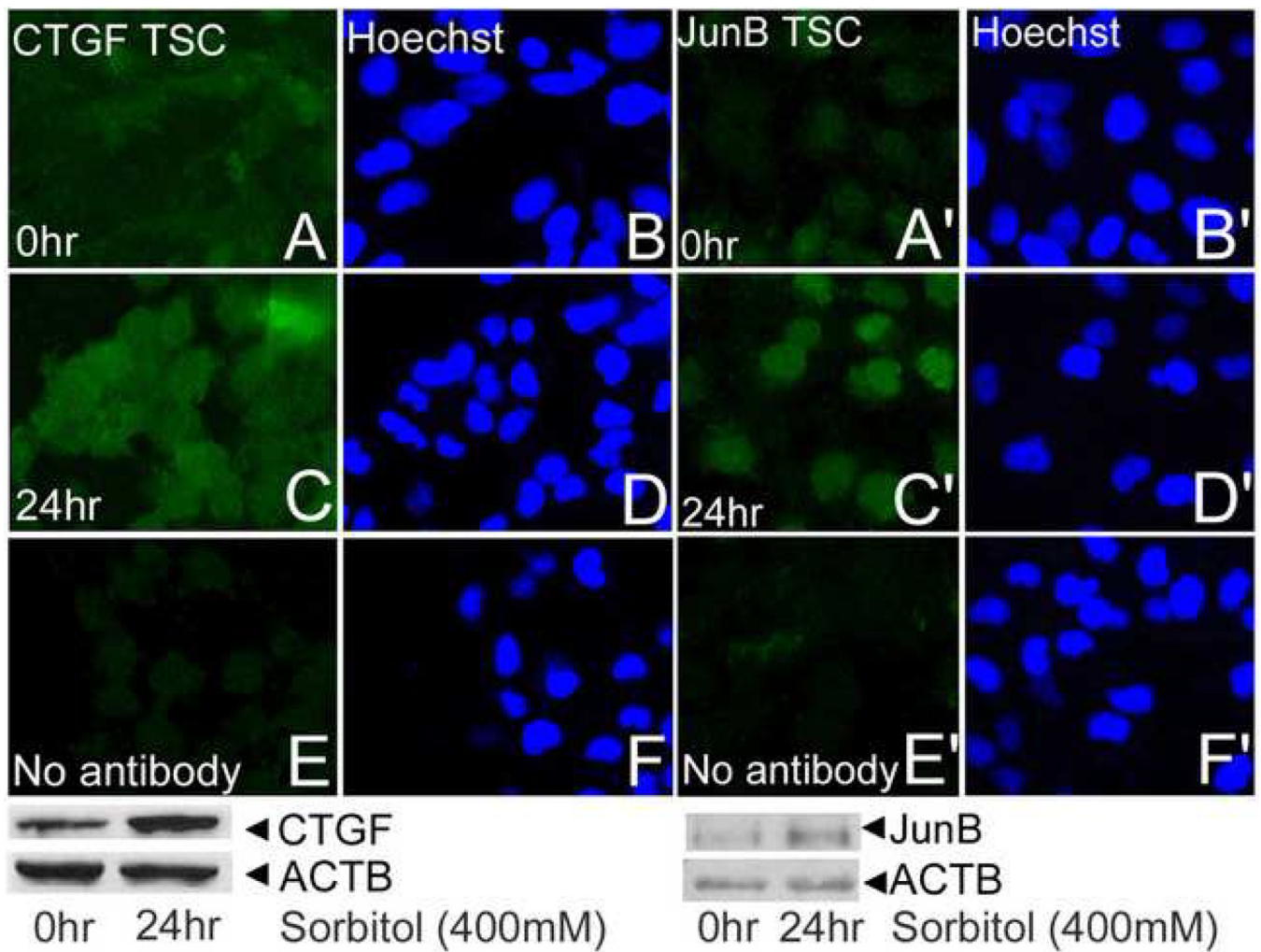
Figure 3.
Two proteins, CTGF/CCN2 and JunB, increase similarly to the increase in their mRNA transcripts 24hr. TSC were cultured without stress (A, B; A', B'), for 24hr with stress (C, D; C', D') and probed with antibodies to CTGF/CCN2 or JunB as indicated. TSC were cultured for 24hr of 400mM sorbitol and then probed with no first antibody (E, F; E', F'). Paired micrographs in the first and second and in the third and fourth columns show protein immunofluorescence and Hoechst-stained nuclei, respectively. At the bottom of each micrograph series is an immunoblot derived from TSC cultured without stress or after 24hr of 400mM sorbitol. Immunoblots were probed with antibodies to CTGF/CCN2 or JunB and then reprobed with antibodies to Actin (ACTB) as a loading control. Bands for CTGF/CCN2 and JunB are both detected at the correct size of 38kDa.

Table 1

Ontology of upregulated and downregulated genes modulated by hyperosmolar stress

A. Gene ontology: up-regulated 158 mRNA at 24hr compared to 0hr.

1. Protein kinase cascade: p=0.0093 (3), 1B. Activation of MAPKK activity: p=0.0005 (2)

2. Regulation of enzyme activity: p=0.0066 (2), 2B. Negative reg. of protein kinase activity: p=0.0012 (3)

3. Regulation of cell cycle: p=0.0002 (5), 3B. Negative reg. of cell cycle progression: p=0.0007(5)

4. Apoptosis: p=0.0036 (6), 4B. Negative regulation of apoptosis: p=0.0087 (4)

5. Reg. of transcription: p=0.0006 (23), 5B. Positive reg. of transcription, DNA-dependent: p=0.0095 (4)

6. Cell differentiation: p=0.0064 (10)

7. Response to heat: p=5.1735E-05 (4)

8. Organ development: p=0.0099 (8). 8B. Blood vessel development: p=0.0009 (4)

9. Protein dimerization activity: p=0.0019 (4)

10. Nucleic acid binding: p=0.0039 (29), 10B. Single-stranded DNA binding: p=0.0050 (2)

11. Transcription reg. activity: p=0.0002 (4), 11B. Transcription factor activity: p=0.0009 (12)

11C. Transcriptional repressor activity: p=0.0004 (5)

12. Inosine kinase activity: p=0.0001 (2)

13. Polyphosphate-glucose phosphotranferase activity: p=0 (2)

14. Cyclin-dependent protein kinase inhibitor activity: p=0.0018 (3)

15. Nucleus: p=0.0029 (38), 15B. Transcription elongation factor complex: p=2.6779E-21 (6)

16. Nucleolus organizer complex: p=0.00001 (2)

B. Gene ontology: down-regulated 130 mRNA at 24hr compared to 0hr

1. Germ cell development: p=0.0064 (2)

2. Chromosome segregation: p=0.0010 (3)

3. Cell cycle: p=6.9944E-05 (7), 3B. M phase of mitotic cell cycle: p=0.0005 (5)

4. Cell organization and biogenesis: p=0.0076 (10), 4B. Mitotic chromosome condensation: p=0.0016 (2),

4C. Telomere maintenance: p=0.0070 (2), 4D. mRNA export from nucleus: p=0.0077 (2)

5. Cell division: p=9.3031E-06 (8)

6. Nucleic base, nucleoside, nucleotide metabolism: p=0.0018 (20), 6B. mRNA processing: p=0.0005 (6)

7. Macromolecule metabolism: p=0.0093 (24), 7B. DNA replication: p=0.0036 (4)

8. Response to DNA damage stimulus: p=0.0033 (5)

9. Phospholipid binding: p=0.0080 (3)

10. Nucleic acid bind p=4.5E-06 (5), 10B. DNA bind p=0.0094 (18), 10C. RNA bind p=8.37E-06 (12)

11. Nucleotide binding: p=0.0002 (7). 11B. ATP binding: p=0.0043 (15)

12. Transferase activity, phosphorus-containing groups: p=0.0024 (8), 12B. Polyphosphate-glucose phosphotransferase activity: p=0.0001 (7).

13. Chromosome: p=1.2486E-07 (11)

14. Cytosol: p=0.0016(7)

15. Nucleus: p=8.1541E-07 (39)

16. MHC protein complex: p=0.0019 (2).

17. DNA topoisomerase IV complex: p=0 (2)

18. Transcription factor TFIID complex: p=0.0049 (2)

C. Gene ontology: down-regulated 31 mRNA at 0.5hr compared to 0hr

1. Nucleo base, nucleoside, nucleotide metabolism: p=0.0054 (3), 1B. RNA processing: p=0.0010 (3)

2. RNA binding: p=0.0002 (4)

3. Intracellular components: p=0.0052 (7), 3B. RNA polymerase complex: p=3.9866E-05 (2)

Table 2

Transcriptional changes induced by hyperosmolar stress in selected homeostatic genes

Functional Group Genes	Ratio of expression; 24hr stress/T0 (unstressed)				Accession number
	0hr (Arbitrary units)	24hr	T24/T0	P	
CDKI					
p15	118	283	2.4	0.004	AF059567
p21	268	1443	5.4	0.03	AK007630
GADD45 and DDIT					
GADD45β	341	1614	4.7	0.002	AI323528
GADD45γ	133	1687	12.7	0.007	AK007410
DDIT3(CHOP10)	100	529	5.3	0.0004	NM_007837
DDIT4	484	1133	2.3	0.022	AK017926
HSP					
HSP22/Hspb8	325	1079	3.3	0.033	AF250139
HSP68/Hspa1a	479	2142	4.5	5.81E-05	AW763765
HSP70.1/Hspa1b	100	518	5.2	0.01	M12573
HSP70.2/Hspa2	100	208	2.1	0.0003	BC004714
AP1					
JunC	210	1243	5.9	0.04	NM_010591
ATF3	159	736	4.6	0.019	BC019946
ATF4	822	1561	1.9	0.023	M94087
Msx2/Hox8	280	949	3.4	0.03	NM_010261

Table 3

Transcriptional changes induced by hyperosmolar stress after 24hr. Assayed by microarray and Real-Time quantitative PCR (QPCR).

Functional Group	Ratio of expression; 24hr stress/T0 (unstressed)					
	Microarray			Real-time QPCR		
Genes	0hr	24hr	T24/T0% (P value)	T24/T0 (P value)	Accession Number (QPCR)	
	(Arbitrrary units)					
Developmental						
TS cell						
Id2	2604	2401	0.9(1)	1.0(0.7)	NM_010496	
Cdx2	630	359	0.6 (1)	0.1 (0.02)	NM_007673	
TGC primary						
STRA13	346	1698	4.9 (0.002)	3.0 (0.001)	NM_011498	
HES1	158	395	2.5 (0.01)	2.2 (0.02)	BC018375	
PL1	100	160	1.6 (0.008)	3.2 (0.003)	BB414739	
TGC secondary						
PLF	108	930	8.6 (0.0005)	4.6 (0.0002)	X75557	
PLP-M	100	401	4.1 (0.004)	10.0(0.003)	NM_019991	
GATA2	655	1574	2.4 (0.02)	3.7 (0.008)	AK004675	
ECM						
CTGF/CCN2	228	1912	8.4 6.04E-05	7.3 (0.005)	NM_010516	
CYR61/CCN1	795	2753	3.5 (0.005)	3.3 (0.004)	NM_010217	
Homeostatic						
GADD45Y	133	1687	12.7(0.0001)	5.9 (0.0003)	AK007410	
HSP68	479	2142	4.5 (0.004)	5.7 (0.0005)	M12573	
AP1 family						
ATF3	210	1243	5.9 (0.02)	3.9 (0.007)	BC019946	
JunC	159	736	4.6 (0.006)	4.4 (0.02)	NM_010591	
JunB	155	483	3.1 (0.0004)	2.7 (0.03)	NM_008416	
Fos-like 2	100	536	5.4 (0.007)	3.1 (0.01)	BM245170	

Table 4

Lack of significant transcriptional changes induced by sorbitol are detected for some markers of pluripotent TSC, primary and secondary TGC, spongiotrophoblasts, and syncytiotrophoblasts."

Functional Group	Ratio of expression; 24hr stress/T0 (unstressed)				
Genes	**0hr** (Arbitrary units)	**24hr**	**T24/T0** (%)	**P value**	**Accession number**
TSC					
Cdx2	630	359	0.6	1	NM_007673
Eomesodermin	1218	649	0.5	1	BB128925
ErrBeta	478	226	0.5	0.22	BB386239
Cyclin A2	1200	714	0.6	1	X75483
Cyclin B1	2159	1351	0.6	0.68	AU015121
Eed	905	965	1.1	1	U97675
Id1	244	285	1.2	1	U43884
Id2	2604	2401	0.9	1	NM_010496
AP2γ	1676	1576	0.9	1	BB550860
Cyclin D3	749	607	0.8	0.08	NM_007632
TGC primary					
HAND1	1607	1581	1.0	1	U43714
PL1	100	160	1.6	1	BB414739
TGC secondary					
PL-II	100	100	1.0	1	NM_008865
PLP-A	100	100	1.0	1	
Spongiotropoblast					
MASH2	100	100	1.0	1	NM_008554
TPBPa/4311	100	100	1.0	1	NM_009411
Flt1	117	307	2.6	0.16	D88690
Placenta specific 8	131	473	3.6	0.15	AF263458
Syncytiotrophoblast					

Functional Group					
	Ratio of expression; 24hr stress/T0 (unstressed)				
Genes	**0hr (Arbitrary units)**	**24hr**	**T24/T0 (%)**	**P value**	**Accession number**
GCM1	100	100	1.0	1	NM_008103
TEF5	100	100	1.0	1	NM_011566
CXCR4	100	166	1.7	1	D87747
Dlx3	100	100	1.0	1	U79738