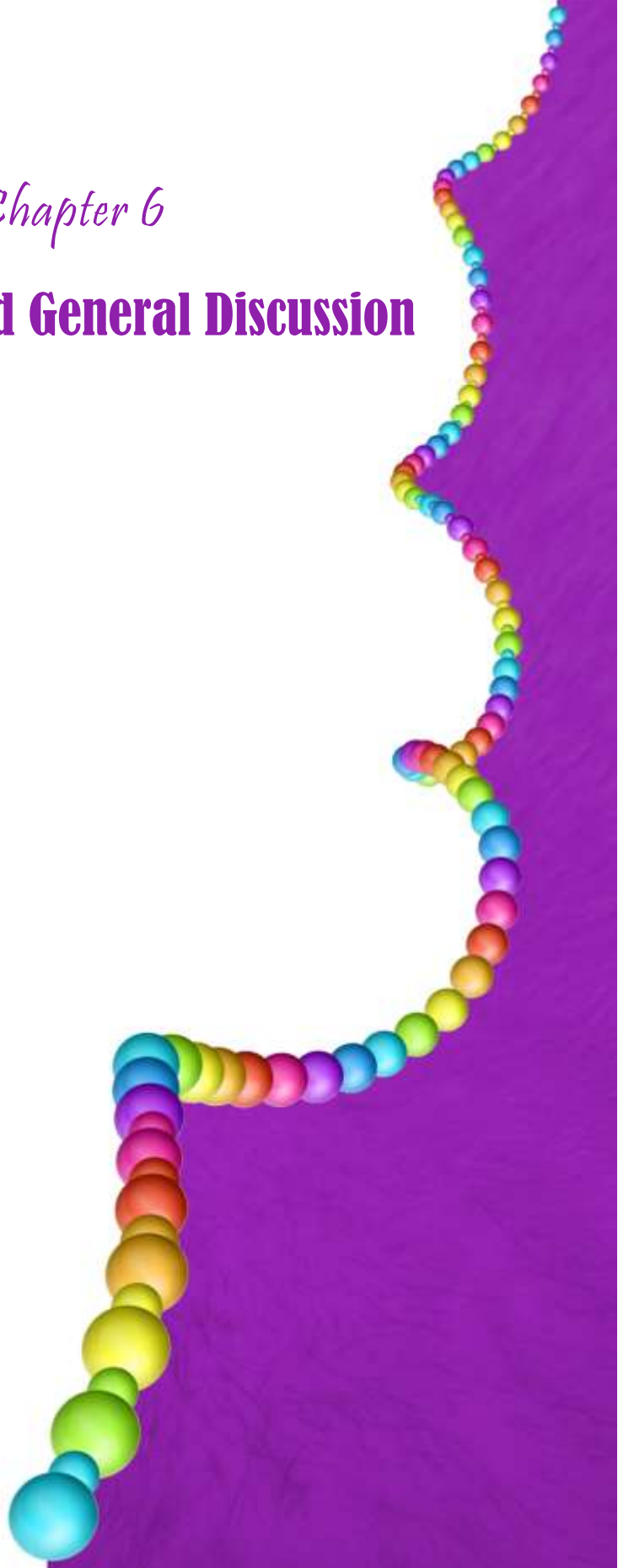
Transcriptomics close to my heart

Chapter 6

Summary and General Discussion



Current drug development and safety testing procedures are time consuming, expensive and not able to detect all human drug-induced toxicity risks, with occasional severe adverse drug reactions as a consequence. The adverse drug reactions also rank high as cause for disease or death[1]. In search for methods to improve *in vitro* toxicological drug safety assessments, we adopted an experimental design better reflecting the human *in vivo* conditions and investigated possibilities to improve toxicogenomics data analysis. Here, we predominantly focused on post-transcriptional mechanisms.

As an example toxicant, we mainly used doxorubicin (DOX), but to some extent also epirubicin (EPI) and idarubicin (IDA) were studied *in vitro* in the human cardiac 3D microtissue model. These chemotherapeutic agents belong to the group of anthracyclines (ANTs) and are known to dose-dependently induce cardiotoxicity[2,3]. The occurrence of cardiotoxicity is difficult to predict, because it can manifest at any stage during the treatment (reversible acute cardiotoxicity) or even months or years after the treatment has ended (irreversible delayed chronic cardiotoxicity) while the involved molecular mechanisms are only partially understood[2-6].

Experimental design

Within toxicology, the experimental design typical includes the use of a solvent like Dimethyl sulfoxide (DMSO) in order make a stable stock solution of the investigated compound. However, in the 1960s DMSO was researched for medical use due to its biological effects. During the clinical trials, high doses of DMSO induced extreme toxicities, especially to the eyes, though. Because the effects of DMSO differ depending on dose and route of administration, the FDA classified DMSO in the safest category with low toxic potential at levels normally accepted in pharmaceuticals[7]. This made the wide use of DMSO possible.

Though it often assumed that effects of DMSO are negligible[8-10], we evaluated the impact of 0.1% DMSO exposure using sensitive high-throughput techniques **(Chapter 2)**. We did this for the cardiac microtissue model relevant for this thesis, but also for the hepatic microtissue model used by other partners of the HeCaToS project.

DMSO effects on cellular processes were analyzed using full transcriptome data supplemented with proteomics data. More than 2000 differentially expressed genes (DEGs, FDR <0.05) and 650 differentially expressed proteins (DEPs, FDR <0.05) were detected in both tissue types, of which more than half were downregulated. Pathway analysis indicated that similar biological processes were affected in both cell types, indicating consistent cross-organ actions of DMSO.

Observed DMSO-induced changes in cellular processes included changes in mitochondrial pathways linked to ROS production and cellular ATP generation. Functional measurements of microtissue ATP content for the hepatic model revealed a small initial decrease (36% decrease in the first 72h), after which ATP content slowly recovered to baseline level. However, a steep decrease over time (87% decrease after 2 weeks of exposure) was observed for cardiac microtissues. This decrease in ATP production may impact toxicological research because changes in the amount of free energy in the cell influences the capability of the cell to deal with induced stresses, thereby potentially leading to erroneous interpretation of results from *in vitro* assays. The DMSO-induced ATP decrease is of even more relevance for the field of assisted reproductive technology, in which DMSO is used for cryopreservation of oocytes and embryos for IVF treatments. Here the ATP content is used as a predictor of embryo viability. Decreased ATP levels, especially in the cleavage-stage, can induce downstream effects that may disrupt cellular function, implantation ability and fetal development[11,12].

While observed tissue-specific differences may be due to tissue type, they may also result from a fundamental difference in the cell model, because the cardiac model contains iPSC-derived human cardiomyocytes that are still maturing, while the hepatic

model contains mature human hepatocytes. Expanding our research to the epigenetic landscape also revealed tissue-specific effects of DMSO. Analysis of miRNAs indicated a complete deregulation of cardiac miRNA biogenesis and miRNA content. Though changes in hepatic miRNA biogenesis were minimal, still 18% of detected miRNAs were significantly changed by DMSO exposure.

Furthermore, we showed, for the first time, the influence of DMSO on the DNA methylation patterns of human cells *in vitro*. Whole genome methylation profiling by MeDIP-seq revealed 66,178 differentially methylated regions (DMRs; q-value <0.05) in cardiac microtissues with 71% hypermethylated, while in hepatic microtissues, no DMRs passed correction for multiple testing. While repetitive sequences appeared preferentially affected, findings also suggested a global deregulation of DNA methylation mechanisms leading to genome-wide changes. It is uncertain if exposed cells can recover from temporary DMSO exposures. While methylation changes have an adaptive nature, they may also be persistent and even heritable by the offspring to induce transgenerational effects[13-17]. This may pose a threat, especially for cryopreserved oocytes and embryos for IVF treatment which are exposed to high concentrations of DMSO during the periconceptional period (before conception until early pregnancy), when epigenetic reprogramming is taking place.

Overall, the extreme changes in microRNAs and alterations in the epigenetic landscape indicated that DMSO is not inert. Therefore, use of DMSO should be avoided where possible. However, for the time being, DMSO is indispensable within biotechnological applications, including toxicological research. To reduce the impact of DMSO, concentrations should be kept as low as possible.

A decrease in DMSO concentration was incorporated within the innovative *in vitro* experimental design which we validated in **Chapter 3**. Next to using the human cardiac microtissue model, which better resembles the human heart *in vivo* due to its 3D spheroid structure and the co-culturing of both cardiomyocytes and fibroblasts, we also included physiologically based pharmacokinetic (PBPK) modelling in order to

achieve a repetitive dosing profile which resembled the *in vivo* drug concentrations in the human heart. The highest exposure was 0.06% DMSO for 2 hours per day, continuing with 0.006% for 6 hours and ending with 0.0025% for 16 hours.

This experimental design was validated by assessing whether our model was able to retrieve the known mechanisms of DOX through pathway analysis and in depth analysis of mitochondrial dysfunction. While DOX mechanisms of action are not fully understood, it is generally accepted that the main mode of action is related to killing dividing cells. DOX has been found to 1) intercalate into DNA, 2) target DNA topoisomerases, and 3) generate reactive oxygen species (ROS)[18-20]. The first two processes inhibit unwinding of DNA, DNA replication, RNA transcription and protein biosynthesis. As a result, proliferation of dividing cells is also inhibited[21,22]. This is thought to be the efficacy of the anti-cancer effects of DOX, while ROS generation is mainly ascribed to toxic effects[23]. The induced oxidative stress may damage cells and cause cell death[19,24]. Other detrimental actions of DOX can be related to death of non-cancer cells or to decreased cardiomyocyte functioning, which may partly result from mitochondrial dysfunction causing an imbalance in cellular energetics[25]. The detrimental actions of DOX, known to occur *in vivo*, can thus be summarized into 3 toxicity processes: cardiomyocyte dysfunction, mitochondrial dysfunction and cell death. We were able to successfully detect biological changes in these three processes in our *in vitro* model by applying next-generation total RNA-sequencing to ribo-depleted RNA samples.

Pathway analysis of differentially expressed genes (FDR<0.05) identified toxicity of DOX by gene expression changes in the pathways of “striated muscle contraction” (= cardiac dysfunction), “TCA cycle & respiratory electron transport” (= mitochondrial dysfunction) and “cellular senescence” (= cell death). A central link between the toxicity pathways was the “formation of ATP by chemiosmotic coupling”. Mitochondrial gene expression changes correlated with ATP measurements, in which the therapeutic dose displayed a relatively stable ATP content, while ATP content during toxic exposure decreased drastically over time. A more detailed analysis of mitochondrial dysfunction revealed gene expression changes in the process of ROS

detoxification, which confirms DOX-induced ROS generation in our *in vitro* model. Furthermore, effects of DOX on the electron transport chain (ETC) and ATP production was observed, with upregulations across time for the therapeutic exposure and a majority of downregulations during the second half of toxic exposures.

Many studies on DOX effects have been performed with high doses in order to observe toxic effects. However, our *in vitro* model clearly demonstrated a dose-dependent effect of DOX exposure, highlighting the importance of applying physiologically relevant dosing profiles during toxicological research. Exposure to the toxic dosing profile reflected processes of acute cardiotoxicity, as observed though a decrease in ATP content and an increase in cell death. On the other hand, therapeutic doses reflected processes underlying the phenotype of delayed chronic cardiac toxicity. The increase in gene expression of mitochondrial and ETC genes was necessary in order to maintain a stable cellular ATP content, indicating an adaptation response. Because genes involved in striated muscle contraction were already showing decreased expression levels, this adaptation response may not be sufficient for maintaining cardiac function. Cumulative toxicity may therefore surpasses the point to which the organ can adapt, thereafter resulting in a delayed phenotype of heart failure, as is observed in patients with chronic cardiotoxicity after DOX treatment[25,26]. These same dose-dependent effects were also observed for the other ANTs (EPI and IDA) analysed in **Chapter 4**.

Advanced transcriptomics analysis

In **Chapter 4**, we went beyond conventional gene-expression analysis by investigation of ANT-induced changes in alternative splicing. Through alternative splicing, a single gene encodes multiple variants of RNA transcripts with distinct functions[27]. This greatly increases the diversity of the transcriptome[28,29], which can be measured using RNA-sequencing technology. It is estimated that 92-94% of human genes are subjected to alternative splicing[27]. During gene expression analysis, the sum of these transcripts is assessed. However, due to differences in biological function of alternatively spliced transcripts, not only changes in gene expression, but also changes

in ratios of expressed transcripts (differential transcript usage; DTU) can result in a distinct phenotype[29,30]. Therefore it is important to make use of technological advances of recent years to analyze DTU for elucidating toxicological mechanisms.

ANT-induced changes in alternative splicing were investigated for sarcomeric genes. The sarcomere is the cellular compartment facilitating contraction and is therefore highly important for heart function and cardiotoxic effects. The most interesting ANT-induced changes were observed for the titin gene (TTN) which is essential for maintaining the structure of the sarcomere because it forms the base for the assembly of the contractile machinery. During relaxation, its protein product unfolds and refolds during contraction. Furthermore, the *in vitro* observed ANT-induced changes could also be detected in human biopsies of heart failure patients with or without prior ANT therapy, confirming the clinical relevance of these observed effects. While the expression of the titin gene (TTN) differed between the ANTs, ranging from no significant changes (IDA) to downregulated at every time point (EPI), DTU analysis identified the most significant change in transcript usage for the TTN-206 transcript in all three ANTs (FDR: $6.96 \cdot 10^{-32}$, $2.04 \cdot 10^{-31}$ & $1.38057 \cdot 10^{-39}$ for DOX, EPI & IDA, resp.). The decrease in TTN-206 transcript usage was correlated *in vivo* with increased left ventricular (LV) mass index (p= 0.002), decreased LVEF (p=0.018) and was the only transcript close to significant (P=0.07) when comparing dilated vs undilated morphologies. The switch form TTN-206 (encoding H7C1P9, 962aa) to TTN-212 (encoding Q8WZ42, 26.926aa) changes the expression of a truncated titin protein variant. These truncated variants have the ability to impact the structural organization of sarcomeres and to disturb regulation of sarcomeric passive force generation and reduce the efficiency of contraction[31-33].

To summarize, while changes in overall TTN gene expression were unable to identify interesting ANT-induced effects, analysis of DTUs identified the decrease of TTN-206 which can be a highly promising as a biomarker for early detection of ANT-induced cardiotoxicity because it correlated with increased LV mass index, decreased LVEF and

can possibly distinguish between dilated and unaffected morphologies. This shows the added value of biological information to be gained from DTU analysis as compared to standard gene expression analysis.

Next to analysis of alternative splicing, other post-transcriptional mechanisms can also be investigated using RNA sequencing technologies. In **Chapter 5**, we focused on the incorporation of microRNA (miRNA) and circular RNA (circRNA) regulatory actions in transcriptomics analysis. MicroRNAs, small non-coding RNAs of approximately 22 nucleotides[1,34], can bind through complementary base pairing to regions within the 3'UTR of the mRNA (known as seed regions). This interaction can inhibit the translation of a mRNA. Furthermore, circRNAs, which are closed continuous loops without 5'caps or 3'tails which are generated through head-to-tail mRNA splicing[35,36], can also bind miRNAs. Because these circRNAs are highly stable and resistant against degradation, the miRNAs are captured and their inhibitory effect on translation is prevented[37].

Because regulatory mechanisms acting on translation are proposed to be the main cause for the notoriously poor correlation between the transcriptome and the proteome[38-40], we postulate that the assessment of these post-transcriptional mechanisms is important. On the short term, this will increase our understanding of biological and toxicological processes. On the long term, we might be able to incorporate the obtained knowledge to design an *in silico* prediction system to estimate the amount of translatable mRNAs, thereby obtaining a highly detailed genome-wide measurement with a biological relevance similar to proteomics data which can be used for toxicological assessments. We therefore designed a scoring system for genome-wide estimation of post-transcriptional miRNA and circRNAs interactions on mRNA translation. This scoring system estimates the fraction of miRNAs which are able to inhibit mRNAs translation because they are not captured by circRNAs. We labelled the obtained score as the miRNA-induced inhibition of translation (MIT) score, of which the calculation was based on the expression values and predicted interactions of mRNA, miRNA and circRNA.

Using the cardiac microtissues exposed to the therapeutic DOX dosing profile, we conducted a pilot study to validate our scoring system. Because a MIT score was obtained for every miRNA able to influence a target transcript, tens to hundreds of MIT scores were obtained per transcript. For interpretation of these MIT scores, we assessed the use of 1) MIT^{Mean}, which reflected the average inhibition efficiency of all miRNAs influencing a transcript, and 2) MIT^{Max}, which reflected the maximum MIT score because we envisioned that this miRNA was responsible for the inhibitory effect while effects of other miRNAs might be negligible in comparison. To determine which of these parameters best disclosed relevant biological processes within the cell, we first estimated the amount of translatable read count (TRC) using the MIT^{Mean} and the MIT^{Max}. Thereafter, we assessed whether the correlation between the proteome and the TRCs was improved compared to the correlation of the proteome with RNA read counts. We found improvements of correlation for 47.5% of cases using TRC^{Mean} values and 45% of cases using TRC^{Max} values. While the absolute improvement of TRC^{Mean} was very low ($\Delta r<0.05$), TRC^{Max} indicated more relevant improvements of correlation ($0.05 < \Delta r < 0.886$). Furthermore, when plotting the protein-abundances and TRC^{Max} values, the obtained graphs were visually very similar, which was not the case for RNA read counts. This demonstrated the ability of the MIT^{Max} and the TRC^{Max} to reflect biologically relevant translation inhibition processes.

Using the scoring system, we identified significant DOX-induced changes in miRNA-circRNA regulation in 60% of investigated transcripts (n=480). Because 63% of the significantly changes comprised an increase in MIT^{Max} score, we concluded that DOX was capable of strongly decreasing RNA translation through changes in regulatory miRNA-circRNA processes. Overall, the ability of the scoring system to identify changes in miRNA-circRNA regulatory systems as confirmed by the proteomics data, highlights the importance for assessment of post-transcriptional mechanisms, not only in toxicogenomics research but in genomics research in general.

Conclusions and Future perspectives

We hypothesized that the use of an experimental design better reflecting the human *in vivo* conditions combined with analysis of post-transcriptional mechanisms would aid the evolution of toxicogenomics research in order to improve drug safety assessments in the future. Using the ANTs as a case study, we indeed showed the possibilities of improving the experimental design to relate more closely to the *in vivo* situation and displayed the vast amount of information gained from studying post-transcriptional mechanisms.

In **Chapter 5** we showed the importance for quantifying post-transcriptional effects of miRNA-circRNA regulation of translation through the use of a scoring system to identify biologically relevant toxicant-changes which may play a role in toxicity.

Because the scoring system is currently restricted to the estimation of miRNAs and circRNAs regulatory effects, expansion to include other post-transcriptional mechanisms (such as alternative splicing) may be considered. Furthermore, the current scoring system may benefit from optimization through the use of more recently developed tools for circRNA prediction approaches (e.g. CIRCexplorer2[41] or CIRI2[42]) and possibly the use of an alternative parameter for optimal interpretation. While our pilot study indicated that the miRNA with the maximum MIT score was better suited for biological interpretation of translation inhibition, compared to the average of the obtained MIT scores, this means that effects of other miRNAs are neglected. Alternatively, the scoring system could be adapted to return the combined effects of all miRNAs affecting a target transcript. Another possibility would be to use a machine learning approach which trains, validates and tests the scoring system in order to improve the precision of the prediction. However, most important would be to validate the scoring system using a multitude of compounds for which the results can be validated using literature or laboratory experiments.

In **Chapter 4**, analysis of DTU revealed highly significant changes for the *titin* gene, which were not detected using observed standard gene expression analysis. This clearly demonstrated the added value for analyzing RNA splicing effects after exposure to a toxicant.

Human patient biopsies were used to validate *in vitro* DTU observations *in vivo*. Because of the low number of biopsies available from ANT treated patients (n=7) and the observed heterogeneity of these human biopsies, the research would clearly benefit from additional validation efforts using more patient samples. Recently we obtained a new batch of human cardiac biopsies which could be included in the analysis, resulting in a total of 15 biopsies available from ANT treated patients. Furthermore, because the biological function of many of transcript isoforms has not been determined, we could only speculate about the biological impact of changes observed for these transcripts. Loss-of-function assays (e.g. RNAi screening[43]) to elucidate the functions of transcript variants are necessary (for both coding and non-coding transcripts) in order to draw reliable biological conclusions. Finally, our cell model contains induced pluripotent stem cells (iPSC)-derived cardiomyocytes which are known to acquire genetic variation during reprogramming of the cells, implying that batches of microtissues were not identical between the different ANT-exposures. Therefore, observed DTU *in vitro* could also have been due to different single nucleotide polymorphisms (SNPs) existing between the batches. Therefore, for the presented study, we regarded findings as reliable when detected in both *in vitro* microtissues and *in vivo* biopsies. For future research on DTU using iPSC-derived cell models, a multitude of iPSC batches should be exposed to obtain results across a variety of genomic backgrounds and the iPSC batches should be identical between exposure studies in order to facilitate comparability.

In **Chapter 3**, our *in vitro* model including 3D microtissues and PBPK-based exposure profiles revealed a clear difference in activated biological mechanisms between the therapeutic and the toxic ANT exposure profile. This clearly indicates that future toxicological research should be performed at doses to which the human population can be exposed in order to obtain relevant safety assessments.

It should be taken into account that here only the effect of DOX on heart microtissues was investigated. For developing a predictive model for repeated dose heart toxicity, future research should thus be performed using additional toxicants and other tissue types (e.g. liver microtissues). Furthermore, next to 3D microtissues, also other *in vitro* systems mimicking the *in vivo* environment have been developed (e.g. organoids) which could also be of interest for toxicological research. Similar to microtissues, organoids are *in vitro* 3D models gained from pluripotent stem cells (either embryonic or induced) or primary tissue[44]. Organoids are physiologically closer to *in vivo* then microtissues because they contain more cell types, are larger in size and are capable of self-renewal. Because of this self-renewal, they can also be cultured for extended time. These features would be beneficial for toxicity assessment. However, culturing techniques for organoids are much more complex compared to culturing of microtissues, which could hamper large scale toxicity assessments.

Chapter 2 revealed that, contradictory to general assumptions, DMSO is not an inert solvent. Even at the commonly used "low" concentration of 0.1%, DMSO was able to induce extreme changes in the epigenetic landscape. With the development of more sensitive analysis techniques, the doses used for toxicological research are also decreasing. In **Chapter 3** we highlighted the need for investigating relevant doses. For DOX, the highest used doses were 0.22µM and 0.65µM for the therapeutic and toxic ANT exposure respectively. Compared to these low doses, these extremely high concentration of 14.1 mM DMSO (=0.1%) could induce changes which may result in erroneous conclusions gained from *in vitro* assays, such as false negative drug toxicity conclusions. Which brings us to a final but very strong message: In the future, avoid using DMSO where possible!

Overall, this thesis demonstrates the benefits of using advanced models & methods for toxicogenomics research. We introduced an advanced experimental design better reflecting the human *in vivo* conditions through incorporation of a 3D cell culture model, exposure to physiologically relevant concentrations and a reduction of the DMSO concentration. Furthermore, we highlighted the added value of advanced toxicogenomics data analysis through assessment of post-transcriptional mechanisms such as alternative splicing and translational regulation by miRNAs and circRNAs. Application of this model and these methods could improve future *in vitro* toxicological drug safety assessments.

References

1 Verheijen, M., Krauskopf, J., Kleinjans, J. C., de Kok, T. M. & Caiment, F. Development and regulatory application of microRNA biomarkers. Biomark Med 9, 1137-1151, doi:10.2217/bmm.15.50 (2015).

2 Bernstein, D. Anthracycline Cardiotoxicity Worrisome Enough to Have You Quaking? Circ Res 122, 188-190, doi:10.1161/Circresaha.117.312395 (2018).

3 McGowan, J. V. et al. Anthracycline Chemotherapy and Cardiotoxicity. Cardiovasc Drug Ther 31, 63-75, doi:10.1007/s10557-016-6711-0 (2017).

4 Maillet, A. et al. Modeling Doxorubicin-Induced Cardiotoxicity in Human Pluripotent Stem Cell Derived-Cardiomyocytes. Sci Rep 6, 25333, doi:10.1038/srep25333 (2016).

5 Cappetta, D., Urbanek, K., Rossi, F. & De Angelis, A. Anthracycline cardiotoxicity: new actors on the stage. Transl Cancer Res 7, S580-S583, doi:10.21037/tcr.2018.04.24 (2018).

6 Chatterjee, K., Zhang, J. Q., Honbo, N. & Karliner, J. S. Doxorubicin Cardiomyopathy. Cardiology 115, 155-162, doi:10.1159/000265166 (2010).

7 FDA. <www.fda.gov/downloads/drugs/guidancecomplianceregulatoryinformation/guidances/ucm073395.pdf> (2012).

8 Galvao, J. et al. Unexpected low-dose toxicity of the universal solvent DMSO. Faseb J 28, 1317-1330, doi:10.1096/fj.13-235440 (2014).

9 Sumida, K. et al. Effects of DMSO on gene expression in human and rat hepatocytes. Hum Exp Toxicol 30, 1701-1709, doi:10.1177/0960327111399325 (2011).

10 Yuan, Y. et al. Efficient long-term cryopreservation of pluripotent stem cells at-80 degrees C. Sci Rep-Uk 6, doi:ARTN 34476 10.1038/srep34476 (2016).

11 Lane, M. & Gardner, D. K. Understanding cellular disruptions during early embryo development that perturb viability and fetal development. Reprod Fert Develop 17, 371-378, doi:10.1071/Rd04102 (2005).

12 Benkhalifa, M. et al. Mitochondria: Participation to infertility as source of energy and cause of senescence. Int J Biochem Cell B 55, 60-64, doi:10.1016/j.biocel.2014.08.011 (2014).

13 Heijmans, B. T. et al. Persistent epigenetic differences associated with prenatal exposure to famine in humans. P Natl Acad Sci USA 105, 17046-17049, doi:10.1073/pnas.0806560105 (2008).

14 Tobi, E. W. et al. DNA methylation differences after exposure to prenatal famine are common and timing- and sex-specific. Hum Mol Genet 18, 4046-4053, doi:10.1093/hmg/ddp353 (2009).

15 Veenendaal, M. V. E. et al. Transgenerational effects of prenatal exposure to the 1944-45 Dutch famine. Bjog-Int J Obstet Gy 120, 548-554, doi:10.1111/1471-0528.12136 (2013).

16 De Rycke, M., Liebaers, I. & Van Steirteghem, A. Epigenetic risks related to assisted reproductive technologies - Risk analysis and epigenetic inheritance. Hum Reprod 17, 2487-2494, doi:DOI 10.1093/humrep/17.10.2487 (2002).

17 Hart, R. & Norman, R. J. The longer-term health outcomes for children born as a result of IVF treatment: Part IGeneral health outcomes. Hum Reprod Update 19, 232-243, doi:10.1093/humupd/dms062 (2013).

18 Damiani, R. M. et al. Pathways of cardiac toxicity: comparison between chemotherapeutic drugs doxorubicin and mitoxantrone. Arch Toxicol 90, 2063-2076, doi:10.1007/s00204-016-1759-y (2016).

19 Sorensen, J. C. et al. Mitochondria: Inadvertent targets in chemotherapy-induced skeletal muscle toxicity and wasting? Cancer Chemother Pharmacol 78, 673-683, doi:10.1007/s00280-016-3045-3 (2016).

20 Burridge, P. W. et al. Human induced pluripotent stem cell-derived cardiomyocytes recapitulate the predilection of breast cancer patients to doxorubicin-induced cardiotoxicity. Nat Med 22, 547-556, doi:10.1038/nm.4087 (2016).

21 Yang, F., Teves, S. S., Kemp, C. J. & Henikoff, S. Doxorubicin, DNA torsion, and chromatin dynamics. Biochim Biophys Acta 1845, 84-89, doi:10.1016/j.bbcan.2013.12.002 (2014).

22 Edwardson, D. W. et al. Role of Drug Metabolism in the Cytotoxicity and Clinical Efficacy of Anthracyclines. Curr Drug Metab 16, 412-426 (2015).

23 Berthiaume, J. M. & Wallace, K. B. Adriamycin-induced oxidative mitochondrial cardiotoxicity. Cell Biol Toxicol 23, 15-25, doi:10.1007/s10565-006-0140-y (2007).

24 Varga, Z. V., Ferdinandy, P., Liaudet, L. & Pacher, P. Drug-induced mitochondrial dysfunction and cardiotoxicity. Am J Physiol Heart Circ Physiol 309, H1453-1467, doi:10.1152/ajpheart.00554.2015 (2015).

25 Tokarska-Schlattner, M., Zaugg, M., Zuppinger, C., Wallimann, T. & Schlattner, U. New insights into doxorubicin-induced cardiotoxicity: The critical role of cellular energetics. J Mol Cell Cardiol 41, 389-405, doi:10.1016/j.yjmcc.2006.06.009 (2006).

26 Chen, B., Peng, X. Y., Pentassuglia, L., Lim, C. C. & Sawyer, D. B. Molecular and cellular mechanisms of anthracycline cardiotoxicity. Cardiovasc Toxicol 7, 114-121, doi:10.1007/s12012-007-0005-5 (2007).

27 Wang, E. T. et al. Alternative isoform regulation in human tissue transcriptomes. Nature 456, 470-476, doi:10.1038/nature07509 (2008).

28 van den Hoogenhof, M. M. G., Pinto, Y. M. & Creemers, E. E. RNA Splicing Regulation and Dysregulation in the Heart. Circ Res 118, 454-468, doi:10.1161/Circresaha.115.307872 (2016).

29 Richard, H. et al. Prediction of alternative isoforms from exon expression levels in RNA-Seq experiments. Nucleic Acids Res 38, doi:ARTN e112 10.1093/nar/gkq041 (2010).

30 de Souza, T. M. Bits and bytes in toxicogenomics: In silico approaches in liver-based mechanistic toxicology; Chapter 3. (2017).

31 Garfinkel, A. C., Seidman, J. G. & Seidman, C. E. Genetic Pathogenesis of Hypertrophic and Dilated Cardiomyopathy. Heart Fail Clin 14, 139-146, doi:10.1016/j.hfc.2017.12.004 (2018).

32 Herman, D. S. et al. Truncations of Titin Causing Dilated Cardiomyopathy. New Engl J Med 366, 619-628, doi:DOI 10.1056/NEJMoa1110186 (2012).

33 Fatkin, D. & Huttner, I. G. Titin-truncating mutations in dilated cardiomyopathy: the long and short of it. Curr Opin Cardiol 32, 232-238, doi:10.1097/Hco.0000000000000382 (2017).

34 Duchaine, T. F. & Fabian, M. R. Mechanistic Insights into MicroRNA-Mediated Gene Silencing. Csh Perspect Biol, a032771 (2018).

35 Liu, J., Liu, T., Wang, X. & He, A. Circles reshaping the RNA world: from waste to treasure. Mol Cancer 16, 58, doi:10.1186/s12943-017-0630-y (2017).

36 Qu, S. B. et al. Circular RNA: A new star of noncoding RNAs. Cancer Lett 365, 141-148, doi:10.1016/j.canlet.2015.06.003 (2015).

37 Memczak, S. et al. Circular RNAs are a large class of animal RNAs with regulatory potency. Nature 495, 333-338, doi:10.1038/nature11928 (2013).

38 Payne, S. H. The utility of protein and mRNA correlation. Trends Biochem Sci 40, 1-3, doi:10.1016/j.tibs.2014.10.010 (2015).

39 Chen, Z. J. et al. Fresh versus Frozen Embryos for Infertility in the Polycystic Ovary Syndrome. New Engl J Med 375, 523-533, doi:10.1056/NEJMoa1513873 (2016).

40 Maier, T., Guell, M. & Serrano, L. Correlation of mRNA and protein in complex biological samples. Febs Lett 583, 3966-3973, doi:10.1016/j.febslet.2009.10.036 (2009).

41 Zhang, X. O. et al. Diverse alternative back-splicing and alternative splicing landscape of circular RNAs. Genome Res 26, 1277-1287, doi:10.1101/gr.202895.115 (2016).

42 Gao, Y., Zhang, J. & Zhao, F. Circular RNA identification based on multiple seed matching. Briefings in bioinformatics, doi:10.1093/bib/bbx014 (2017).

43 Mohr, S. E. & Perrimon, N. RNAi screening: new approaches, understandings, and organisms. Wires Rna 3, 145-158, doi:10.1002/wrna.110 (2012).

44 Fatehullah, A., Tan, S. H. & Barker, N. Organoids as an in vitro model of human development and disease. Nat Cell Biol 18, 246-254, doi:DOI 10.1038/ncb3312 (2016).