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Endoplasmic Reticulum Stress Signals in Defined Human Embryonic Stem Cell Lines and Culture Conditions

Miguel Angel Blanco-Gelaz · Beatriz Suarez-Alvarez · Gertrudis Ligero · Laura Sanchez · Jose Ramon Vidal-Castiñeira · Eliecer Coto · Harry Moore · Pablo Menendez · Carlos Lopez-Larrea

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Abstract Human embryonic stem cells (hESCs) are especially resistant to several cellular stresses, but the existence and induction of Endoplasmic Reticulum (ER) stress by culture conditions are unknown. Using qPCR, here, we investigated the behavior of the principal sensors of ER stress and their relation with the feeder layer, the type of conditioned media used in feeder free systems and the upregulation of several differentiation markers. We observed the preservation of pluripotency, and detected differential expression of differentiation markers in HS181 and SHEF1 hESCs growing on Adipose-derived mesenchymal stem cells (ASCs) and feeder-free system with different conditioned media (HEF-CM and ASC-CM). Taken together, these results demonstrate evidence of ER stress events that cells must resolve to survive and maintenance of markers of pluripotency. The early differentiation status defined could progress into a more differentiated state, and may be influenced by culture conditions.

Keywords hESCs · Conditioned media · ER stress · Feeder-free culture system

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M. A. Blanco-Gelaz · B. Suarez-Alvarez · J. R. Vidal-Castiñeira · C. Lopez-Larrea (✉)
Histocompatibility and Transplantation Unit,
Hospital Universitario Central de Asturias,
33006 Oviedo, Spain
e-mail: inmuno@hca.es

G. Ligero · L. Sanchez · P. Menendez
Andalusian Stem Cell Bank, Instituto de Investigación Biomédica,
University of Granada,
Granada, Spain

E. Coto
Department of Molecular Genetics,
Hospital Universitario Central de Asturias,
Oviedo, Spain

H. Moore
Centre for Stem Cell Biology, University of Sheffield,
Western Bank,
Sheffield, UK

E. Coto · C. Lopez-Larrea
Red de Investigacion Renal—REDinREN,
Instituto de Salud "Carlos III",
Madrid, Spain

E. Coto · C. Lopez-Larrea
Fundacion Renal "Iñigo Alvarez de Toledo",
Madrid, Spain

Introduction

The capacity of Embryonic Stem Cells (ESCs) for self renewal and differentiation into any cell type requires competent repair systems to ensure genomic stability with a low mutational rate over multiple generations and powerful mechanisms of defense against multiple cellular stresses. In fact, mouse and human ESCs exhibit higher stress tolerance and potent responses than differentiated cells against oxidative and UV-induced DNA damage [1, 2]. However, the response of ESCs to other stress triggers which involve DNA damage and apoptosis in final steps is unknown. Endoplasmic Reticulum (ER) chaperones are efficient sensors that contribute to signaling and transcriptional regulatory pathways in differentiated cells by modulating cellular processes via protein conformational and protein complexation state [3]. Over one-third of all newly synthesized polypeptides in eukaryotes interact with the membrane or luminal space of the ER. This interaction is

essential for folding, post-translational modification, and assembly of these proteins, but sometimes protein biosynthesis becomes inefficient or is aborted [4]. Similarly, excessive production of specific proteins can induce their accumulation in the ER and activate a pathway called the Unfolded Protein Response (UPR). UPR is closely related to another pathway triggered during ER stress, ER-associated degradation (ERAD). Both adaptive responses regulate the transcription and activation of some factors (transcription factors and chaperones) which can protect the cell from apoptotic processes and finally dying [5, 6]. We proposed that distinct culture conditions may activate specific genes involved directly in the transcriptional and apoptotic branch of Unfolded Protein Response and ER-associated degradation. The most sensitive sensors of ER-anomalies related with protein overload are Immunoglobulin heavy chain binding protein (BIP/GRP78/HSPA5), Activating Transcription Factor 6 (ATF6) and the atypical spliced form of X-box binding protein (XBP1s). These sensors continuously monitor the ER environment and activate downstream signals to avoid protein overload and cellular exhaustion.

From the first established stem cell line obtained from the inner cell mass of mouse blastocysts, and later from primates and human blastocysts, one of the major, accomplished technical feats was establishment of the optimal culture conditions that avoid differentiation and maintain their self-renewal status [7–9]. Indeed, in view of the therapeutic potential of human embryonic stem cells (hESC), efforts have focused on development of defined synthetic media to support the growth, expansion and the self-renewal properties without the use of animal products, feeder cells, MEF or human embryonic fibroblast (HEF)-conditioned media [10, 11]. Other developments recently described have been focused about the use of human derived-extracellular matrix components to reduce the exposure to feeder layers and animal ingredients [12]. The differentiation of the hESC in culture is one of the principal challenges faced by the cell biologists. To obtain specific xenobiotics-free culture media and feeder layers, several laboratories have studied the effect of feeders derived from their own hESC [13], the CD105+/CD24 hESC-derived mesenchymal stem cell line, HuES9.E1 [14], human marrow stromal cells [15] and adult human mesenchymal stem cells derived from various tissues [16]. Adipose tissue is a well-defined human tissue, easily obtained and with a great potential of utilization in regenerative medicine of the future [17, 18]. Adipose-derived stem cells (ASC) have been phenotypically and functionally characterized and, at present, are being used for tissue engineering successfully [19–22].

The general hallmark of self-renewal in hESC, and the sufficient and necessary transcription factors that maintain the pluripotent state have been described [23, 24]. However, most previous studies examined hESC-derived embryoid bodies (hEB) and their differentiation events elicited in long-term culture without passage. In addition, hESCs maintained in MEF-conditioned media differentiate into autologous hESC-derived fibroblast-like cells (hdFs) to support hESCs culture homeostasis by releasing IGFII and TGFβ in response to bFGF [25]. These differentiation events collaborate to maintain the self-renewal status and survival. However, the "potential status of differentiation" in hESC cultures grown on Matrigel and other feeder layers has not been reported.

We hypothesize that the gene expression profiles of hESCs (HS181 and SHEF1) grown on Matrigel and ASC could be different. We chose quantitative real-time PCR and real-time PCR array (qPCR array) to evaluate the activation state of several ER stress sensors and the transcription profile of selected embryonic stem cell differentiation/lineage markers in two hESC (HS181 and SHEF1) growing on Matrigel and ASC compared with basal level on feeder layers where they were derivate (HEF). A comparative analysis of the activation state of ER stress sensors suggests their potential relationship with growth on Matrigel and ASC and their lineage specificity.

Materials and Methods

Maintenance of hESCs

Human ESC lines HS181 and SHEF1 (kindly provided by Prof. Outi Hovatta, Karolinska Institute, Sweden and Prof. Harry Moore and Peter Andrews, University of Sheffield, UK, respectively) were maintained in a feeder-free culture over Matrigel-coated T25 flask (BD Biosciences) in ASC conditioned media (ASC-CM) or HEF-CM. The basal media used to prepare de conditioned media consisted of 80% KO-DMEM supplemented with 20% KO serum replacement, 1% non-essential amino acids, 1 mM L-glutamine, 0.1 mM β-mercaptoethanol and 8 ng/ml of bFGF (all from Invitrogen, CA). ASC-CM and HEF-CM were prepared and collected as described in detail for mouse embryonic fibroblast conditioned media [10, 26]. Human fetal fibroblasts were purchased from ATCC (SCD-1112SK). Human mesenchymal stem cells were obtained from post-natal adipose tissue from healthy donors upon informed consent as described previously [27, 28]. During routine maintenance, HEF and ASC were grown in IMDM and advanced-DMEM respectively, plus 10% FCS and 2 mM L-glutamine and split in a ratio of 1:2 when they reached 80–90% confluence. ASC were fully characterized and showed typical fibroblast-like morphology, immunophenotype ($CD44^+$, $CD90^+$,$CD73^+$,$CD105^+$,$CD45^-$, $CD34^-$, $CD14^-$, HLA-DR^-) and in vitro differentiation capacity into

osteoblasts, chondrocytes and adipocytes [29, 30]. HS181 hESC line was maintained onto HEF for 82 passages and ASC for 13 passages, and was fed with ASC-CM and HEF-CM for 6 passages. SHEF1 hESC line was on HEF for 64 passages, ASC for 8 passages, and ASC-CM and HEF-CM for 5 passages. Morphology of undifferentiated hESC colonies were maintained as in those cultures on feeder cells (Fig. 1).

RNA Isolation and cDNA Synthesis

Total RNA from hESCs in different culture conditions was isolated using TRIZOL reagent (Invitrogen. CA).

For quantitative real-time PCR experiments, equal amounts of total RNA (3 µg) were reverse transcribed in triplicate from each cell line and each culture condition described above by using RT^2 First Strand Kit (SA Biosciences), according to the manufacturer's protocol for qPCR array. One cDNA sample was used for reference gene selection and endoplasmic reticulum stress (ER stress) qPCR analysis, and two samples for qPCR array in duplicate.

qPCR Analysis

Each quantitative PCR reaction used 30 ng of cDNA, 1 µl of PrimerMix and SYBR Green PCR Supermix 2× (BIO-RAD) in a final volume of 25 µl according to its instructions. The assays were performed in duplicate including blank control (without cDNA) and retrotranscription control (cDNA synthesis without RNA). Product identity was confirmed by a single pick in the melt curve and a single band in agarose gel.

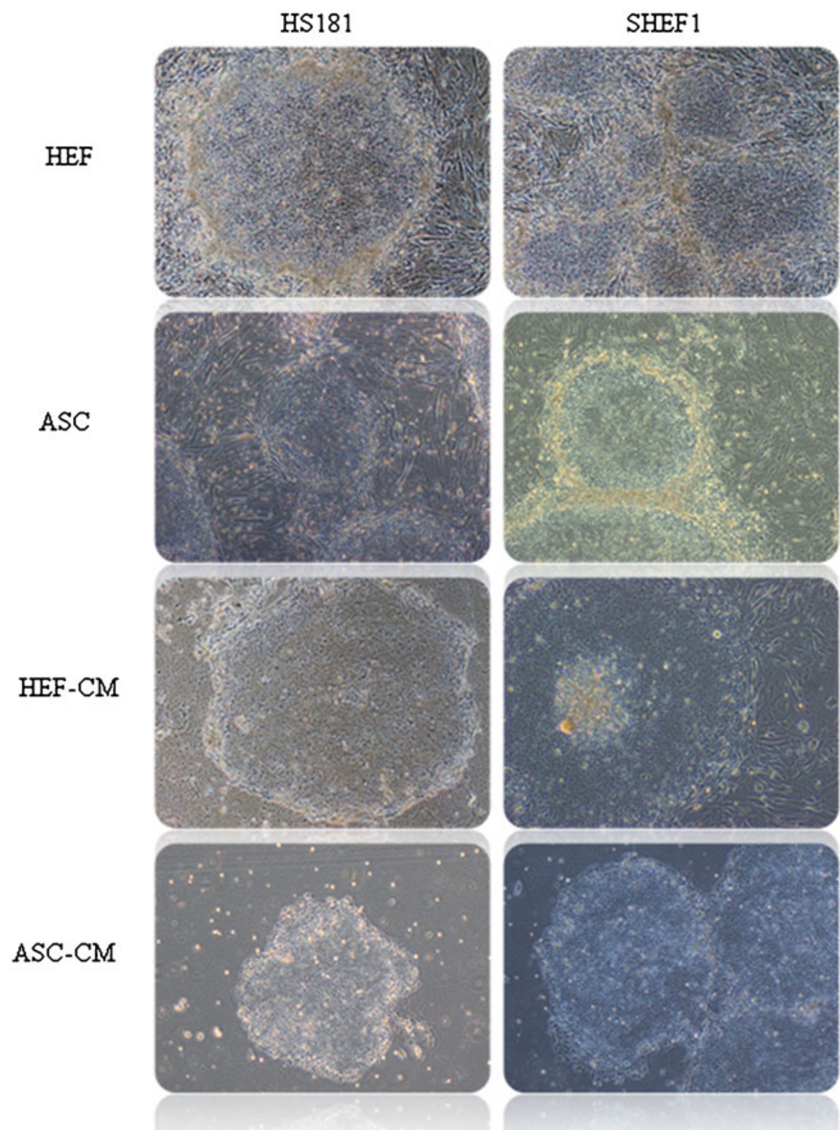

Fig. 1 Morphology of undifferentiated hESC HS181 and SHEF1 colonies. hESC were grown onto human embryonic fibroblasts (HEF), adipose-derived mesenchymal stem cell (ASC), conditioned media derived from HEF (HEF-CM) and conditioned media derived from ASC (ASC-CM), as described in Materials and Methods

Reference Gene Selection

We performed qPCR analysis of twelve reference genes using the Human Endogenous Control Gene Panel supplied by TATAA Biocenter (Goteborg, Sweden) to obtain the most stable gene for each hESC in the aforementioned conditions. The genes tested were: GADPH, TUBB, PPIA, ACTB, YWHAZ, RRN18S, B2M, UBC, TBP, RPLP, GUSB and HPRT1. The included *GenEx Light* software (*NormFinder* software) [31] calculated each gene's expression stability value in the aforementioned samples and ranked them for identification as the optimal reference genes among the set of candidates.

Endoplasmic Reticulum Stress Analysis

We analyzed 5 genes involved directly in the transcriptional and apoptotic branch of Unfolded Protein Response (ATF6, XBP1s, BIP, CHOP) and ER-associated degradation (HERP) to study the cellular response to ER-overload using an analogous qPCR reaction in duplicate with the following primer pairs: XBP1s For 5′CGG AAC CCA AGG GGA ATG AA3′, XBP1s Rev 5′CTG CAC CTG CTG CGG ACT3′, CHOP For 5′TTG CCT TTC TCC TTC GGG AC3′, CHOP Rev 5′GCT CTG GGA GGT GCT TGT GA3′; HERP For 5′CTG GGA AGC TGT TGT TGG3′, HERP Rev 5′CAT GTA GTA CTG TCG TGC3′; BIP For 5′ AAG ACA AGG GTA CAG GGA AC3′, BIP Rev 5′CTT TCC AGC CAT TCA ATC TTT TC3′; ATF6 For 5′TTG CTG GGG GAG TCA CAC3′ and ATF6 Rev 5′TGG ACT AGG GAC TTT AAG CC3′. The assays were performed by duplicate including blank control (without cDNA) and retrotranscription control (cDNA synthesis without RNA). PCR efficiencies were calculated from a standard curve of serially diluted cDNA reverse transcribed from RNA of untreated HeLa cells and ER-stressed HeLa cells by 1 h incubation with 1mM DTT. Controls and product identity were performed as described above.

Quantitative Real-Time PCR Array Assay

Quantitative PCR array was performed using RT² Profiler PCR Array-Human Embryonic Stem Cell (SA Biosciences) according to the manufacturer's protocol. This qPCR array analyzes 84 genes involved in the maintenance of pluripotency and differentiation markers to monitor the early events of hESC differentiation. The design criteria of PCR array assay ensured that each qPCR reaction generated single, gene-specific amplicons and prevented the co-amplification of non-specific products. cDNA (30 ng) from each test condition was mixed with the adequate qPCR Master Mix specifically designed for BIO-RAD MyIQ thermocycler in duplicate following manufacturer's instructions. Ct analysis was performed with on-line software provided by SA Biosciences for each condition. The ΔCt, $2^{(-\Delta Ct)}$, fold of change and fold of regulation for each of the 84 genes were obtained.

Results

Reference Gene Selection

Quantitative real-time PCR was used to measure the RNA transcription level of 12 genes included in the Human Endogenous Control Gene Panel described in Materials and Methods in hESC HS181 grown under the following conditions: (1) on human fibroblast (HEF); (2) on adipose-MSC (ASC); (3) on Matrigel in human fibroblast-conditioned culture media (HEF-CM) and (4) on Matrigel with adipose-MSC—conditioned culture media (ASC-CM). Analogous studies were performed with the SHEF1 cells (HEF, ASC, HEF-CM and ASC-CM). The expression of the genes was analyzed by the software NormFinder. As shown in Fig. 2, our results indicated the clear need of this type of analysis to define the reference gene for each hESC for later qPCR array analysis. Variations among the genes analyzed for each cell line and culture condition were ordered according to their stability values (SD) for graphical representation. The most stable single reference gene in HS181 was YWAHZ with a SD of 0.07 and the least stable was B2M with a SD of 1.07. In SHEF1, GADPH was the most stable single gene (SD=0.15), and ACTB the least stable single gene (SD=1.04). In conclusion, YWAHZ and GADPH were the most stable reference genes for the hESCs HS181 and SHEF1 respectively, and were used for normalization in comparative analyses of mRNA expression levels in these hESC.

Endoplasmic Reticulum Stress Analysis

The status of ER stress genes in HS181 and SHEF1 cell lines were compared under the different culture conditions by normalizing to their reference gene YWAHZ and GADPH. As shown in Fig. 3, the mRNA levels of ATF6, BIP and XBP1s vary in defined conditions in comparison to HS181 and SHEF1 grown on the original feeder layer (HEF). For these experiments on ER stress, the mRNA level in this original culture condition was set to 1. HS181 (Fig. 3a) growing in HEF-CM exhibited 6 fold rise in BIP transcription levels over baseline but only a modest rise in ASC (1.8 fold). In contrast, XBP1s was 4.42 fold higher in ASC-CM and 3.19 fold in ASC. ATF6 transcription level was stable. In SHEF1 (Fig. 3b), the transcriptional levels of ATF6 and BIP were stable, but XBP1s was upregulated in cells grown on Matrigel with HEF-CM (16.31 fold) and

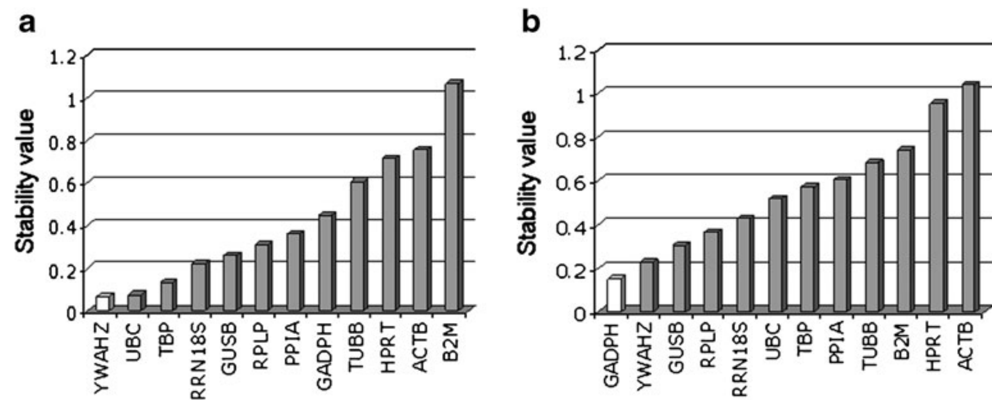

Fig. 2 Stability values of 12 potential reference genes in hESC HS181 (**a**) and SHEF1 (**b**). hESC were grown on HEF, ASC, HEF-CM and ASC-CM as indicated and described in Materials and Methods

ASC-CM (4.48 fold). The results indicated a relationship between the growth on Matrigel and XBP1 activation by alternative splicing. In addition, the difference observed between ASC-CM and HEF-CM suggested the involvement of culture media-factors in this upregulation (HEF-CM 16.31 vs ASC-CM 4.48, as the SHEF1 cells were grown on Matrigel with different conditioned culture media). We chose Homocysteine-induced Endoplasmic Reticulum Protein (HERP) and GADD153/CHOP as sensors of ER stress-resolving pathway. HERP has been extensively involved in ERAD and CHOP is studied as pro-apoptotic factor, both in relation with protein accumulation and folding into ER. As shown in Fig. 3a, HERP was upregulated in HS181 grown on ASC (7.11 fold) and was suppressed by growth on Matrigel (HEF-CM 0.3 and ASC-CM 0.59). CHOP transcription level varied 2.16 fold on ASC, 0.5 in ASC-CM and 1.18 in HEF-CM. In SHEF1 on Matrigel (Fig. 3b), HERP was upregulated 2.18 and 3.11 folds with HEF-CM and ASC-CM respectively, but CHOP was stable in all culture conditions tested.

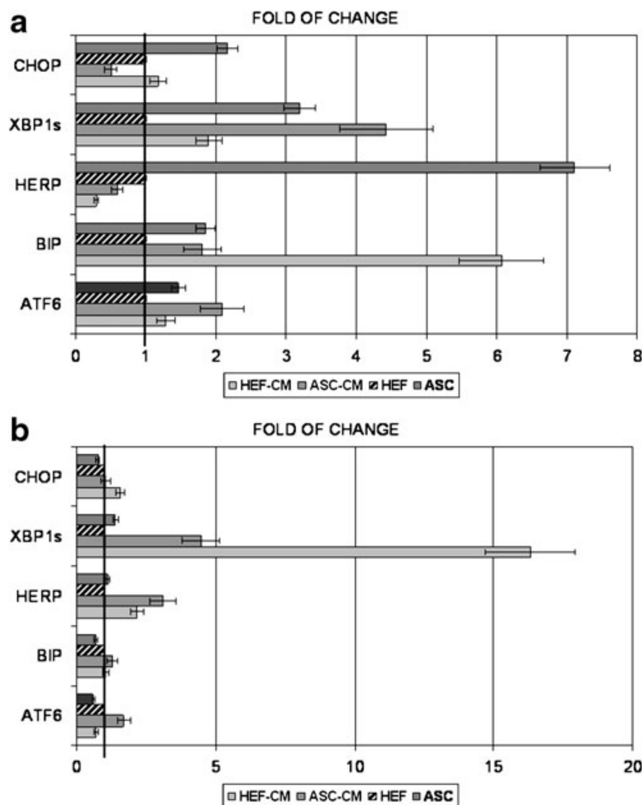

Fig. 3 Analysis of endoplasmic reticulum stress-sensors. hESC HS181 (**a**) and SHEF1 (**b**) were grown on HEF, ASC, HEF-CM and ASC-CM as indicated and described in Materials and Methods. Graphics represent fold of change (FC) of ATF6, BIP, XBP1s, HERP and CHOP comparing conditions indicated versus HEF (HEF=1)

qPCR Array Analysis of Pluripotency and Differentiation Markers

We detected mRNA level modifications in several markers of ER-stress associated with growth on specific feeder layers. These observations could indicate a cellular signal of protein overload in the ER. To further explore this potential effect of feeder layers, an extensive comparative analysis of transcription of 84 genes involved in the maintenance of pluripotency and differentiation markers [see supplemental data 1] in hESC was done under various culture conditions in comparison to the most stable endogenous control for each hESC. First, assay reproducibility was examined by correlation coefficients between duplicates and in all the conditions studied. The high correlation coefficients observed between duplicates (from 0.9867 to 0.9966 in all pairs comparison, see supplemental data 2) indicated good intra-sample reproducibility. Similarly, web-based software which subsequently analyzed qPCR array data provided the high correlation coefficients independently, confirming the reproducibility. In contrast, we had observed some inter-sample differences, which seemed to indicate differences in the expression of the analyzed genes.

Then, we analyzed the differences in mRNA levels of these 84 genes in HS181 and SHEF1 hESC grown on HEF

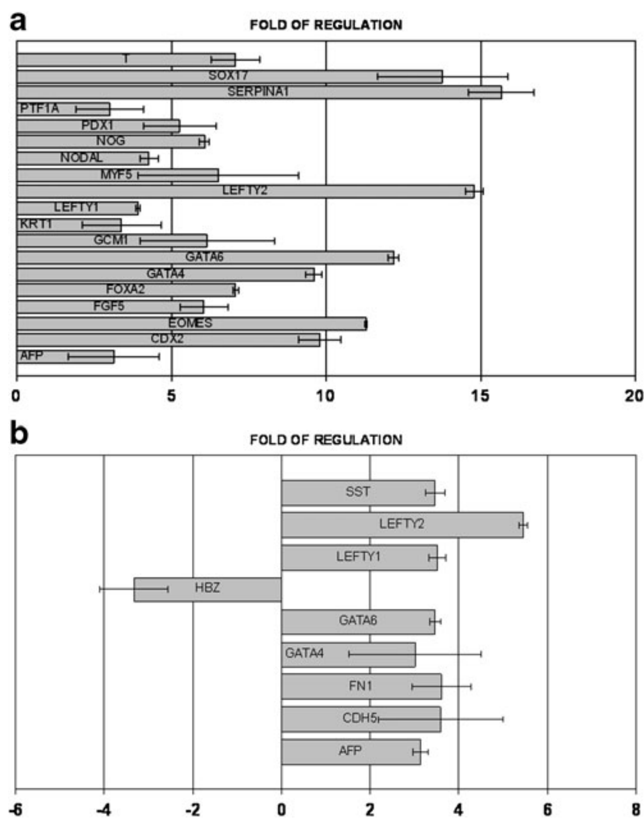

Fig. 4 Genes regulated in hESC growing on adipose-derived mesenchymal stem cells (ASC). HS181 (**a**) and SHEF1 (**b**) hESCs were expanded during 13 and 8 passages, respectively, onto adipose-derived mesenchymal stem cells, as described in Materials and Methods. Graphics represent fold of regulation (FR) positive or negative (−3≥FR≥3) of given genes when cells grown on ASC (complete data analysis listed in Supplemental data 3 and 4, and gene names on Supplemental data 1)

and ASC, as described in Methods. A gene was considered significantly upregulated if the average "fold of regulation" (FR) calculated using the ΔΔCt method and the duplicate samples was greater than 3. Conversely, a gene was classified as downregulated if the average "fold of regulation" was less than −3. Among the 84 genes tested, 21 genes were upregulated and none downregulated by changing the feeder layer of HS181 from its originally derived HEF to ASC (Fig. 4) during 13 passages. In SHEF1, only 9 genes modified their expression levels by culturing on ASC during 8 passages, and all these variations were near in magnitude with the cut-off imposed (±3). The higher expression observed in HS181 hESC occurred in extra-embryonic endoderm markers (FOXA2 +7.0817 and GATA4 +9.6110), visceral endoderm (AFP +21.0237 and SERPINA1 +15.6738), and trophoblast markers (CDX2 +9.8095, EOMES +11.2927 and GCM1 +6.1675). Variations in only 5 genes were common between HS181 and SHEF11: AFP, GATA4, GATA6, LEFTY1 and LEFTY2, being more pronounced in HS181 hESC (complete list of FR on supplemental data 3 and 4). These data suggested a relative differentiation of HS181 cell line growing on ASC, while SHEF1 seemed to be more stable.

Following the comparative study, we next investigated the expression of genes affected by hESC culture on Matrigel with conditioned media. First, HS181 was cultured during 6 passages and SHEF1 during 5 passages on Matrigel with HEF-CM. Figure 5 showed the fold of regulation of genes from HS181 and SHEF1 grown on Matrigel with HEF-CM. As shown, more variations of mRNA levels analyzed were detected in SHEF1 hESC than in HS181 hESC. The majority of common variations in HS181 exhibited suppression (inverse sign). The following genes exhibited opposite modulations in HS181 and SHEF1: (HS181 vs SHEF1) CDH5 −54.3392 vs +233.2228; CDX2 −16.6073 vs +109.6606; EOMES −18.4101 vs +30.6051; FOXA2 −22.5372 vs +229.7976; GATA4 −12.4614 vs +131.7232; GATA6 −12.8930 vs +77.7034; OLIG2 −5.8724

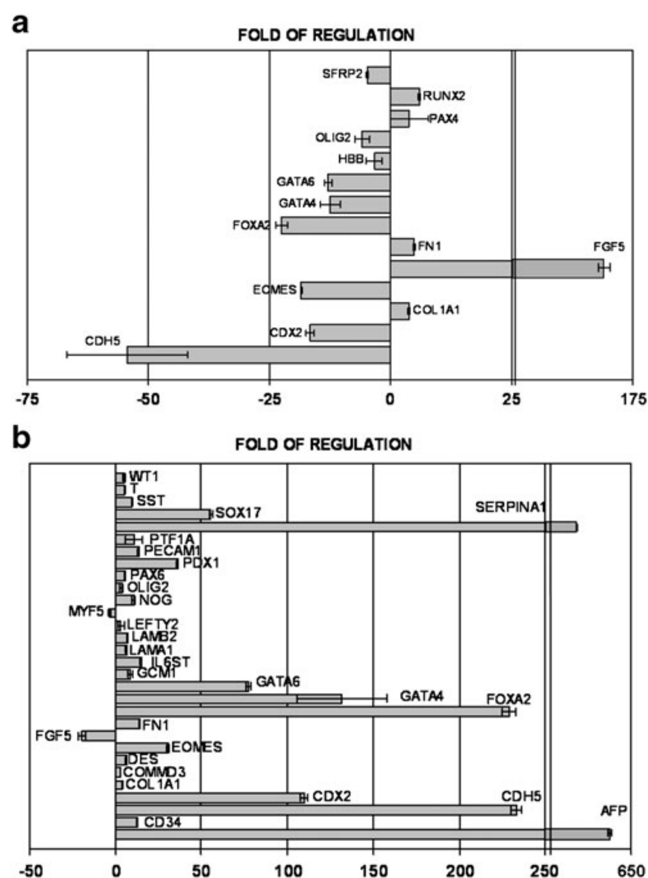

Fig. 5 Genes regulated in hESC grown on Matrigel with human embryonic fibroblasts-conditioned media (HEF-CM). HS181 (**a**) and SHEF1 (**b**) hESC were expanded during 6 and 5 passages respectively onto Matrigel with HEF-CM as described in Materials and Methods. Graphics represent fold of regulation (FR) positive or negative (−3≥FR≥3) of indicated genes when cells were grown on Matrigel with HEF-CM (complete data analysis on Supplemental data 3 and 4, and gene names on Supplemental data 1)

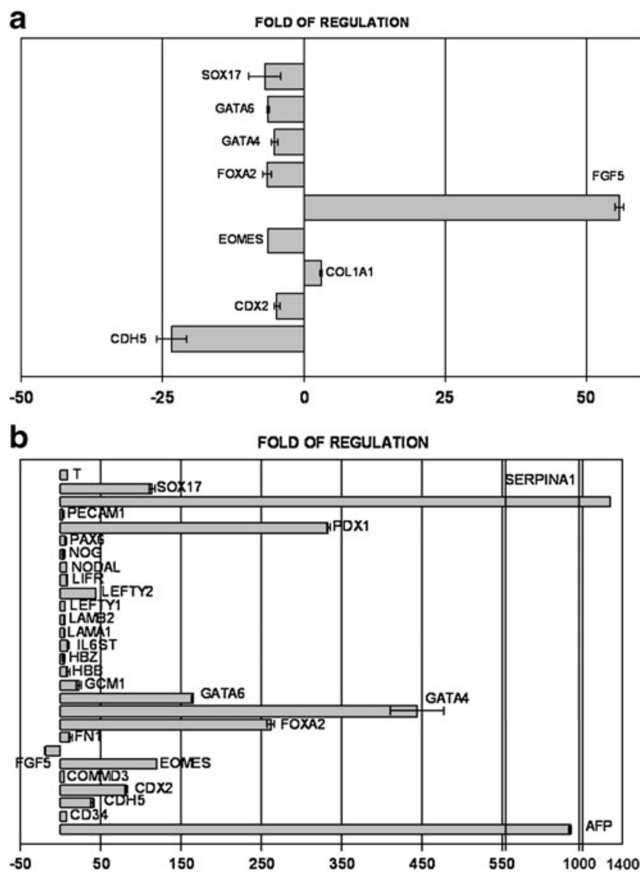

Fig. 6 Genes regulated in hESC grown on Matrigel with adipose-derived mesenchymal stem cells conditioned media (ASC-CM). HS181 (**a**) and SHEF1 (**b**) hESC were expanded during 6 and 5 passages, respectively onto Matrigel with ASC-CM as described in Materials and Methods. Graphics represented fold of regulation (FR) positive or negative (−3≥FR≥3) of given genes when cells grow onto Matrigel with ASC-CM (complete data analysis listed in Supplemental data 3 and 4, and gene names on Supplemental data 1)

vs +3.4553; FGF5 +170.7628 vs −19.8626). Modulation of 3 markers was detected only in HS181: PAX4 +3.8542, RUNX2 +6.0065 and SFRP2 −4.8044. In contrast, 20 markers exhibited significant modulation in SHEF1. High FR variations in SHEF1 were detected in visceral endoderm markers (AFP +614.2073 and SERPINA1 +541.1627), parietal endoderm (FN1 +14.1888, LAMA1 +6.3708, LAMB1 +7.1240 and SOX17 +55.5622) and endothelial differentiation markers (CD34 +12.7124 and PECAM1 +13.4470). To distinguish the effects of Matrigel we performed an analogous study with samples of cells grown on Matrigel with ASC-CM and we comparared to HEF-CM. If these conditions modulate the same genes to the same extent, then the effect is more prominent because of culture on Matrigel, independently of conditioned-media added (complete list of FR on supplemental data 3 and 4). As shown in Fig. 6, this is the correct sentence. In HS181 ASC-CM analysis, 60% genes observed are the same that observed in HEF-CM and their mRNAs level variations are equivalent (correlation coefficients from 0.9806 to 0.9891, see supplemental data 2). Likewise, 80% genes observed in SHEF1 in this analysis maintain the variations and similar level of modification (correlation coefficients from 0.9685 to 0.9745, see supplemental data 2).

The principal factors involved in “pluripotency status”, Oct4, Sox2 and Klf4 did not modify their mRNAs level (see supplemental data 5). Stem cell pluripotency is the result of a complex transcriptional network characterized by the expression of a set of transcription factors including the master regulators of pluripotency Nanog and Oct4/POU5F1 among others. Induced pluripotent stem cells (iPS) with characteristics similar to hESC were obtained by expression of four factors (Oct4, Sox2, Klf4 and c-Myc) from mouse and human, suggesting the important control of these factors over the process of pluripotency [32–34]. Figure 7 has depicted the effects of four experimental growth-conditions for HS181 and SHEF1 on several main factors involved in the undifferentiated status. As shown, the

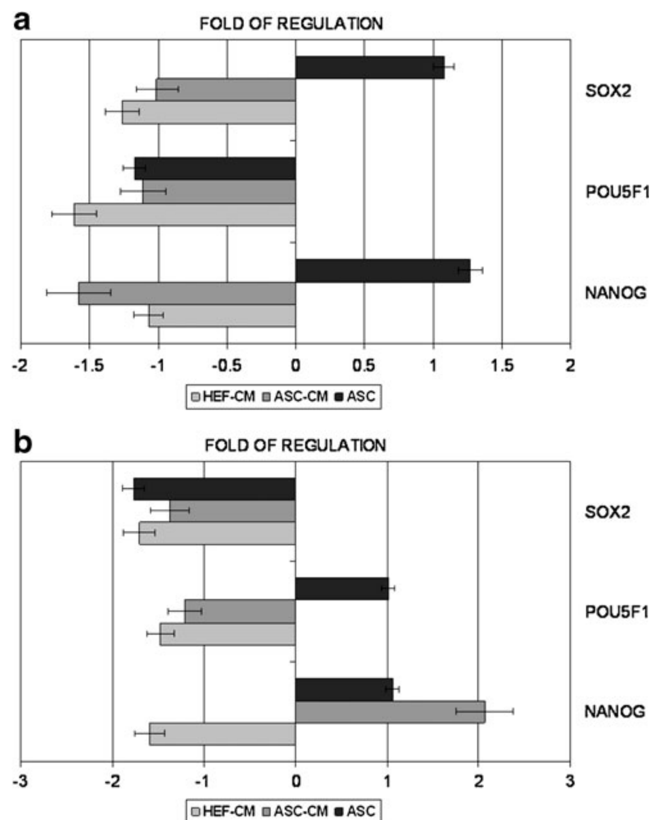

Fig. 7 Analysis of embryonic stem cell-specific factors in hESCs HS181 (**a**) and SHEF1 (**b**) which were growing on HEF, ASC, HEF-CM and ASC-CM as described in Materials and Methods. Graph represented fold of regulation (FR) positive or negative of Nanog, POU5F1 and Sox2 comparing indicated conditions versus HEF. Note that all FR are between 3 and −3. (see complete Embryonic stem cell-specific factors—comparative analysis on supplemental data 5)

transcriptional level of POU5F1, Sox2 and Nanog was maintained throughout the conditions and strongly indicates the maintenance of a pluripotency status, but with a trend to diminish when cells were grown on Matrigel in both hESCs. The overall increase of mRNA level in several markers of differentiation does not indicate cellular differentiation definitively, but taken together, they would be marker of a predisposing state of differentiation. This status could progress towards further differentiation status, and may be influenced by culture conditions.

Discussion

We have described the importance of controlled culture conditions in the maintenance of pluripotency in hESC, and the identification of stress signals in the ER and their association with increases in several markers of differentiation. As a first step, we identified a stably expressed reference gene in each cell line for normalizing the expression of genes and markers of interest. Quantitative real-time PCR is a widely used, highly reproducible technique that requires minimal amount of RNA, and the accurate quantification of a true reference gene to allow normalization of the gene of interest [35, 36]. Some usual housekeeping genes as glyceraldehyde 3-phosphate dehydrogensase (GADPH), β-actin (ACTB) or 18S rRNA are used extensively as references to account for experimental differences in gene expression assays. Nevertheless, recent reports indicated that they were demodulated in different diseases, animal models, or even under varied experimental conditions, which may lead to skewed results and consequently misinterpretations. Genes expressed at stable levels within the cells in the studied experimental system need to be identified and used as reference [37–40]. To ensure stable RNA transcription in different conditions, we accessed 12 candidates for reference gene under our different conditions and chose YWAHZ for HS181 hESC and GADPH for SHEF1 using NormFinder software. Their coefficients of variance were also minimal.

Some authors [41, 42] have identified potential regulators of human and mouse ESCs differentiation by analyzing proteomic profiles of embryonic stem cells versus embryoid bodies. These analyses identified significant changes in expression levels of a wide range of chaperones and co-chaperones including heat sock proteins (HSPs). HSP members are regulated by well defined cellular stress events, and its fundamental role was defined as protein folding assistant. The continued interaction

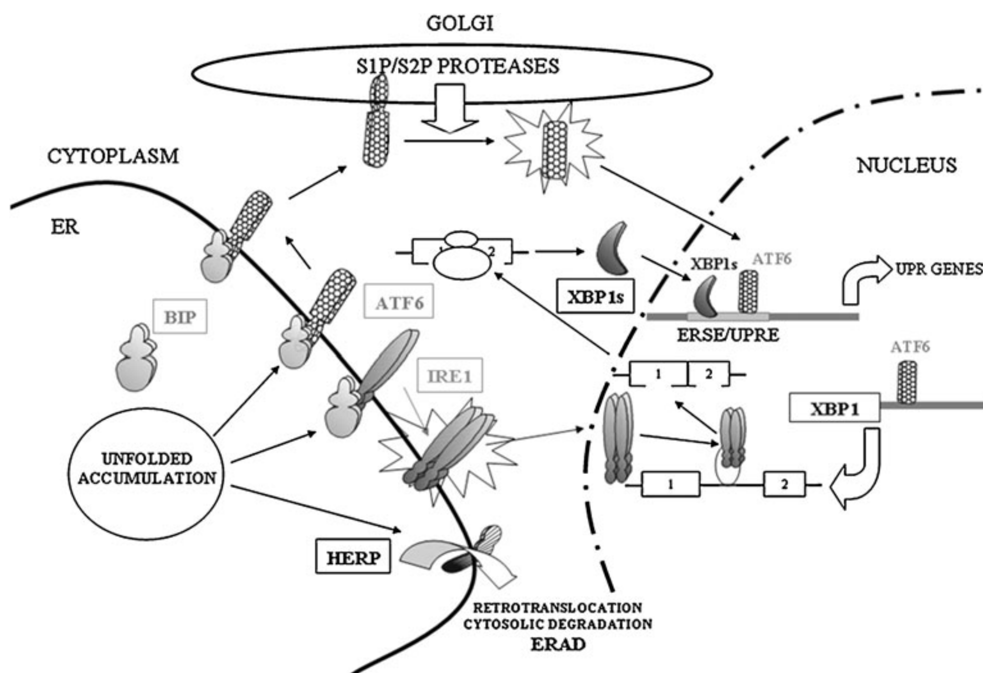

Fig. 8 Unfolded protein response (UPR) pathway: transcriptional branch. BIP protein serves as sensor for unfolded proteins or excessive production of proteins into ER lumen that can induce an accumulation of proteins and activate UPR and ERAD pathways. BIP binds to ATF6 and IRE1 in the ER and promotes their activation. In the final steps, IRE1 mediates an unconventional splicing of XBP1 to produce a potent transcription factor (XBP1s), and activated ATF6 together with XBP1s regulates the expression of several genes named UPR genes (for instance BIP and HERP). This step closes the autoregulatory loop. HERP links UPR signaling to the ERAD pathway by binding members of the ubiquilin family. Ubiquilin functions as a retrotranslocation factor to deliver ubiquitinated substrates to the proteasome for cytosolic degradation

described between HSP90 and two ER transmembrane kinases (IRE1 and PERK) involved in UPR is required for the stability and activity of these proteins [43]. GRP94, the ER-resident paralog of HSP90, is involved in the process of protein chaperoning and folding [44]. However, little has been known about ER stress in hECSs, the transcriptional level of ER stress sensors included in the UPR pathway [45] and their variations in different and defined culture conditions. We described the upregulation of the principal ER stress sensor, BIP in HS181 growing over Matrigel with conditioned media derived from human embryonic fibroblasts HEF-CM (6 folds) compared to the same cell line growing on HEF. Growth on ASC and ASC-CM upregulated BIP mRNA level modestly. BIP has associated with ATF6 and IRE1 in the ER, and its functionality is based on binding to misfolding proteins and release from ATF6 and IRE1 [46–48]. In turn, activated IRE1 catalyzes an atypical alternative splicing in XBP1 mRNA (Fig. 8). XBP1 levels were elevated only in HS181 growing on ASC-CM and ASC (4.42 and 3.14 folds respectively). The complexity of BIP transcription provides us an alternative explanation of this deregulation, given the factors involved on the transcriptional branch of UPR, and its relationship with the apoptotic and translational branch of UPR. Conversely, SHEF1 analysis revealed a large rise of XBP1s levels when cells were grown with HEF-CM and ASC-CM. These culture conditions in SHEF1 (growing on Matrigel) were the most potent inducers of differentiation markers, specifically from parietal endoderm, visceral endoderm and endothelial markers. This strong upregulation in markers of differentiation was not seen in HS181 growing onto neither HEF-CM nor ASC-CM. Its XBP1s mRNA levels were elevated when cells grew onto ASC for several passages, matching the increase in differentiation markers. A coherent explanation is that SHEF1 hESC is more sensitive to early differentiation when they grow onto synthetic layer like Matrigel, independently of media source (ASC or HEF), i.e., SHEF1 appeared more dependent on synthetic feeder layer for growth enhancers than HS181 based on differentiation events. In contrast, HS181 seemed to be more affected regarding ER stress and differentiation events of culture media from adipose-derived Mesenchymal Stem Cells. Based on these data, we proposed a general scheme in which these phenomena of ER stress identified may be directed by XBP1s and not ATF6 in some cases. Our future attention will be directed towards ERAD pathway to resolve the potential loss of intracellular homeostasis more that detected in the UPR pathway because HERP is directly implied in ERAD. ERAD depends exclusively on XBP1s via UPR elements (UPRE) in the promoter of several known and unknown genes (Fig. 8) [49]. Our searches using public software to identify UPRE and ERSE elements in the promoter of the most upregulated markers of differentiation yielded negative results. The relation between HERP and XBP1 is possibly indirectly mediated by other factors.

hESCs used in this study were maintained in "pluripotency or undifferentiated states" in spite of growing on Matrigel or ASC for several passages, but variations observed in differentiation markers could be suggestive of an "early differentiation status" or "predisposing status" to differentiation. Simultaneously, we have detected ER-stress associated phenomena that can indicate a cellular state of alert that the cellular system must settle to survive. The genetic signature of each hESCs may finally drive all cellular processes. hESC cultures are heterogeneous, composed of cells at various stages of differentiation and in the absence of feeder layers on an extracellular matrix, such as Matrigel and other, respond by generating their own support cells. These cells have a fibroblast-like morphology and also arise from hESC colonies cultured on feeder layers [50]. This way, hESCs generate cells to support their own expansion and proliferation, act as an in vitro niche. These fibroblastic cells possess the supportive role in clonogenic self-renewal of hESCs [51]. These new determinants that begin to form a part of the routine hESCs-culture, add to the already well defined genetic heterogeneity in hESCs. Variability observed in numerous studies among hESCs can be partly explained by the fact that each hESC derives from a single embryo that carries specific genetic variants of the human genome. DNA sequence-variations provide the "genetic sign" and are common in the human genome [52]. The identified status described could evolve into a differentiation state that is influenced by the interplay among culture conditions, high stress response potential, feeder layer and genetic signature of hESCs. The reversibility of these states is unknown.

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