SZENT ISTVÁN UNIVERSITY

PhD School of Animal Husbandry Science

STUDYING THE DEVELOPMENTAL GENE EXPRESSION PATTERN OF MAMMALIAN EMBRYOS PRODUCED BY BIOTECHNOLOGY PROCEDURES

Thesis

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1. INTRODUCTION AND GOALS

The possibility to freeze mammalian reproductive cells has revolutionized the fields of animal husbandry, biotechnology and human medicine by allowing to determine the place and time of fertilization and embryo development. Despite the breakthrough that this new technique caused, still it encounters problems as a result of the freezing injuries of cells that decrease both the quantity and the quality of frozen-thawed gametes and embryos. The significance of the difficulties is highlighted by the fact that for the majority of the mammalian species only the sperm-freezing is solved until now. Consequently, most of the strategies to increase the cryopreservation success rates focus on imitating the natural physiology (e.g.: appropriate temperature, pH and medium components) and reducing the inevitable damage caused by the manipulation (Saragusty és Arav 2011).

In contrast to this principle, the efficiency of cryopreservation could be improved by preconditioning the gametes and embryos with precisely adjusted and applied sublethal stress treatment. The studies performed by the research group of Szent István University, Faculty of Veterinary Science, Department of Animal Breeding and Genetics were based on the hypothesis that optimized sublethal stress can have protective effect. In their experiments the high hydrostatic pressure treatment (HHP) was chosen as the stressor, by which the gametes and embryos were treated just below their tolerance limit. The HHP treatment was then followed by cryopreservation of the cells and their freezing-tolerance was investigated.

The developed method was so successful that it has already been shown to have an advantageous effect on different domesticated species (e.g. porcine, bovine and ovine embryos, oocytes and spermatozoa) not only in combination with different freezing methods but also with other assisted reproductive techniques (Pribenszky és Vajta 2011). Despite the observation of a biological effect of the HHP technique, a fundamental understanding of the underlying molecular mechanisms is still lacking. Therefore, it is indispensable to reveal the molecular basis of the beneficial effect of the HHP, because it would allow us to further improve the cryopreservation protocols.

My aim was to reveal as many details as possible about the HHP effect on the gene expression during the early (preimplantation) development to study how HHP protects embryos against freezing injury. Since the high hydrostatic pressure has been shown to be effective for other assisted reproductive techniques such as somatic cell nuclear transfer (Pribenszky és Vajta 2011), I sought

to gain insight into a general mechanism that helps the gametes and embryos to tolerate different *in vitro* stresses.

Objectives of the thesis were:

1. First, to demonstrate that the HHP-technique affects the transcription of certain genes in the chosen model system.

2. Next, by analyzing the impact of the HHP-treatment on murine oocytes to study whether it affected the RNA abundance of oocytes. We performed gene expression microarray experiments on matured oocytes to study the response of the whole mouse transcriptome to the applied HHP stress, to reveal the intracellular processes activated or inhibited by the treatment. As until the embryonic genome activation (EGA) the maternal RNA is indispensable, in the first part of the experiments we studied whether the HHP stress affected the RNA abundance of oocytes

3. To study the HHP effect after the embryonic genome activation (EGA), we also analyzed the global gene expression pattern of four-cell stage embryos developed from HHP-treated oocytes after fertilization with ICSI. The embryos were also analysed by whole mouse genome microarrays.

4. The extensive raw data that were collected by the transcriptome profiling experiments was further analysed by different bioinformatics software to gain insight into the processes that might help the oocytes and embryos to overcome the freezing stress.

2. MATERIALS AND METHODS

2.1. Preparation of embryos and oocytes

2.1.1. Production and culture of blastocyst stage embryos, HHP treatment

In the first part of our experiments the goal was to study the transcriptional response of mouse blastocysts to the HHP treatment. Early blastocysts were obtained at Day 3.5 post-hCG administration by flushing the uterus then the blastocysts were cultured till the expanded blastocyst stage.

For the gene expression study, the pools of 10 expanded blastocysts were loaded into 0.25-mL plastic straws (IMV, L'Aigle, France). After loading, the embryos were treated with 60 MPa pressure for 30 min on room temperature. Hydrostatic pressure treatment was performed by a computer controlled hydrostatic pressure device HHP 100 (Cryo-Innovation Ltd., Budapest, Hungary), then treated and control groups were frozen for RNA isolation. To study the prolonged response to the HHP stress further groups were collected after 120 min of *in vitro* culture in KSOM (Millipore, Billerica, USA) on 37ºC, following the treatment.

2.1.2. Collection and HHP treatment of mouse oocytes, fertilization by intracitoplasmic sperm injection, embryo culture

In the second part of our experiments the aim was to analyze the impact of HHP treatment on the global gene expression during mouse early preimplantation development. First, we studied the response of HHP-treated oocytes using gene expression microarrays.

For this, ovulation of B6D2F1 female mice was induced and oocytes were collected by flushing the oviducts. Cumulus-oocyte complexes were loaded into 0.25 ml standard embryo straws (IMV, France). Straws were then placed into the chamber of a computer controlled, hydrostatic pressure generating device (Cryo-Innovation Ltd., Budapest, Hungary) and the sublethal stress treatments were performed by generating 20 MPa HHP for 60 min at 37°C. Treated and control oocytes were freed from cumulus cells by exposing them to 0.1% hyaluronidase (Sigma-Aldrich, St. Louis, USA) in CZB-Hepes medium for 1 min. Thereafter, oocytes were either frozen for RNA isolation (-80°C) or transferred back into CZB medium and returned to the incubator for ICSI.

All micromanipulations were performed using an Olympus IX 71 inverted microscope (Olympus Optical, Japan) equipped with a Piezo-driven micromanipulation system (PMAS-CT150, Prime Tech, Ibaraki, Japan). The injection was made in CZB-HEPES. Application of a few piezo-pulses separated the head from the tail (Kuretake et al., 1996). Isolated sperm heads were injected immediately into mouse oocytes.

After ICSI, mouse oocytes were further incubated then examined with an Olympus IX microscope with Hoffman differential interference optics (Olympus Optical, Tokyo, Japan) for evidence of activation. Zygotes exhibiting two pronuclei and a second polar body were cultured further till the 4-cell stage. 78% of the treated and 82% of the control oocytes survived the ICSI procedure, 76% and 65% out of them developed to the 4-cell stage, respectively, then they were frozen at -80°C for the gene expression study.

2.2. RT-qPCR analysis of the HHP-treated mouse blastocysts

The mRNA from 10-pooled blastocysts groups was isolated using Dynabeads mRNA DIRECT Micro Kit (Dynal A.S, Oslo, Norway), according to the manufacturer's instructions. The eluted mRNA was reverse transcribed using M-MLV RT Kit (Invitrogen, Carlsbad, CA) in a 20-µL final reaction volume.

For the real-time PCR studies primers were designed for the selected nine target genes, which were then optimized. Before normalization, the stable expression of the two best preimplantational endogenous control genes (Mamo et al. 2007) were preliminary validated under HHP treatment by using the $2^{-\Delta Ct}$ method (Schmittgen and Zakrajsek 2000).

For quantification of the mRNA 3-3 biological replicates of 10-pooled embryos were analysed, additionally positive and negative controls were included to every run. The reactions were performed using the Rotor-Gene 3000 real-time PCR machine (Corbett Research, Mortlake, Australia) with SYBR Green JumpStart Taq Ready Mix (Sigma-Aldrich, St. Louis, USA). Relative Expression Software Tool - 384 version 2 (Pfaffl et al. 2002) analysis was applied to determine the alterations of internal control and target genes.

2.3. Microarray analysis of HHP-treated oocytes and 4-cell stage embryos developed from the treated oocytes

Total RNA was isolated from treated and control groups of oocytes and 4-cell stage embryos using RNeasy Plus Micro Kit (Qiagen, Hilden, Germany). The RNA Integrity Number (RIN) was

determined with an Agilent 2100 Bioanalyzer instrument using RNA 6000 Pico Chip Kit (Agilent Technologies, Palo Alto, CA, USA).

TrueLabeling-PicoAMP kit (SABiosciences, Frederick, MD, USA) that utilizes an advanced two-round RNA amplification procedure was applied to amplify an appropriate quantity of cRNA from the total RNA of each sample. CyDye Post-Labeling Reactive Dye Pack (GE Healthcare, Waukesha, WI, USA) was used to generate labeled cRNA target, which was hybridized to Agilent 4×44K whole mouse genome chips (GPL4134, Agilent Technologies, Palo Alto, CA, USA). For microarray hybridization the manufacturer's Two-Color Microarray-Based Gene Expression Analysis protocol was followed. After scanning the high-resolution image files, they were processed by the Feature Extraction (version 9.5.1.1, Agilent Technologies, Palo Alto, USA) software. The data of the whole mouse genome chip was subjected to LOWESS normalization, then further analysed by GeneSpring GX 11 (Agilent Technologies, Palo Alto, CA, USA). To validate the gene expression profiling results, data of three biological replicates were analyzed by RT-qPCR.

For the validation of the microarray results 12 and 8 differentially expressed genes were selected from the oocyte and 4-cell stage microarray study, respectively. Primers for representative genes were designed and their gene expression was determined by RT-qPCR. The H2afz, Hprt1 and Ppia genes served as endogenous controls as they have been established to be reliable housekeeping genes during *in vivo* and *in vitro* preimplantation development (Mamo et al., 2007). The isolated RNA from the oocytes and embryos were reverse transcribed using a SuperScript VILO cDNA Synthesis Kit (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions. QPCR reactions were set up using a QIAgility liquid handling robot and performed on Rotor-Gene Q cycler (Qiagen, Hilden, Germany), using SYBR Green JumpStart Taq ReadyMix (Sigma Aldrich, St. Louis, USA). Data of three biological replicates were analyzed by RT-qPCR for each gene, using the Relative Expression Software Tool 2008 V2.0.7 (Pfaffl et al., 2002). The similarity between the results of the microarray measurement and the RT-qPCR validation was assessed by Pearson correlation analysis.

GenesisWeb Hierarchical Clustering analysis with Spearman rank distance calculation was used to group the biological replicates based on the significantly regulated genes (Rainer et al., 2006). Pathway and annotated clustering analysis were performed using the functional annotation tools of DAVID Bioinformatics Resources (Dennis et al., 2003; Huang et al., 2009b) to determine the significantly overrepresented cellular mechanisms. All microarray data were deposited to the NCBI Gene Expression Omnibus database (GEO accession #GSE28443).

3. RESULTS

3.1. The stable expression of the endogenous reference genes after the HHP-treatment

For the appropriate normalization of RT-qPCR experiments stable expressing reference genes are necessary, thus it is indispensable to prove that the applied treatment of the respective experiment does not affect their expression (Bustin et al. 2009). Stable expression of endogenous control H2A histone family, member Z (H2afz) and peptidylprolyl isomerase A (Ppia) genes were preliminary validated under HHP treatment, by using the $2^{-\Delta Ct}$ method (Schmittgen and Zakrajsek, 2000). The expression of these genes was evaluated during a 120 min timescale at 4 different time points. One-way ANOVA analysis demonstrated that both H2afz and Ppia endogenous reference genes had constant expression during the 120 min culture after pressurization, compared to the untreated control. Accordingly, we normalized the expression values of the target genes to the geometric average of these reference genes to obtain reliable relative expression data.

3.2. The immediate and prolonged transcriptional response to HHP stress in mouse blastocysts

Based on the results no downregulation was detected immediately after the pressure treatment among the studied stress related genes ($P>0.05$). However, the antizyme inhibitor 1 (Azin1), with a relative expression close to 2-fold, and the superoxide dismutase 2, mitochondrial (Sod2) were significantly upregulated immediately after HHP treatment.

The general trend shows reduction in transcript quantity following 120 min culture for all genes, which was significant for the soluble superoxide dismutase 1 (Sod1) gene. The exceptions were the growth arrest and DNA-damage-inducible 45 gamma (Gadd45g) and the RNA binding motif protein 3 (Rbm3) by significantly increasing their expression during the culture. In conclusion, we observed significant transcriptional alterations (both up and downregulation) for certain stress-related genes as a result of the HHP treatment.

3.3. Global gene expression changes in mouse oocytes and 4-cell embryos in response to HP treatment

To achieve a comprehensive, transcriptome-scale analysis of the HHP effect, we performed global gene expression profiling experiments on: (i) untreated and HHP-treated mouse oocytes, and (ii) mouse embryos at the 4-cell stage that were developed from ICSI-fertilized untreated or HHP-treated oocytes. As the p-values were not corrected by Benjamini Hochberg multiple test, only those experiments were subjected to further analysis that passed the extensive RT-qPCR validation.

The result of the RT-qPCR validation of 12 studied genes did not confirm the expressional changes detected in the oocyte microarray-experiment (Pearson correlation coefficient, r=0,02). Moreover, for all the studied genes it showed unchanged transcript level. In conclusion, true significant changes could not be observed at the oocyte level following HHP treatment. These results along with the high RNA Integrity Numbers of the RNA isolated from HHP-treated oocytes (8.975±0.206; n=4) indicated that the optimally adjusted HHP treatment did not induce global RNA degradation. Based on our results the HHP treatment did not perturb the transcriptome of oocytes.

However, the same stress did result in a marked effect on transcription at the 4-cell stage showing a transcriptional reminiscence of the HHP-effect during the preimplantation development. At the 4-cell stage the RT-qPCR validation confirmed the expression of all the 8 differentially expressed genes selected for the analysis ($p \leq 0{,}015$; Pearson correlation coefficient, r=0,93), clearly indicating that the changes detected by the 4-cell stage microarray were true expressional alterations. The analysis identified 505 differentially expressed genes ($p<0.05$; fold change≥ 1.5), out of the 250 and 255 were up- and down- regulated, respectively.

During the next steps, the differentially expressed genes of the 4-cell stage microarray analysis were further processed by bioinformatics software. First, GenesisWeb hierarchical clustering showed that the various biological replicates of the sample and control groups at the 4-cell stage are consistent as they were classified into two distinct clusters based on the regulation of the 505 identified genes.

3.4. Major downregulation of translation related genes in preimplantation embryos

After the initial validation steps performed by RT-qPCR and hierarchical clustering analysis we aimed to study the function of the differentially expressed genes by identifying the affected pathways using the functional annotation tools of DAVID Bioinformatics Resources. We looked for Gene Ontology (GO) terms which contain significantly more genes than expected by random allocation. The false positive results were filtered out by statistic method (FDR < 0,05). We found

21 Gene Ontology categories to be significantly over-represented, mostly in the Cellular Component group. Translation was the only identified GO Biological Process, showing an extremely low multiple test corrected p-value, and consistent with this result, structural constituent of the ribosome ($p \leq 2.20E-19$) and ribosome ($p \leq 1.60E-21$) were the highest significantly enriched Molecular Function and Cellular Component categories, respectively. Several additional translation-related GO categories were enriched in genes with altered expression, suggesting that protein synthesis was markedly affected by the HP treatment. Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis further supported this notion as the ribosome was the only identified significant pathway. Furthermore, the functional characterization revealed that the overrepresented categories contained mostly downregulated genes. The highest percentage (90-100%) of the downregulated genes was observed in the translation related categories. Overall, the analysis strongly suggested that the protein synthesis machinery is inhibited in preimplantation embryos developed from HP-treated oocytes.

4. DISCUSSION

Today, there is a growing literature about the effect of high hydrostatic pressure on the cellular processes of mammalian gametes and embryos. Nevertheless, when I begun my work only the results of Huang et al. (2009a) were available in this field.

Primarily, my aim was to examine whether the high hydrostatic pressure has any impact on the expression of selected genes in mouse blastocysts. For this study candidate genes that might be HHP-responsive were selected based on references available for microbes. We also made use of the previous results of our research group about the global transcirptome profiling experiments on vitrified 8-cell stage embryos (Mamo et al. 2006). As a result of the selection we included genes into our study that might play a role in the cellular response to freezing stress. To represent several stress related pathways, we included genes related to growth arrest, oxidative stress, apoptosis and cold-shock.

Based on the analysis, the HHP induced the immediate upregulation of Azin1 and Sod2 genes and the delayed upregulation of Gadd45g and Rbm3. The further upregulation of Gadd45g and Rbm3 following 120 min *in vitro* culture shows that they are participating in the long-term response to HHP stress. After 120 min culture the general trend showed downregulation, out of which the change of superoxide dismutase 1 gene expression was significant. In agreement with this, Siqueira Filho et al. (2011) observed upregulation of the investigated genes after 1 hour of the HHP treatment of bovine blastocysts, thereafter diminishing expression of them. The Rbm3 is a cold-shock activated gene and its major role is to assist the protein synthesis when temperature of the cells decreases below the physiological value (Dresios et al. 2005). The elevated level of the Rbm3 mRNA indicates that the HHP treatment might activate certain cell responses that are participating in protection against the freezing/thawing stresses. The different expression pattern of the superoxide dismutase 1, soluble (Sod1) and the superoxide dismutase 2, mitochondrial (Sod2) genes may indicate that the cytoplasm is more prone to the oxidative stress than the mitochondria during the pressure stress. The Gadd45g is a negative regulator of cell growth (Zhan et al. 1994), and the Azin1 also regulates cell growth through the polyamine synthesis (Tang et al. 2009). The overexpression of these genes at distinct time points is a possible indication that cells protect themselves against pressure stress by delaying their growth till normal conditions are restored. Recent studies identified Gadd45g, Azin1 and Gas5 as putative endoplasmic reticulum (ER) stress response genes (Belmont et al. 2008, Williams and Lipkin 2006). The fact that all these genes were affected by the HHP treatment supports the finding that the ER membrane is hydrostatic pressure sensitive (Mentré et al. 1999; Frey et al. 2008). The ER stress causes the accumulation of unfolded proteins in the lumen of the rough endoplasmic reticulum. To alleviate the stress protein folding and

degradation pathways in the ER are activated, that inhibit protein synthesis (Rutkowski and Kaufman 2004). The fact that these genes were affected by the HHP treatment suggests that some of the HHP provoked transcriptional changes detected in the embryo is connected to the stress response of the ER. Those changes may result in the slowdown of cell growth and translation. In agreement with this, the GADD gene family is presumed to be activated by the ER stress and thereafter it regulates the cell division and growth (Outinen et al. 1999).

Based on the results of the RT-qPCR assay, the expression of certain growth-arrest, cold-shock and oxidative-stress related genes were significantly altered as a consequence of the treatment. Later on further research groups reported similar findings (Bogliolo et al. 2011; Siqueira Filho et al. 2011). Although we demonstrated, that the high hydrostatic pressure treatment has an effect on the gene expression of mouse blastocysts, this study was limited to the expression analysis of only a few selected genes. Accordingly, to comprehensively investigate the cellular processes affected by the pressure-stress, a study covering the whole transcriptome was needed. For that purpose gene expression microarrays are widely used. However, studying very limited number of cells (e.g. oocytes and embryos) by microarray was not feasible for a long time, due to the high RNA requirement of this technique (Bermudez et al. 2004; Kalisky and Quake 2011). Nowadays different exponential and linear amplification techniques provide solution for this limitation: (Eberwine et al. 1992; Van Gelder et al. 1990) the most prevalent methods are based on polymerase chain reaction (PCR) and *in vitro* transcription (IVT; Chiang and Melton 2003, Luo et al. 1999), which allow studying the whole transcriptome even of a single cell. Using IVT amplification in our study sufficient amount RNA could be obtained for the microarray experiments.

We performed gene expression microarray experiments initially on mouse oocytes immediately after the HHP stress and then on four-cell stage embryos developed from HHP-treated oocytes after fertilization by ICSI, and our aim was to reveal which intracellular processes are activated or inhibited by the treatment. The mouse was chosen as the model system since the advantageous effect of HHP has already proven for this species (Pribenszky et al. 2010b; Pribenszky et al. 2012) and its genome is broadly studied and well characterized (Stamatoyannopoulos et al. 2012).

We performed gene expression microarray experiments initially on matured oocytes immediately after the HHP stress. As until the embryonic genome activation (EGA) the maternal RNA is indispensable, in the first part of the experiments we studied whether the HHP stress affected the RNA abundance of oocytes, e.g. by inducing selective degradation (Sirard 2012). Since the EGA occurs at the 2-cell stage in mouse embryos, initiating *de novo* RNA synthesis (Bolton et al. 1984; Li et al. 2010), treatment effects generated in the oocyte may become apparent at the transcriptional level in the subsequent stages of embryo development. Therefore, we also analyzed

the global gene expression pattern of four-cell stage embryos developed from HHP-treated oocytes after fertilization with ICSI. This way, we were able to follow the effect of HHP stress during mammalian preimplantation development.

The transcription profiling study of the HHP-treated oocytes and four-cell stage embryos that developed from these oocytes showed distinct responses to the applied stress. HHP treatment did not perturb the transcriptome of oocytes, and the high RNA Integrity Number values of the RNAs isolated from the HHP-treated oocytes indicated that the treatment did not induce RNA degradation. Importantly, this observation indicates that regarding the RNA pool of the oocytes the HHP technique is safe and does not affect RNA integrity.

However, the same stress did result in a marked effect on transcription at the 4-cell stage. The result of the microarray analysis suggests that the HHP has a significant outcome on the intracellular processes of the oocytes that are not immediately visible on the transcriptional level after the treatment. Nevertheless, after EGA the HHP treatment evokes marked changes on the expression of certain genes.

To gain insights into the molecular mechanisms involved, we used functional annotation clustering to identify the genes showing significantly altered expression in 4-cell embryos. A large number of translation related genes were affected by the HHP treatment, exhibiting massive downregulation. The robust repression suggested that the HHP stress inhibited ribosome assembly and thus transiently reduced the rate of the protein synthesis during preimplantation development.
Our results are consistent with the well-known phenomenon in microbes, where high pressure induces ribosomal dissociation (Schulz *et al.*, 1976; Gross and Jaenicke, 1990; Gross *et al.*, 1993, Niven *et al.*, 1999, Alpas *et al.*, 2003), and is suggested to be one of the principal causes of pressure-induced growth inhibition (Gross *et al.*, 1993). Nevertheless, it should be noted that in *E. coli* the pressure-induced ribosome disassembly is completely reversible for pressures under 100 MPa; after releasing the pressure protein synthesis is resumed (Mackey and Mañas, 2008). Similarly, the inactivation of rat liver ribosomes after HP treatment *in vitro* was reported which was almost completely reversible when pressures under 120 MPa were used (Lu et al. 1997). Thus it is possible that optimized HHP treatment transiently inhibits the protein synthesis in mammalian oocytes and embryos.

Ribosome biosynthesis is the most energy-consuming process in eukaryotic cells (Warner, 1999; Warner *et al*, 2001). Consequently, the transient arrest upon HP treatment might result in intracellular energy saving that might help the cells to adapt to the altered environment. This hypothesis is further supported by the finding that frozen-thawed human embryos arrested prior to the blastocyst stage are metabolically more active in terms of amino acid turnover than their developing counterparts (Stokes *et al.*, 2007). These results and our theory is in agreement with the

Quiet Embryo Hypothesis, which suggests that preimplantation embryos with lower overall metabolism are more viable (Leese *et al*., 2008; Leese, 2012). In addition to HHP, optimized hydrogen-peroxide treatment of bovine oocytes has been shown to enhance the efficiency of *in vitro* embryo culture (Vandaele et al. 2010). Similarly, osmotic stress treatments on porcine oocytes resulted in improved cryotolerance of the cells (Lin et al. 2009). As oxidative and osmotic stress transiently reduce the rate of protein synthesis in mammalian cells (Wiese et al. 1995; Morley and Naegele 2002), it cannot be excluded that these stressors evoke a similar response to the HHP for enhancing the stress tolerance of preimplantation embryos.

4.1. Hypothesis regarding the beneficial effect of HHP on mammalian gametes and embryos

The presented data have shown that optimized high hydrostatic pressure treatment results in the transcriptional inhibition of protein synthesis in mouse embryos. Based on the reference literature it can be assumed that ribosomes of mouse oocytes disassemble as pressure increases. Nevertheless, this is a completely reversible process if the pressure level is optimal. After pressure is restored to the physiological level the ribosomes assemble and protein synthesis can be continued. Temporal slowdown of the cellular metabolism is not beneficial per se, however, when the cells encounter a further (e.g. freezing) stress they are already in a preconditioned state in which the damages can be better tolerated. Based on our hypothesis the higher developmental competence of the pressure-treated oocytes was supported by reduced metabolism rate and the accumulation of metabolites, which allows the cells to be more flexible to a further shock than cells grown in normal conditions. Thereby the effect of HHP may contribute to the better stress-tolerance of mouse oocytes that could help them to overcome to the harmful effect of cryopreservation. These results are in agreement with previous observations that optimized high hydrostatic pressure does not impair the oocyte development; moreover, if it is combined with another stress treatment it could improve the developmental and blastocyst rates of the embryos.

4.2. Further suggestions

The global gene expression profiling of mouse oocytes and embryos allowed studying the whole transcriptome of the cells, hence our results provide a comprehensive picture on the transcriptional effects of HHP. These results suggest a potential mechanism for how HHP preconditions the reproductive cells and embryos, which might open new ways for studying and further developing the cryopreservation techniques. Nevertheless, in the future it would be

important to study the HHP effect in combination with another (e.g. freezing) stress. This way, further details on the protective mechanism of the HHP treatment could be revealed.

My hypothesis presumes a general effect, thus it would also be interesting to study later embryonic stages during the preimplantation development. The hypothesis could be further strengthened if other embryonic stages would show similar translational inhibition as a result of pressure treatment. When appropriate embryonic stages are chosen then the recovery of the protein synthesis might be followed as well.

Another question raised by our results, whether the reversible translational inhibition in gametes and embryos can be triggered by other methods. If such an effect could be achieved, it would be possible to investigate whether or not it is beneficial for the cells. It has already been shown that optimized hydrogen-peroxide treatment can have some protective effect (Vandaele et al. 2010). It is also known that this compound transitently inhibites the protein syntheis (Wiese et al. 1995) accordingly, it might also be possible that if we hinder translation using such chemicals, this evokes a protective effect on gametes and embryos. For this purpose antibiotics could be used that affect eukaryotic protein synthesis (e.g. cycloheximide, puromycin). Indeed, it was shown that optimized cycloheximide treatment has a favourable effect on the development of parthenogentically activated embryos (Mori et al. 2008).

I think that by using appropriate compounds under optimized conditions positive effects could be achieved, however, its degree wouldn't exceed the effect of HHP. The main obstacle is that it would be more difficult to optimally apply chemicals than pressure. Unlike HHP, chemical compounds do not act instantly and uniformly at every point of the sample, and therefore optimization would be much more challenging. Moreover, the relative speed of action in case of HHP is much faster than for chemical treatments. Pressure can be rapidly applied as well as removed from the system, therefore it affects only a minor period during preimplantation development. Considering that chemical inhibition has a completely different mechanism compared to HHP, it could be successfully applied only if its effect would be reversible (like hydrogen-peroxide) without harmful side-effects.

It is also important that sometimes there is no clear correlation between the RNA and protein expression of a gene. Accordingly, the protein-level validation of our result could strengthen the delineated hypothesis on the protective effect of this optimized stress treatment. If our result could be further verified on protein level, then they could be used to improve protocols of different assisted reproduction techniques.

5. NEW SCIENTIFIC RESULTS

1. It was the first time to show that high hydrostatic pressure affects the mRNA level of certain genes in mouse embryos.

2. It was the first time to study the changes of the transcirptome in mouse oocytes and four-cell stage embryos after treating them with high hydrostatic pressure.

3. Based on my results, optimized high hydrostatic pressure treatment does not perturb the transcriptome of mouse oocytes, and the treatment does not induce RNA degradation. Thus, I provided evidence regarding the safety of the technique.

4. I have shown that in four-cell stage embryos the expression of numerous genes involved in protein synthesis is downregulated as a result of the optimized high hydrostatic pressure treatment.

5. Based on my result and the available literature, I established a hypothesis describing that optimized high hydrostatic pressure treatment cause transient inhibition of cellular protein synthesis. Consequently, certain metabolites are accumulated, which might help the cells to better tolerate the harmful effects of a further stress.

6. PUBLICATIONS

Articles in international journals with impact factors

Bock I., Raveh-Amit H., Losonczi E., Carstea A.C., Feher A., Mashayekhi K., Matyas S., Dinnyes A., Pribenszky C. (2014): Controlled hydrostatic pressure stress downregulates the expression of ribosomal genes in preimplantation embryos: a possible protection mechanism? *Reproduction, Fertility and Development* (in press) [IF: 2,56]

Bock I., Losonczi E., Mamo S., Polgar Z., Harnos A., Dinnyes A., Pribenszky C. (2010): Stress tolerance and transcriptional response in mouse embryos treated with high hydrostatic pressure to enhance cryotolerance. *CryoLetters* 31:401-12. [IF: 1,12]

Kobolak J., Kiss K., Polgar Z., Mamo S., Rogel-Gaillard C., Tancos Z., **Bock I.**, Baji A.G., Tar K., Pirity M.K., Dinnyes A. (2009): Promoter analysis of the rabbit POU5F1 gene and its expression in preimplantation stage embryos. *BMC Molecular Biology* 10:88. [IF: 2,89]

Furhter articles in international journals with impact factors

Lovrics A., Gao Y., Juhasz B., **Bock I.**, Byrne H.M., Dinnyes A., Kovacs. K.A. (2014): Boolean Modelling Reveals New Regulatory Connections between Transcription Factors Orchestrating the Development of the Ventral Spinal Cord. *PLoS ONE* 9: e111430. [IF: 4,24]

Rungarunlert S., Klincumhom N., **Bock I.**, Nemes Cs., Techakumphu M., Pirity M.K., Dinnyes A. (2011): Enhanced cardiac differentiation of mouse embryonic stem cells by use of the slow turning lateral vessel (STLV) bioreactor. *Biotechnology Letters* 33:1565-1573. [IF: 1,68]

Conference abstracts in international journals with impact factor

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Pribenszky C., Matyas S., Losonczi E., Stanca C., **Bock I.**, Vajta G. (2010): Stress for stress tolerance: improving cell survival by sublethal stress treatment of eggs before vitrification – pilot study. 66th Annual Meeting of the American Society for Reproductive Medicine, Denver, 23-27.10.2010. *Fertility and Sterility* 94:S32. O-106. oral presentation, abstract. [IF: 3,12]

Bock I., Mamo S., Polgar Zs., Pribenszky C. (2008): Changes in gene expression of mouse blastocysts treated with high hydrostatic pressure pulse. 16th International Congress on Animal Reporoduction, Budapest, 13-17.07.2008. *Reproduction in Domestic Animals* 43:145-46. poster, abstract. [IF: 1,6]

Presentations, posters in international conferences

Polgar Zs., Tar K., Rungarunlert S., Muenthaisong S., **Bock I.**, Pirity M., Dinnyes A. (2009): Improved derivation of embryonic stem cell lines from inbred c57BL/6J mouse strains. 7th Annual Meeting of the International Society for Stem Cell Research, Barcelona, 8-11.09.2009. p. 104.

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