Modulation of ER stress in HSCs

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Thesis for the degree of Bachelor of Science
University of Iceland
Faculty of medicine
School of health science
May 2014

HÁSKÓLI ÍSLANDS

Modulation of ER stress in HSCs

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ABSTRACT

Background: Hematopoietic stem cells (HSCs) give rise to mature blood cells and are used in bone marrow transplantation (BMT) to treat various blood malignancies. However, substantial mortality and morbidity is still related to BMT both in terms of engraftment failure and graft versus host disease. Some patients do not have the option of BMT because suitable donors can not be found or are identified too late. Ex vivo expansion of HSCs would counter against these problems but HSCs show limited growth capacity *ex vivo*, with drastic reduction in their reconstitution potential after a short time in culture. Knowledge in HSC regulation is vital in the development of HSC expansion from human cord blood enabling these samples to be used for effective BMT of adult patients. Recently this lab found that DPPA5 RNA binding protein plays a role in lowering ER stress and increasing HSC capacity after ex vivo culture. Sensing of ER stress relies on posttranslational changes in low abundant proteins but studying this in detail is not possible in primary HSCs so we asked if we could use the LHX2 HSC-like cell line as a model for ER stress in the hematopoietic system.

Methods: ER stress was induced in LHX2 cells with tunicamycin (TM) and thapsigargin (TG). They were then analyzed with fluorescence activated cell sorter (FACS) and ER stress proteins were analyzed with Western Blot. Lentiviral knockdown (KD) of DPPA5 was performed in the cells and RNA was analyzed with qRT-PCR.

Results: LHX2 cells induced with TM and TG showed dose dependant lowering in both total cell number and in multipotent progenitor cell numbers. Cells that were treated with the highest dose of TM had an increase in CHOP (late time point ER stress inducer) which promotes cell apoptosis. DPPA5 KD cells showed marked decrease in LSK cell number. The DDIT3 gene, that codes for the CHOP protein, was upregulated in these cells suggesting that they were more sensitive to ER stress.

Disscusion: The LHX2 cell line is very sensitive to induced ER stress. The LHX2 cells express DPPA5 and they may be used to look at specific targets of DPPA5. We showed that the DPPA5 KD cells have increased expression of genes related to ER stress but to see the full extent of the effects we would need to further analyse the effects of the DPPA5 KD and effects on ER stress.

ABBREVIATIONS

ATF 4	Activating transcription factor 4
ATF 6	Activating transcription factor 6
BM	Bone marrow
BMT	Bone marrow transplant
CHOP	CCAAT-enhancer-binding protein homologous protein
CLP	Common lymphoid progenitor
CMP	Common myeloid progenitor
Dppa5	Developmental pluripotency associated 5
ER	Endoplasmic Reticulum
ERAD	ER-associated degradation
ES cells	Embryonic stem cells
FACS	Fluorescence activated cell sorter
FBS	Fetal bovine serum
GRP78	78 kDa glucose-regulated protein
HSCs	Hematopoietic stem cells
IL-6	Interleukin-6
KD	Knock down
Lin^-	Lineage negative cells
LSK	$Lineage^- Sca\text{-}1^+ c\text{-}Kit^+$
LT-HSCs	Long term hematopoietic stem cells
MPP	Multipotent progenitor cells
PERK	Protein kinase R-like ER kinase
qRT-PCR	Quantitative real time polymerase chain reaction
SCF	Stem cell factor
Scr	Scrambled sequence
shRNA	Small hairpin RNA
ST-HSCs	Short term hematopoietic stem cells
TG	Thapsigargin
TM	Tunicamycin
UCB	Umbilical cord blood
UPR	Unfolded protein response
XBP1s	X box binding protein-1 spliced

1 Introduction

1.1 Hematopoiesis

The hematopoietic system consists of a wide selection of specialized cells that are critical for life. These cells have a whole range of different function such as the transportation of oxygen (and CO_2) by the red blood cells (erythrocytes), fighting infection and the removal of abnormal cells (T-cells, B-cells and granulocytes) and blood clotting (platelets) (1), (2). These cells are all produced throughout the lifespan of an individual in a process called hematopoiesis (3). Because most of the mature blood cells are short lived there is a need for them to be replenished continuously with the average human needing approximately one hundred billion new hematopoietic cells each day (4). These mature cells come from various progenitor cells that all come from the hematopoietic stem cells (HSCs) which, in the adult, reside in the bone marrow (3), (4). The hematopoietic cells have a hierarchical arrangement with long term HSCs (LT-HSC) and short term HSCs (ST-HSC) at the top (4), (5). The LT-HSCs enable long term reconstitution of all blood lineages and can form colony forming units in the spleen after transfer to lethally irradiated recipients while the ST-HSCs only allow transient reconstitution (6). These cells then give rise to multipotent progenitors that can form all lineages with further specialized progenitors below them (figure 1).

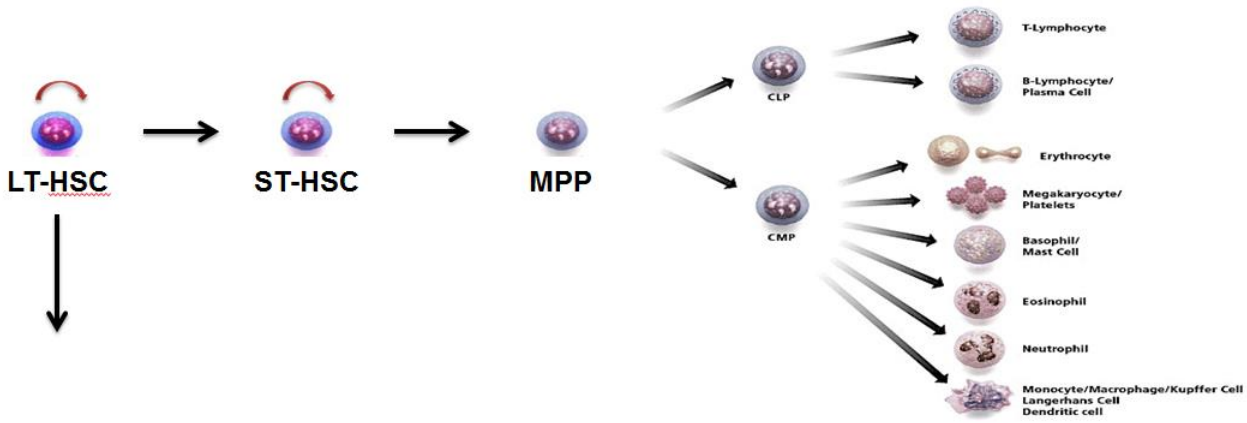

Figure 1. The hematopoietic hierarchy. The long term and short term HSCs are at the top of the apex and can self renew and differentiate into multipotent progenitors (MPP) that can differentiate into all hematopoietic lineages. These cells can then commit themselves to either the lymphoid or myeloid lineage via the common lymphoid progenitor (CLP) or the common myeloid progenitor (CMP) cells. The cells then become more specialized and restricted further down the pyramid.

1.2 Hematopoietic stem cells

Strong evidence for the existence of HSCs came in the aftermath of the bombings of Hiroshima and Nagasaki in 1945 (7). The lethal radiation that the inhabitants of these cities suffered caused bone marrow failure but when they were injected with cells from the bone marrow or spleen of unradiated donors the lethal consequences could be prevented (4). Although these findings did not show whether there were multiple stem cells or if a single multipotential stem cell existed they did confirm that there were indeed blood forming cells in the bone marrow and spleen and this paved the way for further research on the HSCs.

Research on the blood forming cells continued throughout the years and with the use of cell surface markers and clonal in vitro assays it was established that the blood system is a hierarchical arrangement with the HSCs at the top (8). The HSCs give rise to early progenitor cells that proliferate and progressively differentiate into more specialized progenitors (lymphoid progenitor- and myeloid progenitor cells) that ultimately give rise to fully mature blood cells. Unlike the HSCs, the progenitor cells do not have the ability to self-renew. In fact self renewal is one of the two main defining features of the HSC with the other being multilineage differentiation (9). Self renewal refers to the fact that after cell division at least one of the two daughter cells should be identical to the parent cell. Self renewal can be both symmetrical where both cells are like the parent cell or asymmetrical where one cell is like the parent cell and the other differentiated. Multilineage differentiation means that a single HSC must have the potential to give rise to all of the mature blood cell lineages in an organism (9), (10).

1.3 Phenotype of hematopoietic stem cells

The hematopoietic system is described as being hierarchically organized with the HSCs at the very top giving rise to various subpopulations of hematopoietic cells finally producing fully mature cells. Furthermore HSCs themselves can be subdivided into long term (LT-HSC) and short term (ST-HSC) cells. The LT-HSC have self-renewal capability and are defined as enabling repopulation beyond 12 weeks after retransplantation in mice while the ST-HSC can generate all lineages but are only capable of transient engraftment (6), (9). As of yet a single cell surface marker that separates HSCs from other BM cells has not been identified so the identification of HSCs relies on a set of cell surface markers (4).

Studies on mouse HSC have always been an integral part of stem cell biology and they remain important today, especially to test the functionality in transplantation which of course is the ultimate test. In the developmental stage of stem cell biology the first HSC markers were discovered in mice (4), (11). Murine HSCs lack a number of cell surface markers that more mature cells express, such as B220 that B-cells express and CD3 that T-cells express. This lack of markers is known as the lineage-negative (Lin^-) subset. Lin^- cells that also expressed c-Kit and Sca-1 were purified and this became known as the LSK-population (12), (13). Within the LSK population, $CD34^-$ cells possess long-term multilineage reconstitution and self renewal (LT-HSCs). In contrast, the first cell marker found to identify human HSCs was CD34, expressed on less than 5% of all blood cells (14). CD34, however, does not only mark HSC but also more differentiated progenitor cells so to further isolate HSCs studies were set out to find more specialized cell surface markers. Over the past decade CD90 (Thy1) has, along with CD34, marked a subset population of cells that contained most multilineage capacity (15). CD45RA and CD38 markers that negatively enrich for HSC were found later. Therefore in the last few years the human HSC phenotype has been established as $CD34^+CD38^-CD90^+CD45RA^-$.

1.4 Hematopoietic stem cell expansion

The aforementioned defining features of HSCs (self renewal and multilineage differentiation) make these cells an exciting option for stem cell based therapy, but ex-vivo expansion of HSC has proven unsuccessful because of the difficulty to generate sufficient stem cell population and the inability of the HSC to remain undifferentiated. It is well known that during homeostasis the number of HSCs is maintained at a relatively constant level but studies have shown that during hematopoietic stress HSCs self-renew to an extensive level (16). Furthermore these studies have demonstrated via serial transplantation in lethally irradiated recipients that the HSC numbers increased 8400-fold after four successive transplantations (16).

The reason that ex vivo expansion of HSCs is important is because these cells are used in bone marrow transplants (BMT) to treat many blood malignancies, with leukemia being the most common one (17). However, substantial mortality and morbidity is still related to BMT both in terms of engraftment failure and graft versus host disease. An alternative approach has been the use of umbilical cord blood (UCB) which has shown to be a rich source of HSC but the stem cell number in a single unit of UCB has not proven enough for transplantation into

an adult (18). In acute leukemia, where there is a need for rapid transplantation, the identification of suitable donors can take too long and in the meantime the patient may deteriorate and eventually die if a suitable donor isn't found. These obstacles, along with the fact that the number of transplanted $CD34^+$ cells is related to patient survival (10), have led to the consensus that clinical application of HSCs would benefit from HSC expansion. For example the purification of HSCs in an allogeneic transplant would bypass the graft-versus-host disease by eliminating immunoreactive T-cells from the graft (19). There is also the possibility of purifying HSCs from cancer patients and reintroducing them after chemotherapy or radiation and thereby eliminating the possibility of reintroducing cancer cells to the patient (20).

The fact that HSCs can expand in vivo suggests that there are specific factors or signals that promote their self-renewal and amplification. The challenge remains to expand undifferentiated HSCs in numbers sufficient for therapy of adult patients. The common goal of HSC research today is to understand how HSCs can self-renew and expand in vivo thereby making it possible to expand HSCs ex vivo. Ex vivo culture is not the optimal environment for HSCs and they seem to be vulnerable to various cellular stress in such surroundings that have negative effects on HSC culture and limits their expansion. One type of cellular stress that HSCs are known to be vulnerable to in ex vivo culture is ER (endoplasmic reticulum) stress (21), (22).

Embryonic stem cells (ES cells) have been used as a model to research which genes and novel factors are important in keeping stem cells undifferentiated in culture (23). One of these factors is Developmental pluripotency-associated 5 (Dppa5). Dppa5 is an RNA binding protein that is highly expressed in HSCs and is a critical regulator, especially in relation to ER stress. A recent study by Miharada et al (21) showed via lentiviral overexpression of Dppa5 in primary cells that Dppa5 plays a role in lowering ER stress in HSCs, increasing their ex vivo expansion significantly. That is the reason why increased knowledge of the role that Dppa5 plays in the regulation of ER stress is important because lowering ER stress in HSC cell culture could help ex vivo expansion of these cells (figure 2).

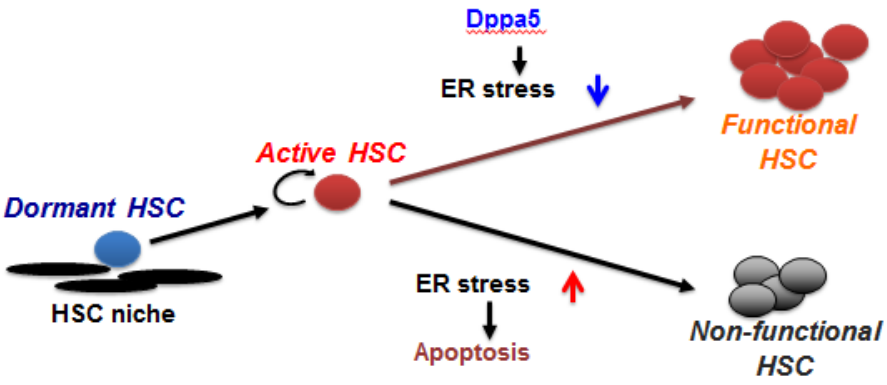

Figure 2. The role of Dppa5 in ER stress. Increased ER stress in ex vivo culture of HSCs leads to apoptosis in the cells and non-functional HSC. Dppa5 has been shown to down regulate ER stress thereby increasing the number of functional HSCs and helping maintain the cells in culture.

1.5 ER stress

The endoplasmic reticulum (ER) is an organelle that is the center of protein management in cells. It's responsible for protein translocation, protein folding and post-translational modification that allows for further transport of the proteins to the Golgi apparatus and then to vesicles for secretion or display on the plasma surface (24), (25), (26). Proteins that are unfolded or misfolded are retained in the ER, translocated to the cytoplasm by the ER-associated degradation (ERAD) pathway and then degraded by the proteasome. When cells synthesize proteins in amounts that exceed that of the folding mechanism or the ERAD, unfolded proteins accumulate in the ER (24). When proteins are unfolded they expose hydrophobic amino-acids that under regular conditions would be located inside the folded protein and they begin to form protein aggregates. These protein aggregates are toxic to the cell (ER stress) and can under enough exposure induce apoptosis. ER stress is observed in a number of diseases including Alzheimer's disease, Huntington's disease and polycystic kidney disease (27).

In the cell, ER stress is induced through three different pathways. Under normal physiological conditions IRE1, ATF6 and PERK are bound to the heat shock protein Grp78 but when unfolded proteins accumulate in the ER they are released from Grp78 and their pathways are activated (24). IRE1 and ATF6 are generally known as pro-survival pathways or early ER stress pathways where the accumulation of proteins is not severe enough to induce

apoptosis but instead the proteins are degraded in order to restore homeostasis (28), (29). The PERK pathway meanwhile is a more pro-apoptotic pathway or late-time point ER stress pathway (30). Through a number of steps PERK increases the expression of the transcription factor CHOP that controls expression of genes involved in apoptosis (28), (31). These are the three different ER stress pathways and together their response is called the unfolded protein response (UPR) (figure 3).

To see how ER stress affects different cells in culture it's possible to use ER stress inducers such as tunicamycin and thapsigargin. Tunicamycin is an antibiotic that prevents N-linked glycosylation and causes misfolding of proteins which activates the UPR (32). Thapsigargin inhibits ER specific calcium ATPase and increases intracellular calcium levels (33). By using these two chemicals it's possible to induce ER stress in ex vivo cell cultures.

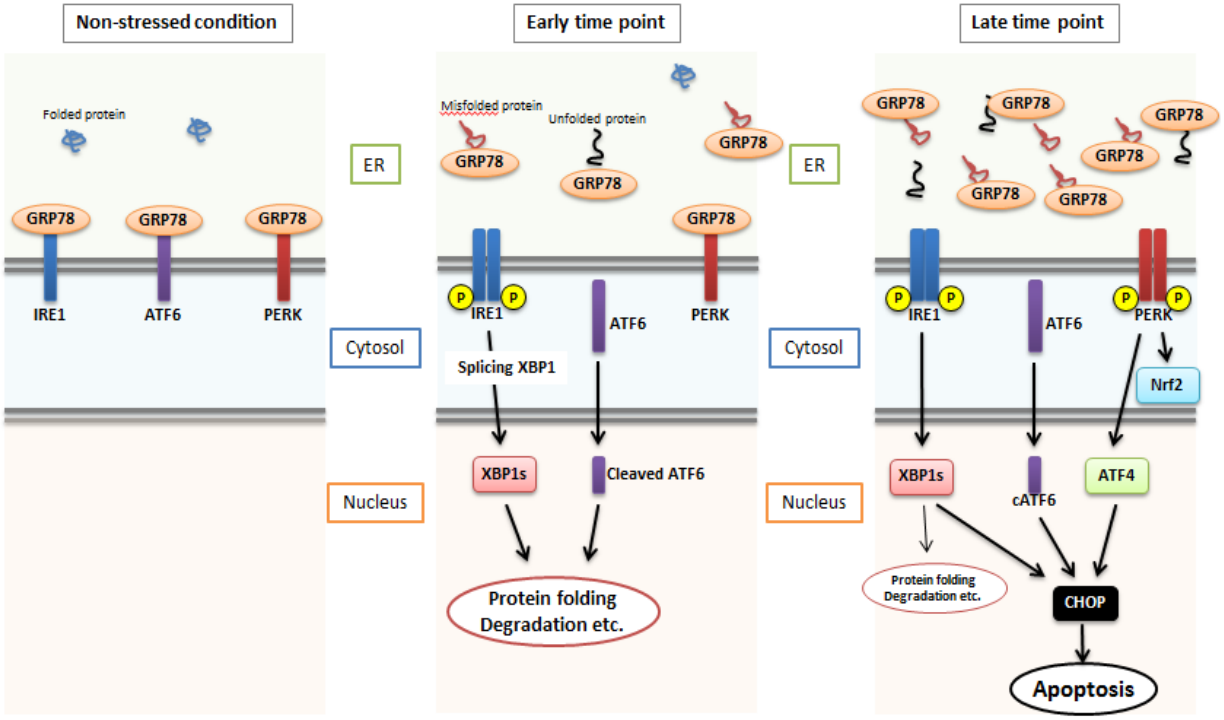

Figure 3. The three different pathways of ER stress (adapted from Luo and Lee, *Oncogene* (2012); Nishitoh, *J.Biochem* (2012)) . When IRE1 is activated it removes a small intron of the gene that codes for X box binding protein-1 (XBP-1) This creates a transcription factor (XBP-1s). Meanwhile ATF6 is translocated to the Golgi apparatus where it is cleaved by proteases which yields the transcription factor cATF6. Both transcription factors, XBP-1 and cATF6, lead to expression of ER chaperone GRP78 and the ERAD factors that are involved in degradation of proteins. These pathways are commonly known as pro-survival UPR (28), (29). Activation of PERK is a more pro-apoptotic response (30). When PERK is activated it phosphorylates eukaryotic translation initiator factor 2α which allows the translation of ATF4 mRNA that encodes the transcription factor CHOP that controls expression of genes involved in apoptosis.

1.6 The aim of this study

As shown before the sensing of ER stress is in part based on post translational protein modifications of low abundant proteins. To study the downstream effects of this we need to do so at a protein level which would not be possible with primary HSCs due to insufficient cell numbers, so the aim of this study was to use the LHX2 cell line (HSC like cell line) as a model for ER stress in the blood system. Furthermore we wanted to see if the LHX2 cells could cope with cell cultured induced stress (ER stress) without Dppa5.

2 Materials and methods

2.1 Cell culture

We used the LHX2 cell line as a model for HSCs. The LHX2 cells display a $CD34^-$ LSK HSC phenotype and are generated via overexpression of the LIM homeobox gene 2 (34). The cells were cultured in IMDM medium supplemented with 5% fetal bovine serum (FBS), mouse stem cell factor (SCF) and human interleukin-6 (IL-6). To induce ER stress we used both tunicamycin and thapsigargin in different concentrations. Tunicamycin was dissolved in 100% ethanol and used in the concentrations of 0.06, 0.15 and 0.3 μg/ml. Thapsigargin was dissolved in 100% ethanol and used in the concentrations 1 nM, 2 nM and 5 nM.

2.2 Western Blotting

Equal amounts of proteins were separated on 10% NuPage Bis-Tris gels (Invitrogen) and transferred to a PVDF membrane (Invitrogen). Antibodies specific for actin, CHOP, GRP78 and XBP1s were used. Membranes were visualized with Supersignal (Thermo Scientific) after incubation with anti-mouse or rabbit secondary antibodies.

2.3 Fluorescence activated cell sorting

Fluorescence activated cell sorting (FACS) was used to sort hematopoietic stem and progenitor cell populations using antibodies for cell surface markers CD34, Lin, c-Kit and Sca-1. The LHX2 cells were stained with CD34, Lin, c-Kit and Sca-1 antibody mix. Hematopoietic stem and progenitor cell populations were sorted and analyzed using FACSCalibur™. The data from the FACS was then analyzed using Flowjo.

2.4 Lentiviral knock down of Dppa5

Dppa5 specific small hairpin RNA (shRNA) lentiviral vectors (MISSION® shRNA) were obtained from Sigma-Aldrich. Scrambled sequence (Scr), where Dppa5 was not knocked down, was used as a control. The puromycin-resistance gene was removed from the vector and replaced by Venus lentiviral vector. LHX2 cells were transfected with lentiviral vectors and integration of the virus visualized with Venus.

2.5 Magna Rip

Five to twenty thousand of LHX2 cells with Dppa5 KD and without Dppa5 KD were directly sorted into the RLT buffer of the RNeasy® Micro Kit (QIAGEN) and total RNA extracted following the manufacturer's protocol. Extracted RNA was reverse transcribed with SuperScript® III (Invitrogen). Real-time PCR reactions were performed on 7900 HT Fast Real-Time PCR System (Applied Biosystems) and the findings were normalized to HPRT.

2.6 Statistical analysis

All statistical analysis was performed using Graphpad prism. Statistical differences between groups were calculated using students t-test where $p < 0.05$ was considered statistically significant.

3 Results

3.1 LHX2 cells show lowering in cell number after *in vitro* TM treatment

To see how the LHX2 cells would handle induced ER stress we used tunicamycin to induce ER stress *in vitro*. 250.000 LHX cells per well were cultured for 24 hours with differing concentrations of TM in each well (0.06, 0.15 and 0.3 µg/ml respectively) and for comparison a control well with no TM was used. FACS analysis showed both lowering in total cell number (figure 3A) as well as lowering in the number of cells with the HSC phenotype (LSK) (figure 3B) after TM treatment, compared to the control cells. The biggest difference seen in both total cell number and LSK cell number compared with the control well was with the highest concentration of TM (0.3 µg/ml).

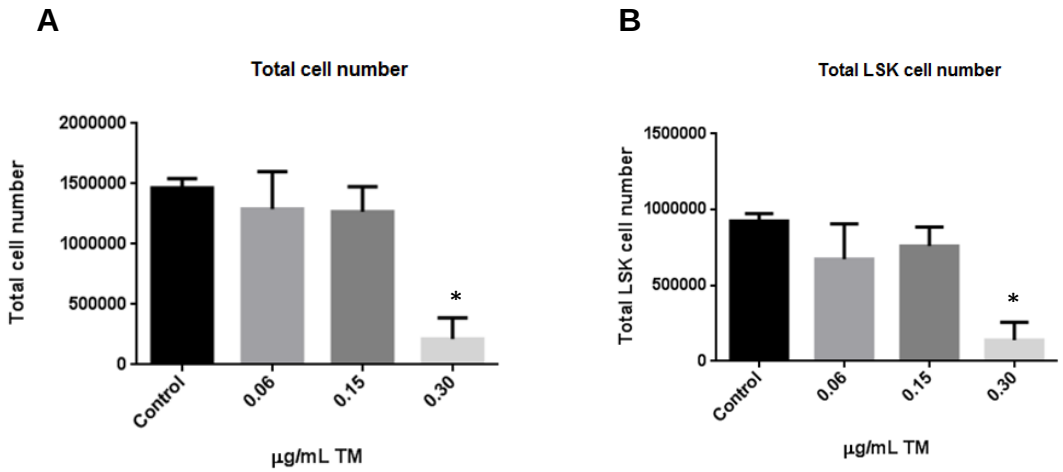

Figure 3. ER stress was induced in 250.000 LHX2 cells/well with differing concentrations of tunicamycin and cultured for 24 hours. A) Total cell number after treatment with TM. The difference between control and 0.30 µg/ml TM was statistically significant ($p < 0.05$) B) Total number of LSK cells after treatment with TM. The difference between control and 0.30 µg/ml was statistically significant ($p < 0.05$).

3.2 LHX2 cells show dose dependent lowering in cell number after *in vitro* TG treatment

To confirm that the induced ER stress was the likeliest reason for the lowering in cell number with TM treatment we also induced ER stress *in vitro* with thapsigargin (TG). As the LHX2 cells seemed more sensitive to TG than TM we used 500.000 cells per well, with three differing concentrations of TG and a control well. The cells were then cultured for one day. The cells showed the same dose dependent trend in both total cell number (figure 4A) and LSK cell number (figure 4B).

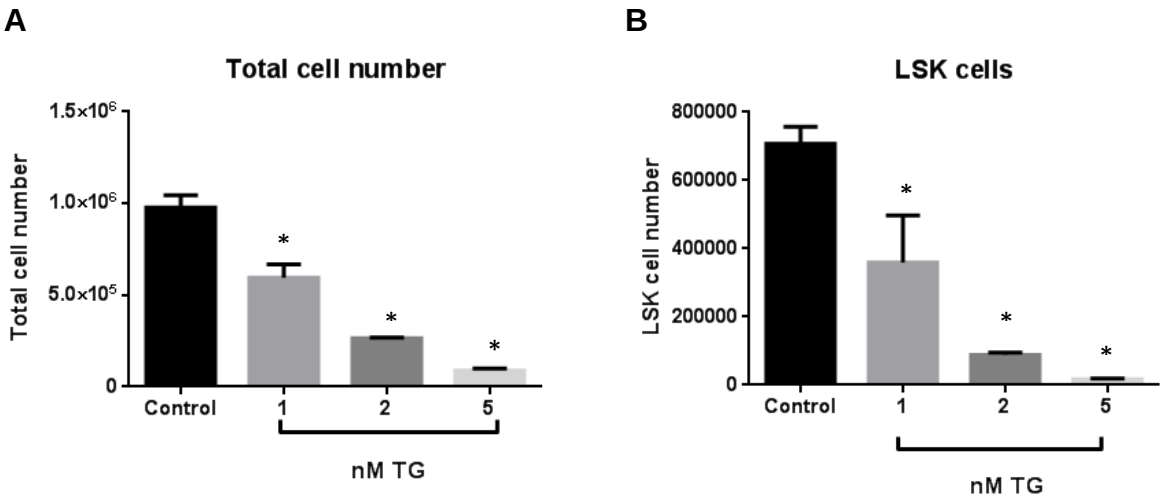

Figure 4. ER stress was also induced in the LHX2 cell with thapsigargin. The cells were induced with TG and cultured for 24 hours. Thapsigargin increases intracellular Ca^{2+} levels and is quite toxic to the cells so we used low doses (1, 2 and 5 nM respectively). A) Total cell number after treatment with TG. The difference between control and all doses of TG was statistically significant ($p < 0.05$). B) Total number of LSK cells after treatment with TG. The difference between control and all doses of TG was statistically significant ($p < 0.05$).

3.3 Late ER stress proteins are highly expressed in LHX2 cells treated with TM

After the LHX2 cells were treated with TM we analyzed ER stress related proteins in the cells using Western Blot to see whether there was any noticeable difference in the expression of these proteins in the different TM concentrations. We analyzed the CHOP (late time point ER stress protein), GRP78 and XBP1s (an early time point ER stress protein) proteins and

compared them with the amount of actin in each concentration and used that as a baseline for the amount of proteins in each TM concentration. In the highest concentration of TM the CHOP protein was expressed 300 times more compared to the control (figure 5B). GRP78 was expressed 20 times more in the highest TM concentration compared to the control (figure 5C) and XBP1s was expressed nearly 30 times more in the next highest concentration of TM, 0.15 µg/ml, compared to the control (figure 5D).

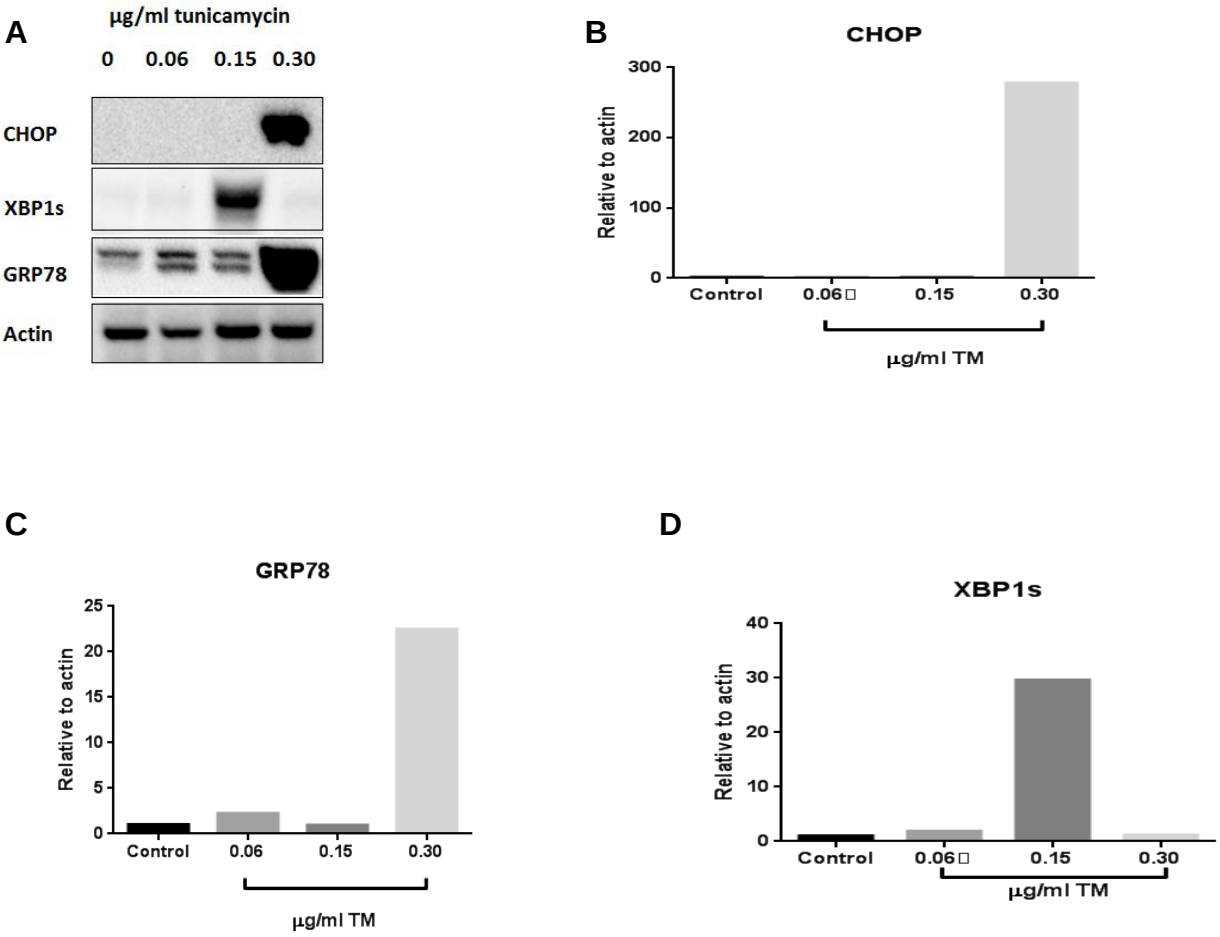

Figure 5. After the LHX2 cells were treated with TM ER stress specific proteins were analyzed with Western Blot. A) The proteins that were measured were CHOP (late ER stress protein that induces apoptosis), XBP1s (early ER stress protein) and GRP78 (heat shock protein that senses misfolded and unfolded proteins in the ER). The concentration of these proteins was then measured relative to the actin concentration as a baseline for the amount of proteins in each well. B) The CHOP protein was expressed nearly 300 times more in the highest concentration of TM (0.30 µg/ml) compared to expression in the control well. C) The GRP78 protein was expressed more than 20 times more in the highest TM concentration compared with the control well D) XBP1s was expressed nearly 30 times more in the next highest concentration of TM (0.15 µg/ml) compared with the control well.

3.4 Cells with Dppa5 KD show decrease in LSK number

The Dppa5 gene was knocked down in the LHX2 cells using a lentiviral approach. Using FACS analysis we saw that in the shRNA 2 well the total number of LSK cells was remarkably lower in cells that had taken the virus in compared with cells, in the same well, that had not taken the virus into their genome (figure 6). There was not a decrease in total cell number between wells but as we could not compared the cells directly between wells we would need to analyze this further.

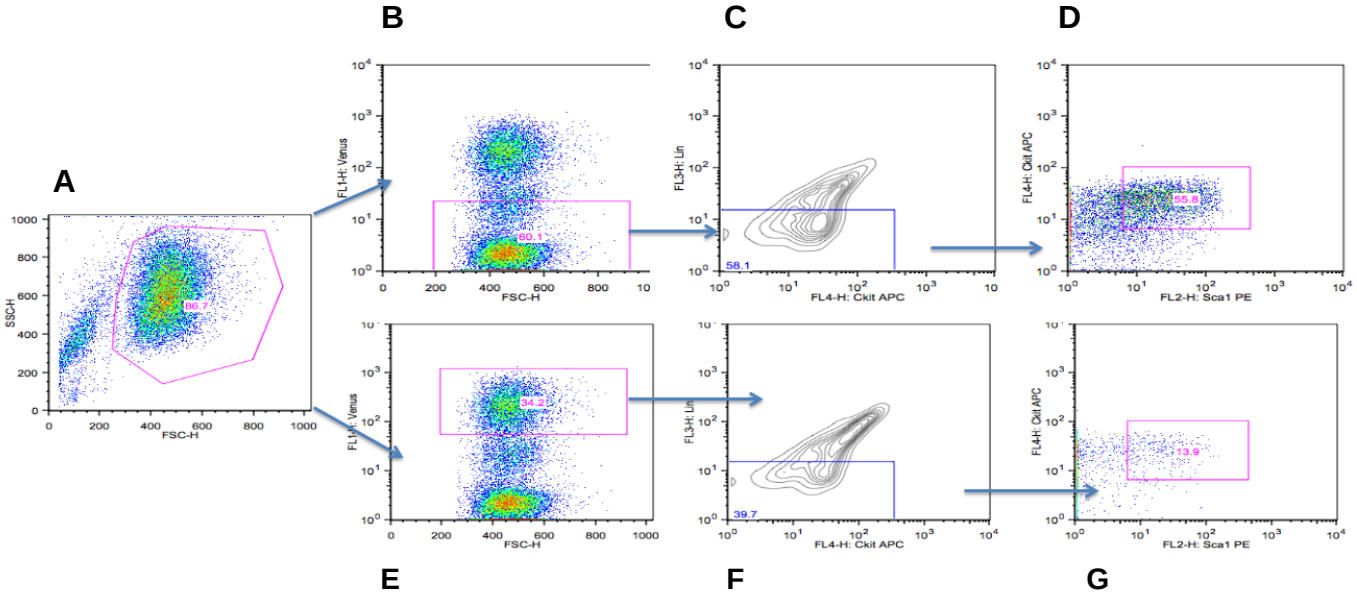

Figure 6. FACS analysis of LHX2 cells that had the Dppa5 gene knocked down. The upper row (B-D) shows the selection of Venus negative cells (cells that did not take the virus in) while the lower row (E-G) shows cells that did take the virus in to its genome and have thereby had the Dppa5 gene knocked down. The cells where Dppa5 was KD showed a lower number of LSK cells (G) compared to the cells were Dppa5 was not KD (D).

3.5 Cells with Dppa5 KD show increased expression of ER stress genes

Using qRT-PCR we analyzed mRNA expression of specific ER stress genes in Sh-2 Dppa5 KD cells and compared the expression to Scrambled shRNA which was our control. We saw marked increase in the expression of the DDIT3 gene, which codes for the CHOP transcription factor and in ATF4 in the Dppa5 KD cells. This indicates that the decrease in LSK like cells within Dppa5 KD LHX2 cells may be due to ER stress induced apoptosis.

However, the cells that were analyzed with qRT-PCR were not sorted for VENUS+ cells from the non-Venus cells and thereby it was difficult for us to analyze how well the KD procedure of Dppa5 had gone in the cells that had taken the virus in. We expect to see an even greater difference when pure VENUS+ cells will be compared.

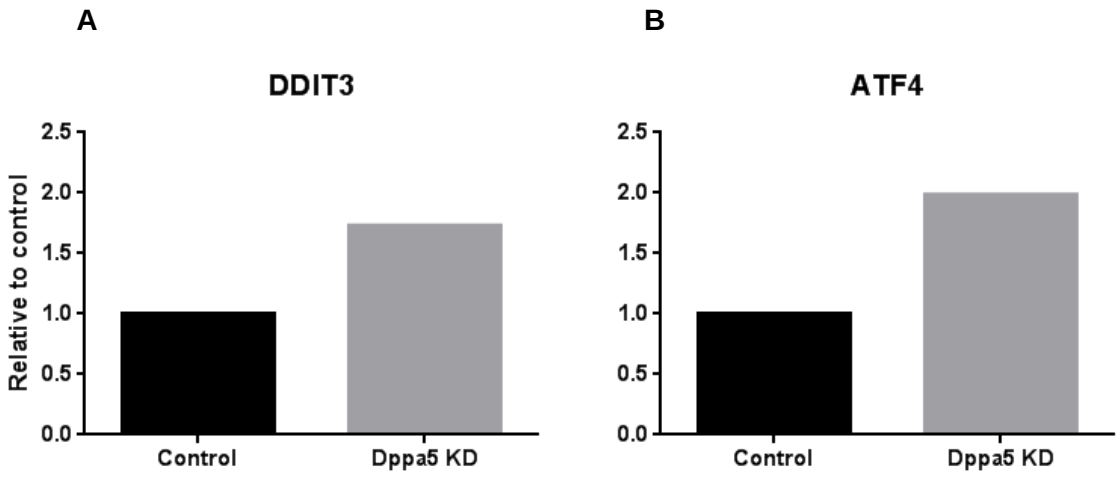

Figure 7. ER stress genes were analyzed with qRT-PCR in Dppa5 KD cells and compared with cells where Dppa5 was not knocked down. A) DDIT3 is the gene that codes for the late ER stress protein CHOP. DDIT3 was expressed 72,9% more in cells where Dppa5 was knocked down indicating that these cells were more sensitive to ER stress. B) ATF4 was expressed 98,6% more in cells where Dppa5 was knocked down, again indicating increased sensitivity to ER stress.

4 Discussion

By inducing ER stress *in vitro* in the LHX2 cell line with TM we showed that these cells are very sensitive to ER stress resulting in cell death and lowering in LSK cell numbers. The biggest difficulty was finding the right concentration of TM to use since the LHX2 cells were very sensitive to this chemical. Due to this sensitivity to TM the dosage we ended up using was lower than had been used before with primary cells in previous studies. There seems to be a shift from 0,15 ug/ml TM where the cells can tolerate induced ER stress to 0,3 ug/ml TM where they start to show drastic lowering in cell numbers. To further confirm that the effect of TM was caused by ER stress we used TG as well to induce ER stress. The LHX2 cells were even more sensitive to this chemical which again shows that the sensitivity of these cells to ER stress. This confirms the fact that they are HSC-like in that regard and on that basis could be used as a model for ER stress in the hematopoietic system.

In the highest concentration of TM (0.3 μg/ml) the LHX2 cells showed increased expression of the late ER stress protein, CHOP. It was expressed nearly 300 times more in that concentration compared with control well. CHOP is a transcription factor that controls the expression of apoptotic genes. The fact that it is expressed in such high quantity in the highest TM concentration indicates that massive apoptosis is taking place in the cells, again pointing to the fact that the LHX2 cells are sensitive to ER stress. The CHOP protein was not significantly increased in the lower concentrations of TM compared to the control because in those concentrations we were inducing milder ER stress. The cells in the two lower concentrations were, in all likelihood, in the pro-survival phase of ER stress, which was supported by the fact that XBP1s, an early ER stress protein, was highly expressed in the 0.15 μg/ml concentration of TM.

When Dppa5 was knocked down in the LHX2 cells we did not notice a decrease in total cell number compared with cells where Dppa5 was not knocked down. We did however see a decrease in the number of LSK cells in Dppa5 KD cells which indicates that ER stress could be playing a role in specializing cells and making them lineage comitant. Dppa5 could also be protecting the cells from the ER stress and thereby keeping the HSC profile intact. However, we only compare the cells in the shRNA2 KD well with FACS on Venus+ (KD) and Venus- (No KD) cells but did not compare them to virus transduced control cells because of low number of cells. This experiment would have to be repeated where pure Dppa5 KD cells are

compared to pure scrambled shRNA transduced cells for correct experimental setup and more accurate result.

When mRNA expression of ER stress genes were analyzed using qRT-PCR we found that the DDIT3 and ATF4 genes were expressed higher in the DPPA5 KD cells than the control cells. These genes are directly related to late ER stress. DDIT3 codes for CHOP and together with ATF4 they promote late ER stress and/or apoptosis. This could indicate that the Dppa5 KD cells are in fact more susceptible to ER stress and would confirm the role of Dppa5 in reducing ER stress. However we would need to analyze this further in the KD cells to get a better indication of the role of Dppa5.

In conclusion these results indicate that LHX2 cells can serve as a good surrogate model for primary HSCs in ER stress research. Two recent papers by Miharada et al (*Dppa5 improves hematopoietic stem cell activity by reducing endoplasmic reticulum stress* (in press, 2014)) and by van Galen et al (*The unfolded protein response governs integrity of the haematopoietic stem-cell pool during stress* (Nature, 2014)) have shown that HSCs are sensitive to ER stress and protein integrity is important in these cells. These findings will hopefully, in the near future, allow for the detection of direct targets of Dppa5, using LHX2, related to ER stress control that could be further tested in primary mouse- and human HSCs.

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