NORTH-WEST UNIVERSITY
YUNIBESITI YA BOKONE-BOPHIRIMA
NOORDWES-UNIVERSITEIT
POTCHEFSTROOMKAMPUS

Evaluation of eukaryotic cultured cells as a model to study extracellular DNA

D.L. Peters (Hons. B.Sc.)

Dissertation submitted in partial fulfilment of the requirement of the requirements for the degree Magister Scientiae in Biochemistry at the North-West University (2011)

Supervisor:

Prof. P.J. Pretorius

School for Physical and Chemical Sciences, Centre for Human Metabonomics, North-West University, Potchefstroom campus, South Africa

The Poetry of Complexity

The most astonishing characteristic of life, is that it cannot be defined. As new layers of its complexity are unravelled and new insights into its being are unearthed, we realize only one absolute truth: we are now farther from understanding it than we were before we started.

D.L. Peters

Acknowledgements

My parents who have supported me, I wouldn't have been able to do this without you.

Thanks is in order to Dr. Oksana Levenets for her advice and aid on the growth cell cultures, Etresia van Dyk, Chrisna Gouws, Lizelle Zandberg and Maniesh Steyn for their help and guidance in and around the lab.

All the people too numerous to mention who have made a these post graduate studies not only educational but fun and are part of the memories I will forever hold dear. Thank you to my friends and family who were always there when I need them.

A special thanks is in order to my supervisor, Prof. Piet J Pretorius for his expert guidance in both my research and life. I certainly tested his patience. He is very patient.

Jesus Christ for giving me hope, showing me the way and teaching me to how to live. His sacrifice is what drives me forward and gives me courage. May we see His kingdom come.

The trend of eoDNA research in this study is greatly descendant and basically carries on, where left off, from the work presented by Dr. M. Steyn, in her post graduate studies, also under the guidance of Prof. P.J. Pretorius. Many of the protocols used in this study are therefore based on this previous work and is referenced accordingly, where necessary.

Lastly, I thank the NRF and NWU for financial support throughout these studies.

Abstract

The diagnostic value of extracellular occurring DNA (eoDNA) is limited by our lack of understanding its biological function. eoDNA exists in a number of forms, namely vesicle bound DNA, histone/DNA complexes or nucleosomes and virtosomes. These forms of DNA can also be categorized under the terms circulating DNA, cell free DNA, free DNA and extracellular DNA. The DNA can be released by means of form-specific mechanisms and seem to be governed by cell cycle phases and apoptosis. Active release is supported by evidence of energy dependant release mechanisms and various immunological- and messenger functions. Sequencing has shown that eoDNA sequences present in the nucleome reflects traits and distribution of genome sequences and are regulated by ways of release and/or clearance. eoDNA enables the horizontal transfer of gene sequences from one cell to another, over various distances. The ability of eoDNA to partake in horizontal gene transfer makes it an important facet in the field of epigenetic variation. Clinical implementation of eoDNA diagnostics requires that all of the subgroups of eoDNA be properly investigated. It is suggested that eoDNA is the result of the metabolic fraction of DNA that is released by the cell. Various observations indicate that eoDNA may also be incorporated into the genome of a cell, from where it may affect cell function. Therefore horizontal gene transfer in higher organisms is a real possibility. In this study, variations and increases in eoDNA levels over time correlate with stressors that are subjected to 143B human osteosarcoma cells. It seems viable to assume that a stressor is met by a change in the molecular machinery of a cell, required to neutralise the onset of metabolic instability. This may be done by amplification of necessary cistrons, producing metabolic DNA, that may then be observed after its release as eoDNA. The presence of hydrolysing enzymes gives an updated real time picture of the state of eoDNA. The eogenics hypothesis emanating from this study, suggests that amplification and horizontal transfer of cistrons affect tissue and organ function over long periods of time, in order for an organism to evolve one or more a specialized genomes.

Opsomming

Die diagnostiese waarde van ekstrasellulêre DNS (eoDNA) is beperk deur ons onvoldoende kennis aangaande die biologiese rol daarvan. eoDNA kom voor is verskeie vorms, naamlik vesikelgebonde DNS, histoon/DNS-komplekse en/of nuleosome en virtosome. Hierdie vorms kan verder ook gekatagoriseer word onder terme soos sirkulerende DNS, selvrye DNS, vrye DNS en ekstrasellulêre DNS. DNS word moontlik vrygestel deur vorm-spesifieke meganismes en mag afhanklik wees van selsiklus fases en apoptose. Aktiewe vrystelling word ondersteun deur bewyse soos betrokkenheid van energie afhanklike vrystelingsmeganismes en immunologiese boodskapper funksies. Geen volgordebepalings wys dat nukluomiese eoDNA volgordes eienskappe en verspreiding van genomiese geenvolgordes reflekteer en ook afhanklik is van gereguleerde vrystelling en verwydering. eoDNA maak horisontale geen oordrag van een sel na ‘n ander moontlik, oor groot afstande. Die vermoë wat eoDNA het om by te dra to horisontale geenoordrag, maak dit ‘n belangrike faset in die veld van epigenetiese variasie. Kliniese implementering van eoDNA diagnostiek vereis dat al die subgroepe van eoDNA deeglik ondersoek word. Dit is voorgestel dat eoDNA die metaboliese fraksie van DNS is wat deur die sel vrygestel word. Verskeie waarnemings stel voor dat eoDNA ook in die genoom van ‘n ontvanger sel geïnkorporeer kan word, waar dit selfunksie affekteer en dus voorstel dat horisontale geenoordrag in eukariotiese organisms ‘n ware moontlikheid is. In hierdie studie korreleer afwykings van eoDNA-vlakke met biologiese stressors, wat op die 143B-selle toegepas word, waaruit dit blyk dat ‘n stressor meegegaan word deur ‘n verandering in molekulêre masjienering, wat vereis word om de megaande stressors op te hef. Dit mag bereik word deur die amplifisering van sistrons, wat dan meegaande metaboliese DNS verteenwoordig en later waarneembaar is as eoDNA. Die teenwoordigheid van hidroliserende ensieme in die ekstrasellulêre ruimte gee ‘n intydse beeld aangaande die toestand van eoDNA. Die eogenetika hipotese wat uit hierdie studie voortvloei, stel voor dat amplifisering en horisontale oordrag van sistrons weefsel- en orgaanfunksie kan beïnvloed oor lang periods, waardeur ‘n organisme een of meer gespesialiseerde genome kan ontwikkel.

Keywords: Circulating, Extracellular, Nucleic Acids, DNA, Horizontal Gene Transfer, Virtosome, Blood Nucleome

Index

Chapter One

An introduction to the cause

1.1 Problem statement and approach

Extracellular occurring DNA (eoDNA) is considered for use as a biomarker by many researchers from various fields. It has proven to be notably elevated in a number of disease conditions. However, variation of eoDNA among individuals, both healthy and ill, have made diagnosis by means of eoDNA concentration virtually impossible. Upon the suspicion that these variations are caused by unknown factors that may be at work in each individual and since unknown variations of factors may be connected to specific cells, tissues and organs, it may prove to be necessary to investigate aspects that influence eoDNA release under isolated conditions. By this is meant, controlling the conditions that possibly affect eoDNA release to as great an extent as possible.

In order to control as much of the potential deviatory biological factors as possible, the model for our study must be a simple one. *In vivo* experiments are not suitable for this purpose, as compartmentalization and the presence of differentiated cells, along with the membrane barriers that may possibly have the ability to selectively transport certain forms or species of eoDNA complexes, may seriously interfere with the collective amount of eoDNA species obtainable from plasma and serum (circulating DNA). Organ cultures, in much the same way are, in most cases, composed of multiple cell types as well and may once again interfere with direct observable eoDNA species. Tissue cultures may be worth investigating, but in relation to even simpler cell culture models, are harder to obtain and maintain.

In this study the use of eukaryotic cell cultures is thus investigated to determine whether a simple model may elucidate some factors that influence the release of eoDNA. Two key aspects concerning the release of eoDNA from eukaryotic cells are considered. These are the nature of eoDNA release over time and the effect of environmental stress on eoDNA release. In this study, the effects of various factors that may have an influence on the release of eoDNA from cultured cells are examined in the light of the eogenics model. The model is described in more detail in Chapter Two, but shortly entails that stress results in amplification of certain genes, producing metabolic DNA that is released as eoDNA. eoDNA may then

once again be the intermediate of horizontal gene transfer and thereby effect a cell after uptake and integration. If stress factors, do in fact, have a direct influence on the release of DNA by cells in culture, the results would once again pose as strong support that eoDNA release is associated with biological functions. If the eoDNA is a representation of what was, prior to its release by the cell, the metabolic fraction of DNA, one would also hope to see correlations in stress adaption by the cells and the release flux of eoDNA.

In this study, the eogenics model, as proposed in section two of Chapter Two, is used for predicting and assessing the outcomes of various experiments. The model requires that certain task-specific genes be activated in response to the molecular requirements of cells. In this study these requirements include maintaining homeostasis and governance of the necessary molecular changes associated with specialization of a cell in its growing state. Genes that are amplified after epigenetic activation, produce the metabolic DNA ultimately responsible for transcription of the required RNA sequences and proteins necessary for normal functionality of the cell. The metabolic DNA is later released, after migration from the nucleus, through the cytoplasm and finally to the extracellular space. The released metabolic DNA, now referred to as eoDNA, can now be quantified. Respective changes in these eoDNA levels with regard to biological stresses or changes to which cells are artificially exposed to, would then serve as an indication as to whether the model is fit for further study. This would be the case if associations are found in eoDNA release patterns and the respective stressors or biological changes administered to the cells.

1.2 Primary Hypothesis

Eukaryotic cells, in culture, can be used to characterise extracellular occurring DNA (eoDNA), by means of relationships between eoDNA release patterns and controllable changes in cell biology.

1.3 Structure of this dissertation

Please note that the reference formats of Section 2.1 and 2.2 are designed to be acceptable for the journals they have been submitted to. The text format however, has been customized to fit that of this M.Sc. dissertation and improve readability. Section 2.1 is comprised of a published critical review article, that serves as the bulk of fundamental literature research that is required to formulate a proper approach to the investigation of extracellular occurring DNA or eoDNA. The review article's main areas of focus are the terminology used in eoDNA research and the characteristics of eoDNA, in terms of molecular associated forms and function. The nature of the article sheds light on some ill defined characteristics of eoDNA which are experimentally addressed in the subsequent sections of the dissertation.

Chapter 2.2 consists of an article addenda, in response to a request from and editor of a the journal, named Mobile Genetic Elements, following the publication of the critical review publication in Clinica Chimica Acta. The article has been submitted on Tuesday, the 10th of May 2011. The formal request, that includes commentary on the critical review publication featured in Chapter 2.1, signed by Dr. Adam P. Roberts, is included in the Addendum. The literature and perspectives of this, first draft, brings together the most important aspects required for formulation of the hypothetical eogenics model, by which the experimental results of this dissertation are to be judged. The literature in this article therefore serves as an important foundation in this dissertation, which is not wholly repeated in order to keep the length of the dissertation to a minimum. Correlations in the results of the main experiments and the eogenics model would ultimately approve the primary hypothesis in this study and also act as standing support the eogenics hypothesis itself.

Prior to the experimentally investigating the primary hypothesis, preliminary experiments were firstly required for the design and standardisation of the protocols used in this study and therefore form part of the materials and methods section of this dissertation. Two eoDNA extraction protocols are first compared to one another and the most appropriate protocol, in terms executability, cost affectivity, precision and accuracy is chosen. The eoDNA content and the storage of medium samples are also discussed in chapter three.

The structure of the study's main experimental layout is illustrated in Figure 1. Forming the experimental basis of this study is the effect that confluence of 143B cells have on the release of eoDNA. As the literature in chapter two describes, various authors find somewhat

conflicting results, which may be due to differentiation of cells, implicating that various cell lines may act in a different manor. After deciding on the appropriate confluence with which 143B cells are to be worked with for the purposes of this study, eoDNA release from the various 143B cells (standard and transfected) are investigated and discussed in chapter five. Adding to chapter five, an important aspect of eoDNA, namely turnover, which is merely speculated on in a recent literature is also, rather adequately, addressed. The influence of severe stress and slight variation of growth conditions on the eoDNA release patterns is also investigated in Chapter Five

For investigating the effects of stress on eoDNA levels, the main experiments that form the backbone of this study include:

- The effect of gene manipulation on the release of eoDNA
- The effect of heat shock treatment on the release of eoDNA
- The influence of subtle differences in nutrition on the release of eoDNA

Each of these experiments have its own aim, approach, customized materials and methods (where necessary), discussion and conclusion, as is necessary to properly explain all the proceedings. In order to keep the flow of information however, certain experiments are cross-referenced with one another and also have some additional aspects of literature that is specific to that experiment.

A final discussion and conclusion, that involves comprehension of all the results, binds the individual experiments together. Lastly the dissertation is rounded off by a personalised view of the future prospects and universal perspectives that is brought to light by the study.

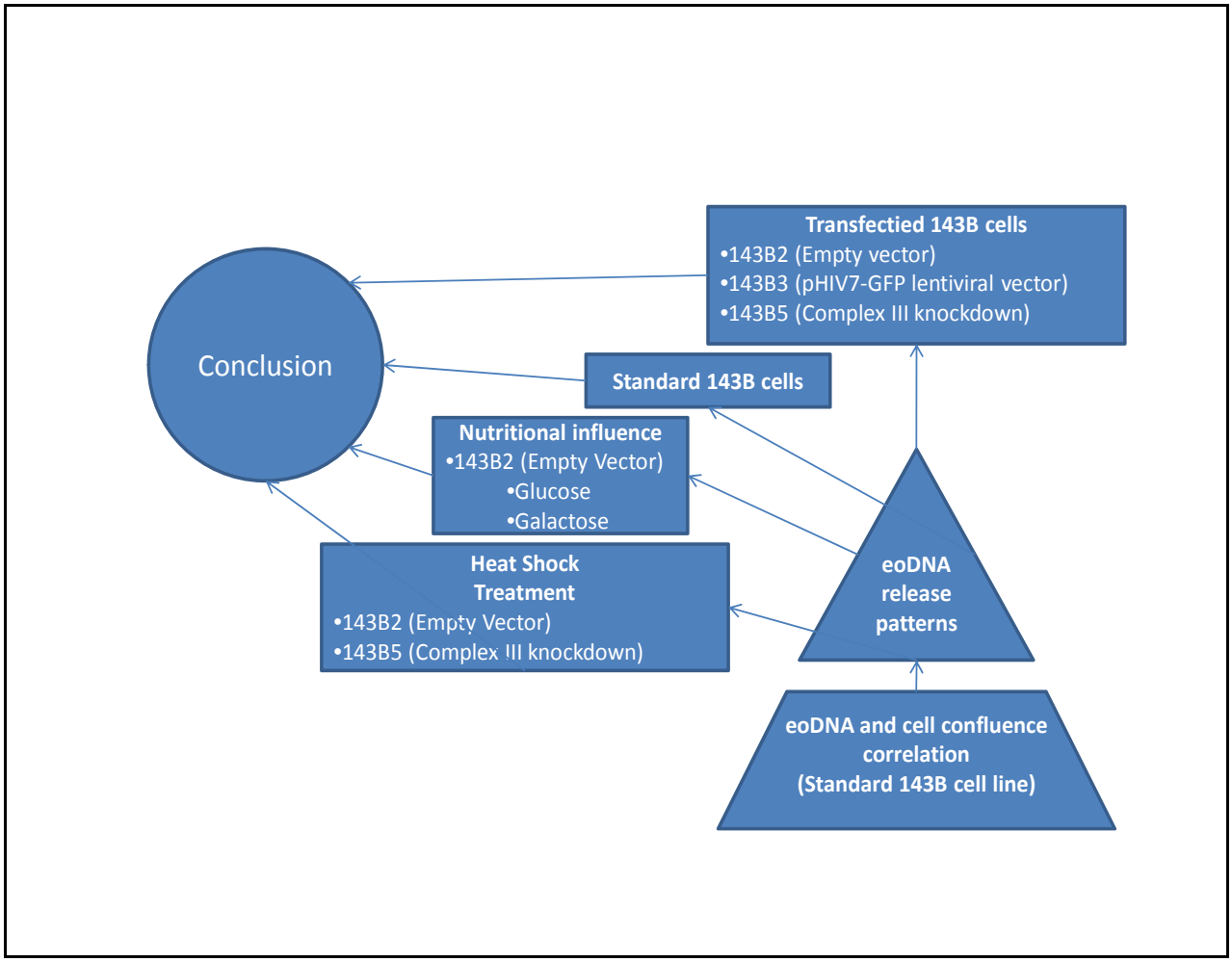

Figure 1. Schematic representation summarising the flow of experiments toward a common conclusion. Shown here are the main sections of which the study is compromised. The foundation of the pyramidal box constitutes the investigation on the appropriate confluence of cells, that is to be applied in the subsequent experiments. The pinnacle of the experiments required for the approval or disproval of the primary hypothesis is based on the release patterns of eoDNA. These release patterns, as observed under varying circumstances are discussed individually, in the light of the experiments performed in each chapter, before all the results are brought into perspective to reach a final conclusion.

Chapter Two

An overview of the present perspective on eoDNA

2.1 Origin, translocation and destination of extracellular occurring DNA — A new paradigm in genetic behavior

D.L. Peters, P.J. Pretorius / Clinica Chimica Acta 412 (2011) 806–811 807
journal homepage: www.elsevier.com/locate/clinchim

We thank the NRF for funding our research.
Corresponding author.
Tel.: +27 18 299 2066;
fax: +27 18 299 2316.
E-mail address: 13152726@nwu.ac.za (D.L. Peters).
0009-8981/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.cca.2011.01.026
Contents lists available at ScienceDirect

Abstract

The diagnostic value of extracellular occurring DNA (eoDNA) is limited by our lack of understanding its biological function. eoDNA exists in a number of forms, namely vesicle bound DNA (apoptotic bodies, micro particles, micro vesicles and exosomes), histone/DNA complexes or nucleosomes and virtosomes. These forms of DNA can also be categorized under the terms circulating DNA, cell free DNA, free DNA and extracellular DNA. The DNA can be released by means of form-specific mechanisms and seem to be governed by cell cycle phases and apoptosis. Active release is supported by evidence of energy dependant release mechanisms and various immunological- and messenger functions. Sequencing has shown that eoDNA sequences present in the nucleome reflects traits and distribution of genome sequences and are regulated by ways of release and/or clearance. eoDNA enables the horizontal transfer of gene sequences from one cell to another, over various distances. The ability of eoDNA to partake in horizontal gene transfer makes it an important facet in the field of epigenetic variation. Clinical implementation of eoDNA diagnostics requires that all of the subgroups of eoDNA be properly investigated.

2.1.1. Brief history of eoDNA

Newly synthesized nucleic acids are spontaneously released by living cells [1] and genetic information can be passed horizontally in this manner [2]. The mechanisms involved in this process are however very poorly understood. Grafting experiments showed the dramatic impact that foreign extracellular occurring DNA (eoDNA) has on the formation of a phenotype [3]. eoDNA has a variety of forms that are present in various extracellular media, including the blood, sputum, urine and even cerebral spinal fluid (CSF) of humans and other vertebrates [4], and must also be present in extracellular circulation in plants [3,5], bound to and/or incorporated in different arrangements of molecular particles. These particles are discussed later in this text.

This paper will attempt to properly define the most commonly used terms (e.g. circulating DNA, circulating-free DNA, extracellular DNA and cell-free DNA) and briefly discuss the nature of the currently known eoDNA containing structures (micro-vesicles, micro-particles, apoptotic bodies, exosomes, histone complexes and virtosomes). We discuss the origin of sequences and various forms of active release in these divergent eoDNA forms, while keeping necrosis and apoptosis in mind as alternative mechanisms of release. Finally, we take into consideration the relationship of eoDNA sequences in the nucleome and genome and also take to account the effects of turnover and the significance thereof as a regulatory feature for horizontal gene transfer.

2.1.2. Clinical implications

Several observations underscore the potential of eoDNA as a potential, less-invasive diagnostic and prognostic biomarker for an ever increasing number of pathologic conditions, ranging from prenatal diagnosis of Mendelian diseases and fetal sex determination, an early indication of Down syndrome and the onset of pre-eclampsia, to the possibility of specific cancer diagnosis and determining levels of damage caused by trauma and stroke. eoDNA also seems to have certain features that directly influence immune response and may play a key role in autoimmune diseases such as Lupus Erythmatosis [4,6]. Furthermore, eoDNA is rapidly cleared from blood circulation [7] and thus provides a real-time overview of the extracellular genomic state.

Diagnosis is done in the following ways: Firstly the amount of eoDNA in plasma and serum of patients with cancer and various other ailments are higher than what is found in healthy individuals [8]. Adding to this point, an increase in eoDNA integrity reflects the likelihood of positive diagnosis [9]. Secondly, alterations in methylation
and mutation, present in the primary tumor can be detected in the eoDNA of the patient. Thirdly, promoter hypermethylation of specific cancer-related genes can be detected in eoDNA of cancer patients, but not in healthy individuals [10].

2.1.3. Nomenclature

Proper terminology has to be considered a very high priority, if we are to thoroughly understand the purpose and nature of these rather enigmatic forms of DNA. In this review we refer to DNA present in the extracellular environment as extracellular occurring DNA or 'eoDNA' and attempt to explain the nature of subcategories.

In addressing the term 'free-DNA'[11,12], one might assume that the reference is toward DNA that is unbound by any and all forms of cellular and non-nucleic structure and is for lack of a better word 'free'. This would also imply that the DNA is reactive toward its environment and is free to associate with structures such as membrane proteins, as described by Laktionov et al. (2008) [13]. DNA in this form would also be subject to higher levels of lability, as there would be minimal interference to enzymatic breakdown.

Referring to the action of receptor activation in the immune system, it may be possible that proteins and other structures associated with the DNA do the exact opposite and may in effect serve as markers for the activation of specific reactions. The associations of eoDNA with other molecules may therefore place the DNA in a state where it is specifically reactive or -nonreactive toward certain reactions.

We shall now attempt to show the degrees in which eoDNA may be further classified.

Circulating nucleic acids in plasma and serum or CNAPS [2] include all forms of circulating nucleic acids, regardless of their level of confinement. 'Circulating DNA' in all obvious nature describes DNA present in circulation of blood. There is, however, conflict when using

acronyms for ‘circulating-free DNA’ [14] and ‘cell-free DNA’ [15], which would both be referred to as cfDNA. In resolving this problem, ‘extracellular DNA’ (exDNA) might have been a possible replacement for the term ‘cell-free’, but seems misleading, as it should rather refer to the origin of the DNA's formation, which may in fact, according to the work of Philippe Anker and colleagues, be in the extracellular environment [16] by means of what is today, decades later, still an unknown process of extracellular DNA synthesis. We propose the use of the term ‘free-circulating DNA’ in order to avoid confusion with ‘cell-free’, when using acronyms, and promote the use of the term extracellular-occurring DNA (eoDNA), to describe the wide variety of DNA present within the extracellular environment.

With additional regard to ‘cell-free DNA’, one might ask whether this implies that the DNA is unbound (i.e. cut off from the surrounding extracellular space) by cell- or vesicle membrane, or both, as it has indeed been shown that nucleic acids are present outside of cells, bound in micro-vesicles [17,18] and exosomes [19]. This DNA may still be bound to nucleosomes and/or associated with other molecular structures that may or may not be capable of interfering with DNA reactivity.

We recommend the use of terms as follows: Cell-free DNA (cfDNA) and free DNA (fDNA) describes eoDNA that is free from any physical, cellular confinement. Free DNA should also describe DNA that is free from association at a molecular level, whereas this may not necessarily be the case for cell-free DNA. Particle-derived DNA (pdDNA) describes all forms of eoDNA that is found to be associated with other molecular structures.

If we are to form a comprehensive understanding of eoDNA and unlock the full potential of its use, certain critical issues need to be addressed by the relevant research community. Alas, in order to differentiate between all these DNA species with regard to structure and function(s), specific isolation methods need to be developed to allow proper characterization.

2.1.4. The distribution of nucleome eoDNA sequences in the genome

In a study done by Beck et al. which involved the sequencing of serum samples of some 50 healthy individuals, found that the relative amount of all the genomic features was approximately 1. This is an indication that the sequences present in the nucleome are essentially mirrored by that of the genome, with the greatest variations found in representation

of CDSs, UTRs and pseudogenes. They found gene sequence representation in serum to be related to genomic gene length and also that there is a strong relationship between the number of nucleotides derived from a specific chromosome and the size of the particular chromosome. The only exception was chromosome 19 that showed only 81% of the expected expression. Gene density showed no observable influence on expression. In comparison to the genome, an overrepresentation of Alu elements (which are short interspersed elements) has been shown by various authors [20–22], with an underrepresentation of long interspersed nuclear elements L1 and L2 [21].

Reports suggest that the nucleome represents abundances of various DNA sequences and that some of these sequences are also unequally represented in individual nucleomes. It may be possible that these sequences are not released in these ratios either, but that they are possibly generated through sequence specific clearance mechanisms [23].

2.1.5. Cyclic release of eoDNA

Are eoDNA fragments the result of a regulated turnover of cellular DNA during active growth and to a lesser extent during quiescence? This process is deemed necessary to maintain a certain level of DNA repair activity that can be deranged by, for example, the transformation of a cell resulting in an increase in circulating DNA in cancer patients. According to the work of M.A. Madine and colleagues, DNA replication is halted in quiescent cells and further describes how this is regulated by various chromosome binding proteins [24]. Additional evidence shows that membrane associated proteins are also capable of inhibition of DNA synthesis [25]. Once again however, it appears that various tissues portray diverse characteristics. An example of this is the observed replication — in quiescent, glial cells in the abdominal central nervous system of Drosophila [26]. S.R. Pelc similarly reported the phenomenon as early 1972, in his work on metabolic DNA in ciliated protozoa, salivary gland chromosomes, and mammalian cells [27].

2.1.6. Translocation mechanisms of fragmented DNA and eoDNA

2.1.6.1. Intracellular transport

In a study done by Hara et al. it was demonstrated that fragmented DNA was transported in apoptotic retinal dendrites. The axoplasmic transport of fragmented DNA was wholly inhibited by coadministration of vincristine, indicating that the observed axoplasmic transport of fragmented DNA is due to microtubule-related active transport. Additionally, the transport of fragmented DNA in CA1 pyramidal neurons was only observed within a very specific time period after ischemia. The authors suggest that the intracellular transport of fragmented DNA has an influence on regulation and maintenance of neuronal networks, that includes the retinal neurons [28]. If this is indeed the case, eoDNA must also be investigated as an influential factor in tissue development and maintenance.

Looking at translocation of fragmented DNA to the extracellular space, there seems to be three main categories of structures that allow such movement to be possible and also shield the DNA from the nuclease active, extracellular environment. The main categories we are aware of are transport of DNA by vesicle based particles, nucleosomes and virtosomes.

2.1.6.2. Contribution of particles as DNA carriers

Instead of occurring free, eoDNA is complexed with protein- and lipid structures to form particles in the heterogeneous extracellular environment that make up the nucleome. While RNA occurs mainly in particulate structures, DNA is described as occurring either soluble or particulate [29]. Particles associated with, or containing nucleic acids can be divided into various classes, with various characteristics, such as being histone based, vesicle-bound or similarly associated with other macro-molecular structures. It also appears possible that the release of these particles is regulated and may be governed by or occurring under certain circumstances in cell cycle and apoptosis. The most important research priorities towards understanding the function and mechanics of the nucleome are to investigate and categorize the points of genetic origin, the mechanisms of translocation and the impact points of such processes.

2.1.6.2.1. Apoptotic bodies and micro-particles

A number of vesicle-like particles in blood have been found to contain DNA fragments, including apoptotic bodies (ABs) and microvesicles (MVs) such as micro-particles (MPs) and exosomes.

Prior to the formation of ABs, apoptotic cells release MPs in a process of cell shrinkage, at the end of which cellular contents are completely fragmented and dispersed within either MPs or ABs, possibly for ease of clearance by phagocytes and other cells. Apoptotic bodies are known to contain the bulk of DNA degraded during apoptosis. DNA and ribosomal RNA may be cleaved within these particles by nucleolytic breakdown, while other nucleic acid species like intravesicular mRNA have been shown to be protected from RNase, within ABs [30].

Cell death seems to progress with the selective loss of macromolecular components that are dependant of specific macromolecular changes and are influenced by the pathway of cell death, which may be either necrotic or apoptotic [31]. During apoptosis, DNA is degraded in a stepwise manner: Firstly chromosomal DNA is cleaved into large fragments of 50 to 300 kB and subsequently into oligonucleosomes and/or mononucleosomes by endogenous Ca2+- and Mg2+-dependent nuclear endonuclease [32]. This is a hallmark of apoptosis [10]. The core particle of a nucleosome consists of an octamer of duplicate copies of the histones H2A, H2B, H3 and H4 — around which 146 bp of helical DNA is wrapped. Individual mononucleosomes are connected by a stretch of linker DNA. The linker DNA has a variable length ranging between 15 and 100 bp containing the histone H1. Negatively charged DNA is electrostatically bound to the positively charged histones [33]. It is believed that the nucleosome structure protects DNA from degradation, thus resulting in the archetypal ladder pattern of fragment sizes associated with apoptosis [34]. The fragmentation of DNA, achieved during apoptosis, can also be characterized by their termini, due to specific degradation by DNaseII, making identification by sequencing a possibility. Necrosis, on the other hand, results in random degradation of the genome, which may be released in some form of eoDNA.

The ladder pattern obtained from electrophoresis with circulating DNA resembles a similar size distribution to that found in DNA degraded by apoptosis. Because of this and the large

amount of DNA required to be degraded due to apoptosis each day (around 1 to 10 g in a human), it is claimed to be the primary source of DNA in plasma [32]. There seems however, to be evidence that opposes this notion.

Apoptotic bodies (AB) are formed in apoptosis during the cellular process of blebbing or shedding [30,35]. During this process DNA and RNA are packed in separate Abs [15,21] and as they are released, they are rapidly ingested by adjacent cells and professional phagocytes, such as macrophages and dendritic cells [36]. It could therefore be possible that the DNA is completely removed and digested to nucleotides by DNaseII in lysosomes, instead of being unconstrained to free circulation. Furthermore, it seems to be common reason that impairment of these removal properties and/or amplification of cell death leads to tissue injury and also triggers autoimmunity [37].

The contribution of apoptosis to the total amount of circulating DNA, under normal circumstances, is therefore in most probability rather insignificant, but can be expected to increase when phagocytic clearing ability is overwhelmed by certain pathological conditions.

While both apoptosis and necrosis contribute to the "blood nucleome", it is achieved by completely different mechanisms. Necrosis results in what would seem the accidental release and rugged breakdown of DNA. Since apoptotic release occurs in a much more orderly and stepwise fashion, as described above, one would be led to consider this to be more of an intentional and controlled form of release, contrasting necrosis — hence one would also expect specific mechanisms of cellular uptake coupled to forms of apoptotic DNA. Interestingly, studies suggest that apoptosis causes a far greater increase in eoDNA concentration, than does necrosis [38].

2.1.6.2.2. Exosomes

The intracellular fusion of the plasma membrane with multivesicular bodies, leads to the release of intraluminal vesicles as exosomes. These particles are the latest addition to the family of 'bioactive vesicles', that are released into circulation where they are either broken down or are capable of performing a number of roles, that are strongly associable with intercellular communication. These particles have been shown to be trafficked over long distances in circulation and have also been found present in body fluids like CSF and urine.

Exosome signaling is achieved by fusion with other cells, releasing their contents and leaving them to the recipient cell's disposal. Specific proteins and lipids are incorporated into exosome membranes, while specific cytosolic proteins and various mRNA and miRNA molecules are uploaded as cargo and their composition is associable with cell type origin [39]. Some research indicates that exosomes carry DNA containing complexes [40], while other reports have shown exosomes containing specifically RNA and no DNA [41], suggesting the possibility of differing functional classes of exosomes.

Comparing to the gel electrophoresis results of Choi et al. [38] with that of Lehman et al. [42], it would seem that the amount of DNA uploaded to circulation by means of exosome formation is insignificant in relation to apoptosis and necrosis, in terms of both quantity and size. It would seem that the contribution of both exosomes and apoptotic bodies to the amount of circulating DNA is very small, although, according to Holmgren in his publication of 1999, horizontal gene transfer is possible when ABs are transferred to recipient cells [43]. The biological significance of vesicular transport of DNA between cells can thus not be undermined and should be addressed to the point of certainty.

2.1.6.2.3. Histones — more than packaging frames for DNA

Nucleosomes are found in the nucleus, the cytoplasm and the cell membrane. J.-J. Choi et al. also showed that circulating DNA levels in mice increases in parallel with circulating nucleosomes after infusion with apoptotic cells. As fragmented DNA and nucleosomes are common molecular constructs found in the nucleus, the notion that the DNA accidentally released is understandable. On the other hand, there may be a whole different process at work that does not imply that the DNA release during regulated cell death (apoptosis) is accidental and that it may serve a diverse composition of genetic and immunological roles [38].

Over the past two decades, we've seen an increase in evidence showing that linker and core histones can directly move across plasma membranes and are present in various cellular compartments. Histones are therefore described as actively mobile complexes [44,45] and, since histones associate with DNA, these histone complexes are likely capable of ferrying DNA across membranes as well. Histone translocation is possible by means of non-covalent ionic interactions with membranes. These interactions are formed by non-specific

associations, instead of being specific receptor-ligand interactions. The process is not saturable and neither is it energy-independent, however different histones vary in ability to cross membranes. H2A and H4, for example, seem to be more potent in penetration of intact cells than H2B and H3, but the formation of histone oligomers is said to improve penetration efficiency altogether [33].

Circulating DNA was shown to include loci that bound histone H3K27me2. Judging from this, it seems that H3K27me2 is possibly involved in the externalization and stability of plasma DNA [21,46]. The most astounding aspects of histones do however not lie in their ability to stabilize and transport eoDNA, but in the functions that the DNA and histone complexes may ultimately serve as a set.

T.J. Kleine et al. showed that histone tertiary structure and its level of positive charge play a significant role in its ability to form interactions with membrane phospholipids [47]. Given the electrostatic nature of DNA, it should be easy to imagine that the placement of DNA with regard to the tertiary structure of a particular histone, would consequently influence the histone's ability to participate in ionic membrane interactions. The configuration of a DNA-histone set may therefore act as a toggle, but may be capable of having far greater effects than simply permitting its own translocation as we shall subsequently point out.

Studies have shown that histones (in particular linker histones) are associated with plasma membrane expression at the time of the innate immune responses afferent phase. Other data show histones to be in indirect contact with apoptotic cell membranes [45] and as it is well known that apoptosis is coupled to a certain extent of DNA release, this is possibly a pre-emptive step to the release of DNA that is sterically protected from nucleases. Purified histones have been proved capable of increasing the permeability of cell membranes, to diminutive monovalent cations and anions. Sequentially, the increase in permeability may lead to swelling and ultimately lysis [47]. All the while the DNA, capable of molecular interaction with both TLR (toll like receptor) and non-TLR sensors, has deliberate access to immune control function [31].

Apart from eoDNA that is found in elevated concentrations in various disease-states like cancer, histones have also been raised to have possible diagnostic value. The histone

H3K27me3, for example, has been shown to be substantially lower in patients with metastatic prostate cancer in comparison to patients with only localized disease or advanced local disease and has in fact also been shown to even have a minor effect on gene expression by functioning as an epigenetic switch [48]. One cannot yet ignore that the alterations in histone activity seen in metastatic prostate cancer have a causal role to play and that this then requires more attention.

All the above been said, it may be well suited to investigate eoDNA alongside its biological counterpart, the histone, as their individual and combined biological functions may very well depend on their association with one another.

2.1.6.2.4. The virtosome

The selective release of newly synthesized DNA by phytohemagglutinin was demonstrated as early as 1972 [49]. Since then, many studies have shown the regulated release of a newly synthesized DNA/RNA — lipoprotein complex that includes DNA-dependent DNA and RNA polymerases. This complex has been termed the virtosome. The mechanism of its release has also not yet been elucidated, but the process is energy dependant [50]. Studies have also shown that the complex is released and incorporated wholly [51] and is released by both dividing and differentiated cells [50–54]. Synthesis can thus not be specifically linked to mitosis and could also take place during G0, G1 or G2 cell cycle phases. Synthesis of this DNA is most likely to take place in the G0 or G1 phase, as differentiated cells tend to be held in either of these phases for prolonged periods [2].

As the cytosolic and extruded complexes separate in an identical manner by means of agarose gel column chromatography [55], it would seem unlikely that virtosomes are released in exosomes and furthermore, unlike exosomes, virtosomes also contain both DNA and RNA [2]. This trait does not disregard release by some form of exocitosis, but the mechanism of initiation, if exosomal release is the case, has yet to be found [2].

2.1.7. eoDNA and turnover

eoDNA has been demonstrated to be a capable intercellular messenger [56] as eoDNA was shown to be taken up, incorporated and expressed by eukaryotic cells. This horizontal gene transfer may have some dramatic implications, visible within the span of even a single generation [3]. As one of the defining features of DNA released by differentiated cells is that its synthesis is reflected by different metabolic processes [57], one may find such an influence on genomic turnover within confined tissues to be capable of regulatory function specific to that tissue. It has however been demonstrated that RNA from one region of an organism can be carried to other regions throughout the body [58], but as lower circulating DNA levels are befitting to healthy individuals, it may be a slightly irregular event for eoDNA to escape into circulation from confined tissues in the first place. Additionally the process may also be regulated, given the various forms in which DNA may be released.

If this is the case, one should expect to find much higher turnover rates in the interstitial space than in circulation. This eoDNA turnover should also be driven mainly by cellular uptake rather than extracellular enzymatic breakdown. A study done by Anker et al. showed that DNA is released up to a certain concentration, at which point it seems to have reached an equilibrium regulated by poorly understood mechanisms, including the possible presence of feedback mechanism and the possibility of a state where release is equal to uptake [2,52]. Non-complex DNA in the medium seems to have no effect on the release, however, a change in the concentration of released DNA is met by an opposed change that once again establishes equilibrium [59].

How great an effect eoDNA has in regulation of specific tissue function, may be illuminated by thoroughly investigating the nature of eoDNA turnover — a field of research that, to our knowledge, has not yet developed. Certain distinctive features of metabolic DNA have been found to correlate to released eoDNA and metabolic DNA may therefore represent the sites of eoDNA synthesis [57]. It would also be important to determine which fractions of the genome is represented by circulating DNA, if it is to be used as a diagnostic marker for a variety of conditions. Sequencing and RQ-PCR [21] [23] has not conclusively shown that the whole genome is equally represented in eoDNA. We strongly support this and have shown corresponding results by means of large scale sequencing, indicating that circulating

DNA originates from all over the chromosomes with minimal indications of sequence clustering, even though there is an incomplete coverage of all chromosomes [22]. In contrast, unequal distributions of genes have been described by Puszyk et al. [23]. This could be ascribed to one or more possible factors, including variation of copy numbers in certain genomic areas between individuals and a possible manifestation of difference in the transport and/or clearing mechanisms. Total circulating DNA is further likely to have a mixture of origins from various tissues, organs and/or compartments, prior to being transported to the circulatory system. This is indeed the very principle that should allow us the benefits of tumor-diagnostics via circulating DNA. If the presence of these sequences is due to some random mechanism, their use as dependable biomarkers can be questioned. Reproducible results of quantified circulating DNA, cognisant of variations in methods and yields have however, in a variety of health-related conditions, indicated that the origin of this DNA is mainly an ordered process [37,60].

Turnover should not only be influenced solely by release but clearance to the same extent. In maternal plasma, circulating DNA has a mean half-life of only 16.3 min [61]. Circulating DNA can be expected to be cleared by the liver in addition to its breakdown by nucleases and intake by cells. As previously stated, the combination of DNA with nucleosomes may have an effect on the reactional activity toward processes of uptake and breakdown. The halflife of eoDNA measured by Y.M. Lo et al. reflects the halflife of the whole nucleome [61], where various constructs of translocation may provide longer or shorter time frames, in which a particular eoDNA complex can survive intact.

Whether the breakdown and release of eoDNA is a controlled or a random process, it is clear that cells are capable of exchanging genetic information by making use of eoDNA as an intermediate [3,62]. Equilibrium of nucleomic- to genomic gene ratios in bodily confinements and the accessibility to exchange, may be considered to be a possible key feature of genetic homeostasis [21,46]. We expect turnover to be directly involved in this process.

We are unaware of any research regarding eoDNA in the interstitial space of tissues and organs. Given the nature of DNA release, its presence in this compartment is highly likely. It would therefore be interesting to see whether these gene ratios differ between various bodily

confinements and tissues. Cell and tissue cultures may pose as promising models for research in this field.

2.1.8. The nucleome as a reflection of the genome

Taken together, we find that the implications of eoDNA can be expected to be much more significant than what it is given credit for by interest of research. As individual cells donate DNA to the nucleome, they also receive DNA from it. In this light, the nucleome functions as a communications matrix, in which there exists a type of homeostatic standard of functional genetic sequences and relationships. Given the nucleome's direct connections with the immune system, one can also imagine how it actively functions to remove factors that may disturb the perfectly kept balance, whether they be pathogens or cells that have lost efficient regulatory function.

We expect the nucleome's ability in influencing individual cellular genomes to be related to the turnover of the sequences present therein. These turnover rates may be influenced by the type of structure the DNA is released in. A particles half-life would also influence the distance it is carried in circulation and therefore the area of effect in which a certain group of cells can have such horizontal genetic influence.

Diagnostic value set aside, we would like to raise eoDNA as a corner stone that is to help fill our understanding of epigenetic variation.

2.1.9. References

[1] Stroun M, Anker P, Maurice P, Gahan PB. Circulating nucleic acids in higher organisms. Int Rev Cytol 1977;51:1–48.

[2] Gahan PB, Stroun M. The virtosome-a novel cytosolic informative entity and intercellular messenger. Cell Biochem Funct 2010;28:529–38.

[3] Gahan PB. Circulating DNA: intracellular and intraorgan messenger? Ann NY Acad Sci 2006;1075:21–33.

[4] Gahan PB, Swaminathan R. Circulating nucleic acids in plasma and serum. Recent developments. Ann NY Acad Sci 2008;1137:1–6.

[5] Stroun M, Anker P. Prehistory of the notion of circulating nucleic acids in plasma/ serum (CNAPS): birth of a hypothesis. Ann NY Acad Sci 2006;1075:10–20.

[6] Wright CF, Chitty LS. Cell-free fetal DNA and RNA in maternal blood: implications for safer antenatal testing. BMJ 2009;339:b2451.

[7] Lo YMD, Zhang J, Leung TN, Lau TK, Chang AMZ, Hjelm NM. Rapid clearance of fetal DNA from maternal plasma. Am J Hum Genet 1999;64:218–24.

[8] Suzuki N, Kamataki A, Yamaki J, Homma Y. Characterization of circulating DNA in healthy human plasma. Clin Chim Acta 2008;387:55–8.

[9] Gang F, Guorong L, An Z, Anne GP, Christian G, Jacques T. Prediction of clear cell renal cell carcinoma by integrity of cell-free DNA in serum. Urology 2010;75: 262–5.

[10] van der Vaart M, Pretorius PJ. Is the role of circulating DNA as a biomarker of cancer being prematurely overrated? Clin Biochem 2010;43:26–36.

[11] Takagi R, Nakamoto D, Mizoe JE, Tsujii H. LOH analysis of free DNA in the plasma of patients with mucosal malignant melanoma in the head and neck. Int J Clin Oncol 2007;12:199–204.

[12] Pathak AK, Bhutani M, Kumar S, Mohan A, Guleria R. Circulating cell-free DNA in plasma/serum of lung cancer patients as a potential screening and prognostic tool. Clin Chem 2006;52:1833–42.

[13] Mal'shakova VS, Pyshnyi DV, Bondar AA, Vlassov VV, Laktionov PP. Isolation and sequencing of short cell-surface-bound DNA. Ann NY Acad Sci 2008;1137: 47–50.

[14] Gormally E, Caboux E, Vineis P, Hainaut P. Circulating free DNA in plasma or serum as biomarker of carcinogenesis: practical aspects and biological significance.

Mutat Res 2007;635:105–17.

[15] Hahn S, Zimmermann BG. Cell-free DNA in maternal plasma: has the sizedistribution puzzle been solved? Clin Chem 2010;56:1210–1.

[16] Anker P, Stroun M, Maurice PA. Spontaneous extracellular synthesis of DNA released by human blood lymphocytes. Cancer Res 1976;36:2832–9.

[17] Halicka HD, Bedner E, Darzynkiewicz Z. Segregation of RNA and separate packaging of DNA and RNA in apoptotic bodies during apoptosis. Exp Cell Res 2000;260:248–56.

[18] Schiller M, Bekeredjian-Ding I, Heyder P, Blank N, Ho AD, Lorenz HM. Autoantigens are translocated into small apoptotic bodies during early stages of apoptosis. Cell Death Differ 2008;15:183–91.

[19] Skog J, Wurdinger T, van Rijn S, et al. Glioblastoma microvesicles transport RNA and proteins that promote tumour growth and provide diagnostic biomarkers. Nat Cell Biol 2008;10:1470–6.

[20] Stroun M, Lyautey J, Lederrey C, Mulcahy HE, Anker P. Alu repeat sequences are present in increased proportions compared to a unique gene in plasma/serum DNA: evidence for a preferential release from viable cells? Ann NY Acad Sci 2001;945:258–64.

[21] Beck J, Urnovitz HB, Riggert J, Clerici M, Schutz E. Profile of the circulating DNA in apparently healthy individuals. Clin Chem 2009;55:730–8.

[22] van der Vaart M, Pretorius PJ. A method for characterization of total circulating DNA. Ann NY Acad Sci 2008;1137:92–7.

[23] Puszyk WM, Crea F, Old RW. Unequal representation of different unique genomic DNA sequences in the cell-free plasma DNA of individual donors. Clin Biochem 2009;42:736–8.

[24] Madine MA, Swietlik M, Pelizon C, Romanowski P, Mills AD, Laskey RA. The roles of the MCM, ORC, and Cdc6 proteins in determining the replication competence of chromatin in quiescent cells. J Struct Biol 2000;129:198–210.

[25] Pereira-Smith OM, Fisher SF, Smith JR. Senescent and quiescent cell inhibitors of DNA synthesis: membrane-associated proteins. Exp Cell Res 1985;160:297–306.

[26] Prokop A, Technau GM. BrdU incorporation reveals DNA replication in non dividing glial cells in larval abdominal CNS of Drosophila. Dev Genes Evol 1994;204:54–61.

[27] Pelc SR. Metabolic DNA in ciliated protozoa, salivary gland chromosomes, and mammalian cells. Int Rev Cytol 1972;32:327–55.

[28] Hara A, Niwa M, Kumada M, et al. Fragmented DNA transport in dendrites of retinal neurons during apoptotic cell death. Brain Res 2004;1007:183–7.

[29] Pisetsky DS, Ullal AJ. The blood nucleome in the pathogenesis of SLE. Autoimmun Rev 2010;10. 810 D.L. Peters, P.J. Pretorius / Clinica Chimica Acta 412 (2011) 806–811

[30] Beyer C, Pisetsky DS. The role of microparticles in the pathogenesis of rheumatic diseases. Nat Rev Rheumatol 2010;6:21–9.

[31] Su KY, Pisetsky DS. The role of extracellular DNA in autoimmunity in SLE. Scand J Immunol 2009;70:175–83.

[32] Bischoff FZ, Lewis DE, Simpson JL. Cell-free fetal DNA in maternal blood: kinetics, source and structure. Hum Reprod Update 2005;11:59–67.

[33] Hariton-Gazal E, Rosenbluh J, Graessmann A, Gilon C, Loyter A. Direct translocation of histone molecules across cell membranes. J Cell Sci 2003;116: 4577–86.

[34] Luger K. Structure and dynamic behavior of nucleosomes. Curr Opin Genet Dev 2003;13:127–35.

[35] Cline AM, Radic MZ. Apoptosis, subcellular particles, and autoimmunity. Clin Immunol 2004;112:175–82.

[36] Viorritto IC, Nikolov NP, Siegel RM. Autoimmunity versus tolerance: can dying cells tip the balance? Clin Immunol 2007;122:125–34.

[37] van der Vaart M, Pretorius PJ. Circulating DNA. Its origin and fluctuation. Ann NY Acad Sci 2008;1137:18–26.

[38] Choi JJ, Reich III CF, Pisetsky DS. Release of DNA from dead and dying lymphocyte and monocyte cell lines in vitro. Scand J Immunol 2004;60:159–66.

[39] Schorey JS, Bhatnagar S. Exosome function: from tumor immunology to pathogen biology. Traffic 2008;9:871–81.

[40] Garcia-Olmo D, Garcia-Olmo DC, Ontanon J, Martinez E. Horizontal transfer of DNA and the “genometastasis hypothesis”. Blood 2000;95:724–5.

[41] Valadi H, Ekstrom K, Bossios A, Sjostrand M, Lee JJ, Lotvall JO. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. Nat Cell Biol 2007;9:654–9.

[42] Lehmann BD, Paine MS, Brooks AM, et al. Senescence-associated exosome release from human prostate cancer cells. Cancer Res 2008;68:7864–71.

[43] Holmgren L, Szeles A, Rajnavolgyi E, et al. Horizontal transfer of DNA by the uptake of apoptotic bodies. Blood 1999;93:3956–63.

[44] Rumore PM, Steinman CR. Endogenous circulating DNA in systemic lupus erythematosus. Occurrence as multimeric complexes bound to histone. J Clin Invest 1990;86:69–74.

[45] Evans DL, Connor MA, Moss LD, et al. Cellular expression and antimicrobial function of a phylogenetically conserved novel histone 1x-like protein on mouse cells: a potential new class of pattern recognition receptor. J Leukoc Biol 2009;86: 133–41.

[46] van der Vaart M, Semenov DV, Kuligina EV, Richter VA, Pretorius PJ. Characterisation of circulating DNA by parallel tagged sequencing on the 454 platform. Clin Chim Acta 2009;409:21–7.

[47] Kleine TJ, Lewis PN, Lewis SA. Histone-induced damage of a mammalian epithelium: the role of protein and membrane structure. Am J Physiol 1997;273:C1925–36.

[48] Deligezer U, Yaman F, Darendeliler E, et al. Post-treatment circulating plasma BMP6 mRNA and H3K27 methylation levels discriminate metastatic prostate cancer from localized disease. Clin Chim Acta 2010;411:1452–6.

[49] Rogers JC, Boldt D, Kornfeld S, Skinner A, Valeri CR. Excretion of deoxyribonucleic acid by lymphocytes stimulated with phytohemagglutinin or antigen. Proc Natl Acad Sci USA 1972;69:1685–9.

[50] Adams DH, Gahan PB. The DNA extruded by rat spleen cells in culture. Int J Biochem 1983;15:547–52.

[51] Adams DH, McIntosh AA. Studies on the cytosolic DNA of chick embryo fibroblasts and its uptake by recipient cultured cells. Int J Biochem 1985;17:1041–51.

[52] Anker P, Stroun M, Maurice PA. Spontaneous release of DNA by human blood lymphocytes as shown in an in vitro system. Cancer Res 1975;35:2375–82.

[53] Adams DH, Gahan PB. Stimulated and non-stimulated rat spleen cells release different DNA-complexes. Differentiation 1982;22:47–52.

[54] Adams DH, Diaz N, Gahan PB. In vitro stimulation by tumour cell media of [3H]-thymidine incorporation by mouse spleen lymphocytes. Cell Biochem Funct

1997;15:119–26.

[55] McIntosh AA, Adams DH. Further studies on the extrusion of cytosol macromolecules by cultured chick embryo fibroblast cells. Int J Biochem 1985;17: 147–53.

[56] Anker P, Jachertz D, Stroun M, et al. The role of extracellular DNA in the transfer of information from T to B human lymphocytes in the course of an immune response. J Immunogenet 1980;7:475–81.

[57] Gahan PB, Anker P, Stroun M. Metabolic DNA as the origin of spontaneously released DNA? Ann NY Acad Sci 2008;1137:7–17.

[58] Anker P, Stroun M. Bacterial ribonucleic acid synthesis in frog organs after intraperitoneal injection of bacteria. Biochem J 1972;128:101P.

[59] Stroun M, Anker P, Gahan PB, Henri J. Spontaneous release of newly synthesised DNA from frog auricles. Arch Sci Genève 1977;30:229–41.

[60] Bastian PJ, Palapattu GS, Yegnasubramanian S, et al. Prognostic value of preoperative serum cell-free circulating DNA in men with prostate cancer undergoing radical prostatectomy. Clin Cancer Res 2007;13:5361–7.

[61] Lo YM, Zhang J, Leung TN, Lau TK, Chang AM, Hjelm NM. Rapid clearance of fetal DNA from maternal plasma. Am J Hum Genet 1999;64:218–24.

[62] Anker P, Stroun M. Immunological aspects of circulating DNA. Ann NY Acad Sci 2006;1075:34–9.

2.2 Introducing the hypothetical model of eogenics for explaining the function of extracellular occurring DNA in genetic adaptation of eukaryotic organisms.

Abstract

It is suggested that extracellular occurring DNA (eoDNA) is the result of the metabolic fraction of DNA that is released by the cell. Various observations indicate that eoDNA may also be incorporated into the genome of a cell, from where it may affect cell function, thereby horizontal gene transfer in higher organisms is a real possibility. It seems viable to assume that a stressor is met by a change in the molecular machinery of a cell, required to neutralise the onset of metabolic instability. This may be done by amplification of necessary cistrons, producing metabolic DNA, that may then be observed after its release as eoDNA. The presence of hydrolysing enzymes gives an updated real time picture of the state of eoDNA. The eogenics hypothesis suggests amplification and horizontal transfer of cistrons affect tissue and organ function over long periods of time, in order to evolve a specialized genome.

2.2.1. eoDNA and its biological function and impact

Extracellular occurring DNA (eoDNA) has been shown to play a role as a messenger in what may be considered to be uptake and effect. eoDNA released by antigen activated human T-lymphocytes, was injected into mice, after which antibodies were produced that bound specifically to respective antigens. Deducting from this, eoDNA may possibly play a key role in the humoral immune response (Anker et al., 1980:475; Anker et al., 2006:34). It seems reasonable to assume that this is accomplished by complex proceedings, involving the expression of specifically functional antibodies from up taken eoDNA messenger sequences, produced by T lymphocytes in response to antigen presentation. eoDNA may also play the central role in a number of serious disease states, such as systemic lupus erythematosus, that seems to be caused by dysfunctions of immunity (Su et al., 2009:175; Rumore et al., 1990:69.).

A very important set of observations, that suggest that eoDNA plays a much bigger role than to perform as an intermediary messenger, involves horizontal gene transfer (HGT) over intercellular space. The genometastasis hypothesis is based on this principal (Garcia-Olmo et al., 2004:575; Garcia-Olmo et al., 1999:1159; Garcia-Olmo et al., 2000:724) and if correct, the hypothesis indicates that genetic information released as eoDNA influences at least a small

fraction of other cells in the eukaryotic organism (Waterhouse et al., 2009:2704). During more advanced states of cancer, the rise in circulating DNA has been shown to represent genetic material of the same mutagenic composition as that of the tumour cells (van der Vaart et al., 2008:18). Experiments also suggest that mutagenic DNA is quite capable of integrating within the genomes of normal, non-cancerous cells (Anker et al., 1994:869), can affect cell behaviour (Adams et al., 1997:119) and even cause oncogenetic transformation of non-cancerous cells(Garcia-Olmo et al., 2010:560). The likelihood of HGT in humans is also strongly supported in a recent review published by leading authors P.B. Gahan and M. Stroun (Gahan et al., 2010:529).

Additional support for the somewhat overlooked possibility that the HGT occurs in humans and is furthermore not restricted to, only, the transfer of oncogenes, are the effects of allogeneic tissue transplantations on the recipient. According to Miguel Waterhouse et. al. HGT may very likely lead to the illegitimate integration of donor DNA in epithelial cells of the recipient, leading to both chimerism and genomic instability after hematopoietic cell transplantation (Waterhouse et al., 2009:2704). Apart from these mentioned, many previous experiments have also indicated that a type of free movement of DNA between eukaryotic cells is possible (Gahan, 2006:21), but unfortunately the significance this holds has apparently eluded much of the research community.

A very important question at the moment is whether eoDNA sequences are specifically released and also incorporated with precision, in specific positions of a receiving genome. One may expect some form of molecular coordination in this process, as illegitimate integration of sequences would have pathological mutations be a much more common phenomenon.

2.2.2 Metabolic DNA

Over the last few years, various authors have insisted on the metabolic fraction of DNA, that is responsible for performing the genome's metabolic functions, to be the precursor of eoDNA, prior to its release. These deductions are based on the work of Pelc et al. who observed that labelled eoDNA is released by non-dividing cells (Gahan et al., 2008:7). As the cell cultures were of non-dividing in nature, the synthesis and release of the newly synthesized DNA could not be accounted for by DNA synthesis required for mitosis, nor

could it be DNA synthesized for repair. Pelc et al. concluded that the DNA observed in his previous work, was the sequences of active cistrons, of which were made supplementary copies. These additional cistron copies are believed to carry out the metabolic functions of DNA. Furthermore, this DNA fraction is also believed to be replaceable and subject to deterioration(Pelc, , 1968:162). More recently it was concluded by Gahan et al. (Gahan et al., 2008:7) that metabolic DNA shares a number of properties with newly synthesized DNA, indicating that there is a likelihood that eoDNA has its origin from none other than metabolic DNA.

An observation made by Adams and Macintosh (1985) using chick embryo fibroblasts, shows newly synthesized DNA first appearing in the cell nuclear fraction, followed, 3 hours later by appearing in the cytosol and finally, a further 5 hours later, making its appearance in the extracellular space (McIntosh et al., 1985:147). Once inside the extracellular space, the metabolic DNA is referred to as eoDNA. If the eoDNA presents itself in circulation, it may be referred to as circulating DNA (Peters et al., 2011:806).

2.2.3 eoDNA turnover

It is believed that an equilibrium is reached when eoDNA reaches a certain concentration in the extracellular space. This is based on various experiments showing the same amounts of eoDNA being released during repeated incubations of similar times, involving the replacement of growth medium between each observation. This equilibrium in eoDNA release is furthermore believed to be maintained by either regulated release by the presence of a feedback system, or by regulated uptake. (Gahan et al., 2010:529). Could these observations be due to a true feedback system, or is it possibly due to competition between release of eoDNA and clearance thereof and could the overlooked capacity of hydrolyzing enzymes also play a role in this process?

Circulating DNA is subject to the hydrolyzing activity of nucleases in plasma that should inevitably be present in all of the body compartments. In plasma, this hydrolyzing activity is mainly driven by DNAse I (Prince et al., 1998:289) and unless the eoDNA is protected from cleavage in some or other way, it would probably not be allowed the benefit of prolonged extracellular existence, hence –activity and integration. There is not much literature available on the turnover of eoDNA in various forms and compartments, but the half-life of circulating

DNA in maternal plasma has been found to be 16.3 minutes (Lo et al., 1999:218). The total turnover is subject to three factors, namely, rate of release, rate of uptake and the rate of breakdown. It is also more than likely that there would be variation in the turnover of various forms in which eoDNA exists. The known forms are basically variations in the molecular packaging of eoDNA. These forms mainly include virtosomes, nucleosomes, other histone-bound DNA complexes and vesicle-bound forms, such as apoptotic bodies, micro particles and exosomes (Peters et al., 2011:806). All of these structures should allow unique extension of times in terms of the bound eoDNA's half-life, with regard to breakdown by hydrolysing enzymes, as well as uptake rates. The rate of breakdown may have an influence on the distance a particular eoDNA complex may be transported via circulation. Understanding turnover is crucial to understanding the nature of the biological impact of eoDNA, as it may directly influence the area of effect, from the point of its release.

2.2.4. Perspectives

If metabolic DNA represents the metabolically active fraction of the genome, also being the precursor to eoDNA before release, it should be possible to investigate gene activation in response to certain stressors and biological changes, by means of sequencing. This should be possible, as interfering factors can be kept to a minimum using cell cultures. The presence of hydrolysing enzymes would assure that only recently released eoDNA is present in the growth medium, thereby allowing proper assessment of the genetic responses that proceed the administered *in vitro* changes.

Apart from being a capable intercellular messenger, that no doubt acts over short periods of time on an existing molecular interface, eoDNA may have a much more dramatic role to play within an organism over longer periods of time, that must be given more than a moment of thought, as it may very well change our understanding of adaptation in higher organisms all together.

In a hypothetical model, referred to as the eogenics, the interactions between the extracellular nucleome and cellular genomes, allow a higher organism to genetically adapt to long term metabolic changes. The genetic output of a cellular genome, transcends the barriers of the cell, as activated replication yields metabolic DNA, later released as eoDNA. In this system, the activation and deactivation of genes by means of epigenetic mechanisms, the

amplification of genes that perform the metabolic functions of DNA and the release-, turnover-, uptake- and genomic incorporation of eoDNA, are all involved in functioning as an interacting system - its ultimate purpose being to conduct the universal genetic functions of all the cells in an area. This would be accomplished by HGT of the most amplified gene sequences and assist in further expression thereof by incorporation into cells. The distance of HGT would depend greatly on the rate of eoDNA breakdown.

Chapter Three

Materials and Methods

3.1 143B human osteosarcoma cells as the eukaryotic study model

As a model for the study of eoDNA release from eukaryotic cells, the 143B human osteosaroma cell line is used. Being that our laboratory already had a range of 143B cell lines that had been genetically altered by transfection with plasmids containing dissimilar vectors, it deemed to be an appropriate place to start investigating the effect of genetic manipulation on eoDNA release, without unnecessary costs. As various other projects are currently being conducted on these cells, cross referencing of data may also lead to additional publishable information. The cell cultures used in this study were originally supplied by the Centre for Human Metabonomics at the North-West University and the cells were originally transfected by Dr. Oksana Levenets. Knockdown cells had been attained by means of RNA interferance, in which mRNA-specific shRNAs, cloned into RNAi-Ready pSIREN-RetroQ-TetP vectors were used for tranfection of standard 143B cells. With the exception of the 143B cell line transfected with pHIV7-GFP lentiviral vector, that relies on GFP for selection, puromycin was used as a selection marker. For a detailed description of protocols used in preparation of cells, please refer to (Levanets et al., 2011:758.). No antibiotics were added as its biological effect may pose a stressor and lead to deviation in eoDNA release patterns. Cells are grown in six well flasks with 2000µl growth medium added to each well. A maximum of 3% cell death was allowed in all the experiments.
In summary, the 143B cell lines used in this study include:

143B	-	No Vector
143B2	-	Empty retroviral vector
143B3	-	pHIV7-GFP lentiviral vector
143B5	-	Complex III knockdown

Culture conditions set to 5% CO2 incubated at 37°C (HERA cell). Culture medium (Thermo Scientific HyClone #SH30243.01): Liquid; Dulbecco's Modified Eagle's medium (DMEM/High); High glucose; 4.5g glucose/L; With L-Glutamine/sodium pyruvate400mg/L glucose (unless otherwise indicated); 10% FBS; no added antibiotics.

3.2 Experimental Design and expression of resulting values

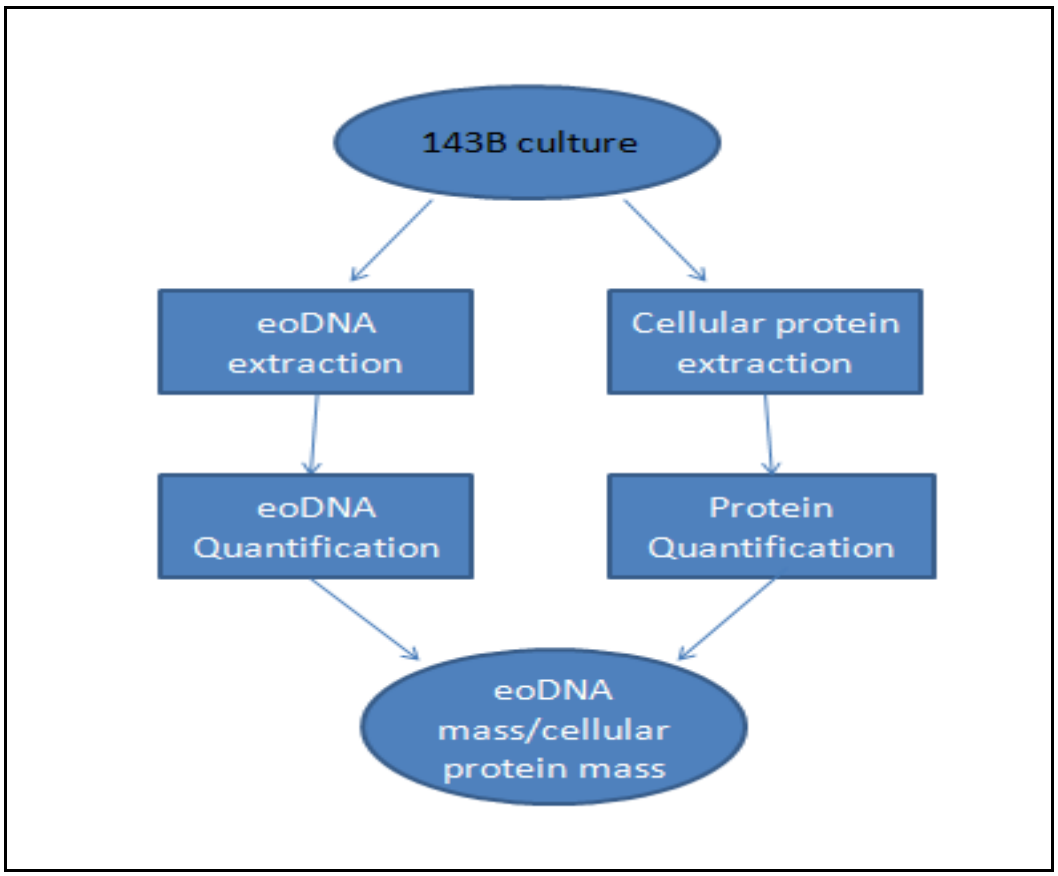

Figure 2. Flow diagram showing the basic design of extraction and quantification of eoDNA and protein used in this study**.** Here is illustrated, a flow diagram of the experimental procedures that finally gives the ratio of eoDNA mass over protein mass. As the diagram indicates, the eoDNA masses and the protein masses are determined in parallel to finally yield the ratio of the two values.

A schematic view of the basic experimental design for acquiring eoDNA mass with relation to cellular protein mass is shown in Figure 2. In order to assess the release of eoDNA by cells in multiple cell cultures, it is necessary to establish some relationship to correlate the values. A classic and very simple way to do this is to make use of cell counts and thus to express eoDNA concentration in terms the amount of cells present in a particular well.

There are unfortunately a few setbacks to this form of standardisation. Cells in the exponential growth state are of dividing and growing nature and the average of cells may not

be of similar size, which may lead to misinterpretations in the meaning of eoDNA levels. This may be explained in the light of Section 2.2. One may expect variation in cell sizes, regardless of equal cell counts, to cause differences in terms of nutritional requirements and also the amounts of cellular machinery required to re-establish homeostasis after subjection to a stressor. Sequentially, gene expression and metabolic DNA production may differ between cell cultures. Since gene expression very likely correlates to eoDNA expression, the differences in cell size very well have an influence on the expression of eoDNA. Additionally, cell counts are not very accurate, leading to rather large standard deviations in either case.

As an alternative, total cellular protein and eoDNA are determined in parallel, as to be expressed in terms of a ratio, establishing a relative observation among cultures. In this study the assessment of cellular protein mass is used to standardise the relationships between eoDNA release in various cell cultures. Preparing cellular protein for quantification by Bicinchoninic Acid assay BCA assay, requires only that cell membranes and structures are thoroughly disrupted prior to quantification, since the remaining cellular contaminants do not interfere with the absorption frequencies of light. The use of glass beads and a shaker is adequate for achieving this goal. The process is simple, cost effective and time efficient. eoDNA extractions are performed directly from growth media by means of NucleoSpin Extract II columns (Macherey-Nagel #740609.250). Cellular protein extract is done by glass beads of approximately 40 MESH (BDH Chemicals Ltd) and shaker (Retsch MM400) set to 30 Hz for five minutes total. The BCA protein quantification procedure is used (Pierce BCA Protein Assay Kit. Thermo Scientific. Instructions Manual. 2161297).

3.3 Comparing the phenol/chloroform extraction method with the silica gel column extraction method

3.3.1 Aim

To determine which eoDNA extraction protocol, phenol/chloroform extraction or silica gel column extraction, is the most appropriate for use in this study.

3.3.2 Approach

In order to have determined which extraction procedure is more efficient for quantification studies of eoDNA, two main extraction procedures were compared. The most effective method would most importantly be precise. Accuracy involved here would not be as important as precision, since various values are to be compared to one another, in order to determine variation in eoDNA among samples. In other words any deductions will be based on the relationships of values among the various samples.

Silica gel columns are internationally used for these purposes, probably since they are exceptionally easy to use and since they are industrially manufactured one may expect each column to have very similar yields (Macherey-Nagel. NucleoSpin Extract II. User Manual October 2007/Rev.6). The more classic phenol/chloroform extraction method is claimed to produce the greatest yields of the extraction procedures (van der Vaart, , 2006:1.), but is very time consuming and is much more subjected to human error, as most of the techniques require great concentration, steady hands and patience. This is especially true when drawing the aqueous (containing eoDNA) phase from the organic (containing residual protein complexes) phase and for the resuspension of invisible, precipitated eoDNA by means of rolling a pearl of TE-buffer across precipitation surfaces.

In order to determine the yields of the two extraction protocols, standard concentrations of genomic DNA is prepared and added to sterile growth medium samples. A standard concentration of human genomic DNA is added to growth medium samples with the standard composition for this study (see Section 3.1). The extraction protocols in question are now performed and the extracted DNA is quantified to determine which, is most suitable for use of eoDNA quantification for multiple samples in short amounts of time from culture media.

3.3.3 Phenol/Chloroform extraction and ethanol precipitation protocol

The protocol used in this study is based on the protocol for extraction of eoDNA from blood plasma, as described by M. van der Vaaart (van der Vaart, , 2006:1.). Since however, the volume of growth medium obtainable from a single well in a six-well plate is not more than 2ml, the protocol had to be optimized for extraction of eoDNA from a very small volume. This was necessary to assure that triplicate extractions could be performed on each individual cell culture, in order to assure accurate results.

Directly after the sample medium is removed from culture, a 20µL volume comprising of 20 mM Proteinase K, 10% SDS is added to every 200µL of sample medium. Concentrated amounts of both Proteinase K and SDS are used to minimize the volumes that are to be worked with, thus ensuring that the extraction may be carried out in micro tubes, exclusively. These samples are now incubated overnight at a temperature of 55˚C, to assure optimum digestion (Proteinase K) and/or denaturing of protein (SDS).

Micro tubes (1,5ml) are respectively marked A and B. A volume of 200µl hydrated phenol and a volume of 200µl of 99% chloroform is added to tubes A and B. The sample (incubated in Proteinase K, SDS) is now added to tube A. Tube A is then vortexed for a period of 30 seconds and is then incubated at -20˚C for 30 minutes to assure proper precipitation of protein, which may prove to be an overwhelming amount, undilutable in the organic phase at room temperature. Following this cooling step, the sample is centrifuged at 12,000 rpm for 10 minutes to separate the organic (phenol/chloroform) and the aqueous phases. Higher rpm values have also proven to aid in removal of large amounts of protein from the aqueous phase. Chloroform is important for increasing the density of the organic phase, which would otherwise, in many cases come to rest on top of the aqueous phase. After the phases are separated, the aqueous phase is moved to micro tube B by means of a pipette. The DNA purification steps are now repeated (vortex, cool down and centrifugation). Purification steps may be repeated if large amounts of protein is hardly removed from the sample. When a clear aqueous phase is obtained, the aqueous phase may be drawn by pipette and moved to a ‘clean’2ml micro tube.

At this point, 1µl of 20mg/ml glycogen is added to the sample, followed by 80µL of 10M Ammonium acetate and finally a 2:1 volume of 100% ethanol is added to the sample, in the sequence as is mentioned. In order to assure proper precipitation the dissolved DNA, the

sample is incubated at -80˚C for 10 minutes. The sample may now be sentrifuged at high rpm, to assure proper precipitation of the small eoDNA fragments. Centrifugation was performed at 22, 000 x g for 1 hour at 4˚C for optimal results. Ethanol is now poured of, while taking care not to disturb the small amount precipitated eoDNA which is hardly visible, if at all. A good idea may be to keep this supernatant, in case one discovers that the pellet has been poured off, along with the ethanol, ammonium acetate and glycogen mixture.

The final washing step requires the addition of a volume of about 1,8 ml (in order to cover the entire precipitation area) of 70% ethanol, to dissolve any precipitated ammonium acetate. After adding the 70% ethanol to the tube, the tube is centrifuged once again at 22, 000 xg for 10 minutes, since the eoDNA can be expected to have been firmly precipitated on the tube walls in the previous centrifugation step.

After once again taking care not to disturb the precipitated eoDNA, the 70% ethanol is poured off and the tube left to air dry, in order to assure that no remaining fluid interferes with the final volume of sample. A 25µL to 50µL 'pearl' of TE buffer (30mM Tris, 0.3 mM EDTA) may now be 'rolled' across the entire precipitation surface, by making use of a pipette, in order to solubilise the precipitated eoDNA.

3.3.4 Silica gel based column extraction protocol

The extractions are performed as described by the NucleoSpin Extract II user maniual. (Macherey-Nagel. NucleoSpin Extract II. User Manual October 2007/Rev.6). Culture media was directly subjected to the standard PCR cleanup protocol.

3.3.5 Real-time Quantitative PCR Protocol

Thefollowing real-time PCR protocol is used throughout all subsequent experiments. The total volume of the reaction mix used in RQ-PCR was 25 µL. This consisted of 2µL DNA and 23 µL mastermix. The mastermix itself was composed of 1µl of 10µM sense and 1 µL of 10µM antisense primers, 0.4µL of 10µM 5'FAM;3'TAMRA Probe, 12.5µL TaqMan Universal MasterMix .8.1µL water. 1,5 mM $MgCl_2$ concentration is used, as was determined by Maniesh Steyn by means of concentration gradients for these primers and probe. The

conditions for PCR were set to denature DNA at 95°C for 10 minutes, this was followed by 45 cycles of denaturation at 95°C for 15 seconds each and annealing at 60°C for 1 minute (van der Vaart, , 2006:1).

β-globin is used for quantification as it is present in all nucleated cells of the human body and could therefore be useful in that results in this in vitro study may correlate to results from other in vivo studies. Moreover β-globin is considered to be a common housekeeping gene present in all nucleated cells of the human body (van der Vaart, 2006:1.). Since the main approach in this study is examining eoDNA release under stressful conditions, and β-globin is considered to be a common housekeeping gene (van der Vaart, 2006:1.), the use of β-globin may prove to be an effective marker sequence for the purposes of this study.

The sequences of the primers and probe used are given in Table 1 and were manufactured by Eurofins MWG Operon. β-globin sequence data is obtainable from GenBank (accession number: U01317).

Concentration of the stock human genomic DNA, used in the creation of a standard curve was accurately determined by PicoGreen Nucleic Acid Quantification (Invitrogen). The concentration gradient for standard series, prepared from whole genomic DNA, was run in triplicate. The standard curve showed R^2 values, no lower than 0.98, which is acceptable for accurate quantification purposes. Samples were run in triplicate. Each run included non template controls. An Applied Biosystems 7500 Real-Time PCR system and accompanying software was used to determine all unknown concentrations by means of the second derivative method obtained from real-time run analysis.

Table 1. Sequences and fragments of β-blobin primers

Fragment 101bp

Forward	5'-GTG CAC CTG ACT CCT GAG GAG A-3'
Reverse	5'-CCT TGA TAC CAA CCT GCC CAG-3'
Probe	5'-(FAM)AAG GTG AAC GTG GAT GAA GTT GGT GG(TAMRA)-3'

3.3.6 Results

The results in Figure 3 are expressed in terms of percentage recovered DNA, with consideration toward the amount of DNA added to the growth medium. That is the mass of DNA recovered, divided by the mass of DNA present in the medium. The amount of DNA present in the growth medium prior to the addition of human genomic DNA is in the order of 10 to the power of -7 or less and is therefore considered to be negligible.

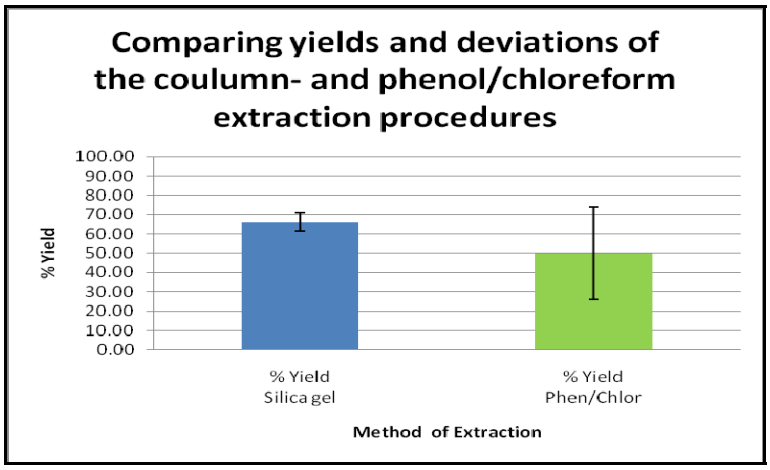

Figure 3. The recovery of a standard amount of human genomic DNA from growth medium, by silica-gel column- and phenol/chloroform extraction method in terms of percentage yield. This figure shows the percentage of DNA recovered from the growth medium samples after extraction with regard to the total volume of DNA that had been added to the medium prior to extractions. Both Silica-gel column extraction yield (left) and phenol/chloroform extraction yield (right) are included. Error bars represent standard deviation.

The silica gel based column extraction proved to be both more accurate and precise than the phenol/chloroform extraction method adapted for growth media samples, as it is clear that a higher percentage yield is thereby gained and also a lower standard deviation acquired.

3.3.7 Choosing the most appropriate extraction protocol - Conclusion

The results clearly indicate that the column extraction protocol produces higher yields of DNA than the phenol/chloroform extraction method. Additionally the column extraction method produces more acceptable standard deviation values than the phenol/chloroform method.

Even though the phenol/chloroform extraction is expected to produce higher yields than the silica gel columns, the results proved differently. This may be due to handling errors when performing the very delicate procedures involved in the phenol/chloroform extraction method, such as rolling a pearl of TE-buffer across the precipitation area of a micro tube to dissolve the µg amounts of eoDNA. Another example would be the delicate pipeting technique of drawing the aqueous eoDNA containing phase from the organic, protein containing phase.

As the column extraction method is far easier to perform and due to the method's additional reliability and repeatability in comparison to the phenol/chloroform extraction this method used throughout the study. In doing so it is assured that all the values of eoDNA obtained may be comparable and in this way all results may be used in delivering a conclusion.

3.4 Extraction of eoDNA and cellular protein in pending experiments

Extractions of various incubation times are performed in separately cultured wells, so as to assure that any effects of lowering the volume, due to multiple extractions from a single well may be overcome. Values are expressed in terms of DNA (ng)/ cellular protein (g) in order to effectively portray relationships in logarithmic graphs.

After removing sample medium from a flask or well by means a pipette, the growth medium is removed from the flask/well and spun down, at 700 x g for 5 minutes. The supernatant is carried over to a new tube, in order to remove any possible contamination by cells that may have broken free from adhesion to the flask/well surface. The required fraction of growth medium is then subsequently subjected to the column extraction method described below.

Following the removal of growth medium, the wells are washed using 2ml of PBS, after which 1ml trypsin (1%) is added for digestion of protein structures attaching the cells to the well surface. After 15 min, the cells can be removed from the well using a pipette. All cells

are transferred to a microtube. The tube once again is centrifuged at 700 x g for 5min. The pellet is then washed using PBS. This is achieved by adding 500µl of PBS to the pellet followed by resuspension. The cell suspension is then centrifuged once again at 700 x g for 5 min, after which the final volume of 100µl of PBS is added. In order to release and dissolve cellular protein, 40 MESH glass beads (BDH Chemicals Ltd) and a shaker (Retsch MM400) is used. The shaker is set to 30Hz for a period of 5 minutes. Protein samples are then stored at -20˚C until quantification may be done using the standard BCA quantification protocol.

3.5 BCA Protein quantification Protocol

The recommended protocol for BCA quantifications was followed (Thermo Scientific. Pierce BCA Protein Assay Kit. Instructions Manual. 2161297). The protocol involves the design of a standard curve with protein derived from a standard concentration of BSA (2µg/µl). Protein masses of 0 µg, 4 µg, 8 µg, 12 µg, 16 µg, 20 µg, were prepared in duplicate to produce optimum results with R^2 values no less than 0,997. Samples containing more than 20 µg of protein, was diluted prior to quantification. Protein quantification is done by BCA Protein Assay Kit (Thermo Scientific #23227) and spectrophotometry (BioTek Synergy HT).

3.6 DNA content in unused culture medium

To be sure that the culture medium and/or the foetal bovine serum (FBS) contains no DNA that would amplify with the β-globin primers and probe that are used for RQ-PCR, a column extraction was performed on the clean growth medium with added FBS , that was used in this study. The results showed no amplification in three out of the six tested media samples and in the remaining three samples concentrations of less than 100 fg/µl were observed, and can be considered negligible. Even though it seems likely that at least the FBS would contain DNA, the very small amount of amplification, or no amplification of sequences all, that is observed is probably due to the fact that the β-globin primers and probe are specifically designed for human genes. These genes are evidently, either not equally well conserved in humans and the subfamily, Bovinae. Another explanation may be that degradation of the DNA sequences present in the FBS (and/or possibly the medium) had rendered the DNA fragments undetectable by RQ-PCR.

Experiments using culture medium, containing FBS can therefore be performed without hindrance of background amplification from the growth medium itself.

3.7 Storage of medium samples for later use

As many samples are to be handled at once during the experiments in this study, it is essential to determine if storage of samples has any effect on the concentrations of eoDNA. Culture media obtained from HeLa cell cultures were used in this experiment due to availability. A fraction of the medium was then subjected to immediate extraction, whereas the remaining fraction was stored at -20°C for a total period of 24 hours and left to thaw at room temperature prior to eoDNA extraction. The eoDNA was then quantified using RQ-PCR The results are shown in Figure 4.

Judging from the results in Figure 4 it is clear that the storage of the medium samples had an extremely negative influence on the eoDNA concentration. Reasons for this coming to be are not clear, as the freeze and thaw of DNA, especially such short fragments as eoDNA, should not be degraded to such an extent. It is possible that nucleatic enzymes, present in the medium, may have an effect on the eoDNA concentrations. Cleavage of DNA would have to take place during the freezing and thawing periods. At the lower temperatures however, the enzyme activity would be greatly stunted. The effect of DNA degradation in the culture medium at 37°C is briefly investigated in Section 5.2 in order to determine its effect on eoDNA turnover. It is unclear whether nucleases could be responsible for the massive amount of degradation observed in the short period of time required for freezing and thawing, especially since the sample would be at room temperature and below. In order to save on expenses, ways of effective storage were not further investigated. If the breakdown of eoDNA is due to cleavage by nucleases, flash freezing and accelerated thawing may possibly lead increase yields of eoDNA.

Due to the extent of deviation seen in sample that were stored at sub zero temperatures overnight, all media samples used in this study were subject to extraction directly following removal from culture.

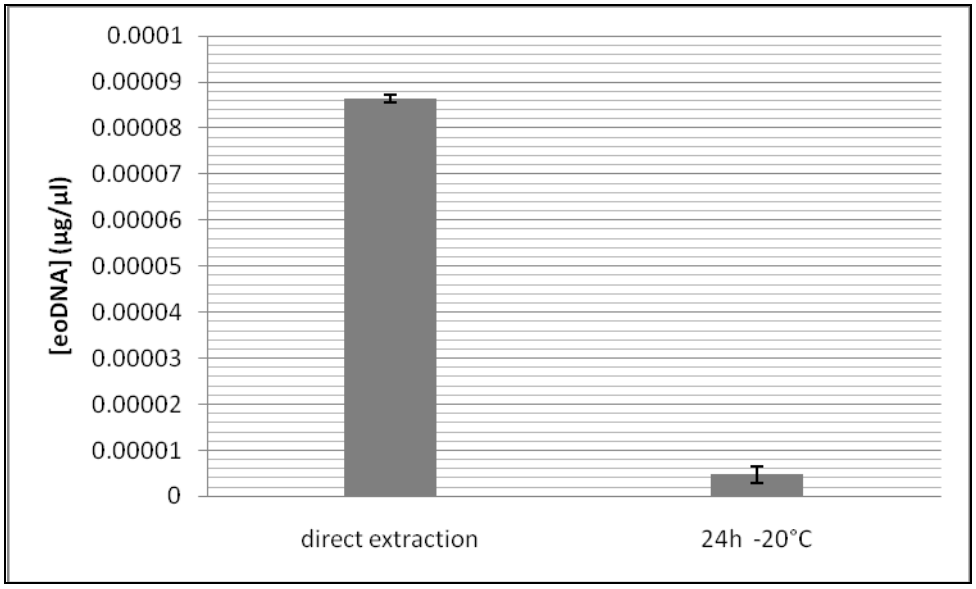

Figure 4 The effect of sub zero temperature incubation for a 24 hour period, on eoDNA. eoDNA concentrations extracted from the culture medium of HeLa cells are shown in this figure. The 'direct extraction' represents the eoDNA that is quantified from an extraction, directly after the medium was removed from the cell culture, whereas, the '24h -20°C' represents eoDNA concentration as measured from the exact same sample medium, incubated at -20°C for 24 hours. Error bars represent standard deviation.

3.8 Assuring medium is not infected

In order to assure that no cultures had been infected, thereby influencing eoDNA levels by microbial activity, fractions of each growth medium sample are incubated for additional and prolonged periods of time (48h at 37˚C) and investigated under magnification by microscope. Infected cultures are discarded from consideration and sequential quantifications not performed.

Chapter Four

The influence of confluence on eoDNA release in 143B cell cultures

4.1 Aim

To determine whether the state of cell confluence in a 143B culture has an impact on the release of eoDNA.

4.2 Approach

It has been shown that eoDNA release may very well be associated with cell cycle phase (Peters et al., 2011:806) (Madine et al., 2000:129) (Prokop et al., 1994:204) (Pelc, 1972:32). As this is at least the case with certain strains of eukaryotic cells, it is important to determine whether the division of 143B cells in culture could possibly effect eoDNA release. These observations would not only give an idea of whether dividing and non dividing cells release eoDNA differently, but would also give a pertinent idea of what the optimal confluence would be to investigate eoDNA release from 143B cells.

One may expect to see some form of variation in eoDNA levels among dividing and non-dividing cells, since various cell cycle phases are expected to be governed by genomic intervention.

4.3 Methods

Cells were incubated in six well flasks in the presence of 400mg/ml glucose, standard 10% FBS (Sigma) and no antibiotics to minimize the possible effect that stressors may play on DNA release. Cells were incubated in volumes of 2ml growth medium per well. Standard (unmanipulated) 143B cells were incubated and grown to the various confluencies, namely 60%, 80%, 90% and 100%, prior to being harvested for DNA and protein quantification.

Cells were initially grown in common 75cm² incubation flasks. The cells were then trypsinated and split in a series of dilutions to produce growth media with 4 distinctive cell concentrations in a descending order. These four cultures mediums, containing descending numbers of cell populations was then each added to an individual well, in a six well plate. The cells were then incubated, until the flask, containing the highest number of cells (initially), reached a 100% confluence. At this point, the remaining wells showed confluencies of 90%, 80% and 60% respectively, which was required for the proceeding extractions.

Following the standard extraction and quantification protocols, the value of total DNA mass is determined by simply multiplying the concentration value obtained from RQ-PCR by 2000µl (the volume of growth medium per well). This value is used to determine the eoDNA mass (µg) /Cellular protein mass (µg) to express the final results. Results are determined from two separate growth medium extractions. Each of these extracted DNA samples are quantified twice using RQ-PCR. Protein content is determined by rupturing the total cellular content and thus performed only once per well. Protein quantification was performed by Pico Green assay using three dissimilar sample dilutions and two repeats of each.

4.4 Results

Referring to Figure 5, the respective eoDNA (µg) / protein mass (µg) of 2.70E-06, 5.32E-06, 5.38E-06 and 6.72E-06 were measured for the 100%-, 90%-, 80%- and 60% confluent cultures. The maximum value of DNA (6.72E-06 eoDNA (µg) / protein mass (µg)) was measured in the 60% confluent well medium, with seemingly lower values measured as confluence increased. There seems to be a sudden drop in eoDNA (µg) / protein mass (µg) once the cells reach 100% confluence, with regard to lower confluencies.

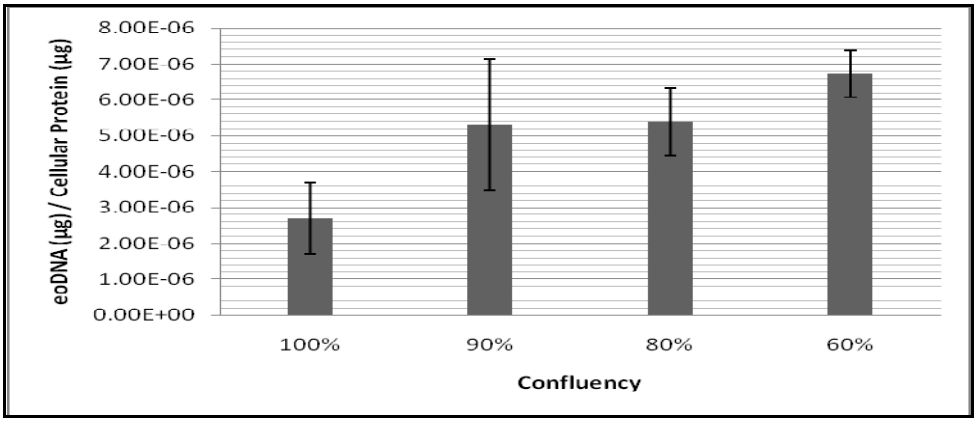

Figure 5. Here is shown the resulting relationships of eoDNA mass (µg) over cellular protein mass (µg) of 143B cells under confluence percentages of 100%, 90%, 80% and 60%. he units of eoDNA and protein mass are so chosen to be easily visualized. Error bars represent standard deviation.

4.5 Discussion and conclusion

Figure 5 shows a clear tendency in eoDNA (µg) / cellular protein mass (µg), for 143B cells of various confluence, to be elevated in cultures that are in the exponential growth state. The results indicate a decrease in eoDNA- / protein mass relationship for cells that are 100% confluent (thus believed to be non-dividing). This concludes that 143B cells show lower levels eoDNA release in non-dividing conditions. These finding do differ from observations suggesting that DNA is replicated during quiescence (Pelc, 1972:327; Prokop et al., 1994:54.), in that 143B cells may possibly release eoDNA as a consequence of mitotic DNA replication. This is certainly not the only possible explanation for a decrease in eoDNA levels observed at 100% confluence and the matter will be further addressed later in this dissertation.

An additional explanation for lower levels of eoDNA at 100% confluence may be as follows: A publication by Rustom et al. (Rustom et al., 2004:1007.) describes how nanotubular structures in mammals, that exist for brief periods of time, connect cells in relatively close proximity to eachother. The structure of these nanotubes are described to have diameters ranging between 50 and 200 nm and may stretch and connect cells lying several cell diameters apart. The nanotubes are capable of transferring cellular components such as genetic material (as well as multiprotein complexes and even organelles) between connected cells. According to a recent review (Rustom et al., 2004:1007., Waterhouse et al., 2009:2704.) this communication system is possibly related to cell fate change, but will require future

studies to determine whether these nanostructures are present in situ and if so, what the biological consequences are.

During the preparation of 143B cell cultures and in the course of the exponential growth states, the cells are seen to form protrusions that seemingly stretch outward in order to make contact with adjacent cells in culture. As the cells come into contact with one another and indeed make contact through structures like nanotubes, one may expect the intercellular communication, by means of eoDNA to be altered (Gahan, 2006:21.), in that these structures form a direct communication link between the cells and henceforth random release and uptake of eoDNA is rendered obsolete and is preferably transported directly to the target cells. This cannot be said with certainty and requires further investigation.

The decrease in eoDNA at 100% confluence may possibly be explained by a decrease in cellular communication (eoDNA included) necessary for the orchestrated growth of a tissue. As the cells come into contact alternative forms of communication come in to being and results in a decrease of eoDNA.

Rustom et al. described the phenomenon of nanotubular transfer of nucleic acids and even structures like organelles between eukaryotic cells. These nanotubes may stretch the lengths of multiple cells and may also be associated with a decrease in eoDNA levels at 100% confluence. (Rustom et al., 2004:1007.). As the 143B cells attach to well surfaces, the cells develop protrusions that seemingly attempt to connect the cells that lie in relatively close vicinity to one another. These protrusions clearly increases the surface area of the cells and if these cells also partake in the forming of nanotubular communication structures (which is very likely indeed), the protrusions would most certainly aid in extending the range of the nanotubes.If the biological function of eoDNA is to serve as a form of intercellular communication, as suggested by Gahan and Stroun (Gahan, 2006:21.), the transfer of eoDNA between cells may be greatly aided in efficiency and specificity by nanotubular trafficking. Once sufficient nanotubular connections are possible, cells may prefer transferring genetic material by means of this system, instead of releasing the material for what is possibly random uptake by adjacent cells. This may explain the decrease in eoDNA present within the culture medium of 100% + confluent cell cultures.

Since there is low variation in the eoDNA levels for 90%-, 80%- and even 60% confluent cell cultures, it seems suitable to perform all subsequent experiments on cell cultures with a 80% confluence. Slight errors in determining confluence would subsequently lead to fairly small deviations in obtainable data. The measurement of eoDNA values at 100% confluence may behave completely differently. This study will not investigate such aberrations in eoDNA levels over time and focuses on the characteristics of eoDNA released by 143B cells during the exponential growth state. It may however be important to address this issue in future studies.

Chapter Five

A simple approach towards investigating real-time release and turnover of eoDNA in culture

5.1 Aim

The aim of the experiments in this chapter is to determine whether it is possible to make use of cell cultures to investigate the nature of DNA release and turnover rates by means of compiling eoDNA release values over time. In doing so, the experiments attempt to show whether variation in release patterns are in some way relatable to controlled subjection of biological factors. The extent of the hydrolyzing capacity towards nucleic acid in culture medium is also addressed, so as to shed light on the nature of eoDNA turnover in cell cultures.

5.2 Approach

5.2.1 Gene manipulation and eoDNA release

The review emanating from this dissertation gives a thorough perspective on the present international scope of eoDNA and discusses critical aspects that must be considered by the relevant research community if we are to better understand the biology of eoDNA. The article stresses that it is very important to understand the nature of eoDNA release and turnover, as the duration of an eoDNA particle's existence may very likely have a significant impact on its bio-functionality, especially *in vivo*. Three factors would ultimately govern such a trait as turnover and includes the rate of release, the rate of uptake by cells and also the rate of breakdown. If the rate of turnover is coupled to feedback mechanisms as mentioned by Gahan and Stroun (Gahan et al., 2010:529.), we would expect to see a type of set-point value which is maintained for homeostasis of eoDNA concentration in the following experimental results.

Experiments carried out by Stroun et al. (1977) (Stroun M et al., 1977:229.), showed that frog heart auricles release similar amounts of eoDNA during 4h incubation periods, repeatedly for a total incubation time of 24h. The deduction made from these findings are believed to be an indication of a possible feedback mechanism involved in maintaining eoDNA concentrations

at a specific concentration, according to Gahan and Stroun (Gahan et al., 2010:529.). Given that these studies were not carried out in a real time analysis fashion (since new culture medium had been added to the cells at each 4 hour incubation), it may be possible that instead of being released and governed by a true homeostatic feedback system, the studies may rather be an indication of a constant release flux of DNA, in which similar amounts are released over a specific period of time. It may be questioned further, whether deviations in the release patterns may be observable when the cells are placed under various conditions of stress as well.

In this study multi-well incubations are used to assess the release of eoDNA by means of a real-time approach, in order to express eoDNA levels in the form of release patterns and to examine whether a homeostatic feedback system is visible under these conditions. Biological factors chosen to provoke changes in eoDNA release patterns include various transfections and heat shock treatment. In order to assess whether overlapping factors may influence eoDNA release patterns, two transfected cell lines were chosen for the heat shock study, that respectively include an empty vector (placing low molecular demands on the cells) and a complex III knockdown (placing high molecular demands on the cells).

5.2.2 Heat shock treatment and eoDNA release

Heat shock treatment puts cells under an enormous amount of stress for the period of time at which the treatment is administered. During this time, normal metabolism is expected to be disrupted, as the increase in temperature seriously effects protein function and also conformation, which may not return to normal as temperature normalises. Cells produce certain heat shock proteins (and possibly some accompanying housekeeping proteins) under such conditions of stress that are said to aid in the correct conformational folding of other protein structures (Houry, 2001:227; Vabulas et al., 2010:a004390.). Additional production of such heat shock proteins, may very well require the amplification of related cistrons representing metabolic DNA, which would later be observable as eoDNA.

As a model for determining whether heat shock treatment has any effect on the release patterns of eoDNA, 143B2 cell lines containing empty transfection vectors and 143B5 cells with complex III knockdowns are used. It may prove useful to investigate how an

overlapping stress factors (being the state of genetic transfection and the biological impact thereof, together with the heat shock stressor) affect the patters of eoDNA release. The control model for the 143B2 and 143B5 cell lines exposed to heat shock, in this experiment, are 143B2 and 143B5 cell lines that were not exposed to heat shock. The degree to which these release patterns differ from those of the transfected cells, not heat shock treated and posing as controls, would also give an idea as to whether the cells are capable of responding (in terms of the eogenic route) to new stressors in addition to previous detrimental conditions, indicating whether overlapping stressors lead to release patterns that also have overlapping traits. If the effect of stresses on the cells compete in terms of severity, with regard to the patterns of eoDNA flux, one may expect to see overlapping effects take place more prominently in the 143B5 culture, as the complex III knockdown has real and serious metabolic implications for the cells, whereas the eoDNA flux patterns of the 143B2 cells containing an empty vector, would probably be easily 'over-writable', since there is no real biological function or demand for expressing the empty vectors.

Since heat shock treatment is exactly as the name says, a shock treatment, one may expect to see a drastic and sudden change in the pattern of eoDNA release during this experiment, instead of an incremental change, as that observed in the other stress-related experiments. If this is seen as a sudden increse in the release patterns of the eoDNA following the time necessary for metabolic to migrate and translocate the cell membrane, it would serve as support for the eogenics model proposed in Chapter 2, Section 2.

5.2.3 Nutrition and eoDNA

In order to form an idea of how sensitive the eoDNA release flux is to the state of nutrition, a simple comparison is made between the release fluxes of 143B2 cells grown in growth medium with galactose and glucose, as respective energy sources. As cancer cells mainly derive energy from glycolysis, the change in nutrition has an impact on metabolism, by shifting metabolism to the Krebs cycle and the respiratory chain (Levanets et al., 2011:758.).

5.2.4 eoDNA breakdown in growth medium

Quantifying eoDNA from a cell culture, incubated in a particular medium for varying amounts of time, would give a good impression of whether the system truly behaves in a homeostatic way. The obtained results may also be subject to the other factors governing turnover and homeostasis of eoDNA, namely uptake and breakdown. In order to determine how great an impact breakdown has eoDNA levels, a fraction of culture media is isolated from the cells while still being subject to further incubation in parallel with sample media in culture. Isolation of media samples eliminates both cellular uptake- and release factors from turnover and thereby highlights extracellular nucleic breakdown.

5.3 Methods

5.3.1 Assessing the impact of genetic manipulation on cells

Standard- and genetically altered 143B cells are used for this experiment. Manipulated cells include the 143B2, 143B3 and 143B5 cell lines that are described in Chapter 3.1. The standard 143B cell line serves as a control to other release patterns. In heat shock experiments, the transfected cell lines that were not exposed to heat shock may be considered as controls to their respective heat shock treated cell models.

A series of parallel eoDNA to cellular protein mass relations are determined at various periods of time in order to formulate a real-time image of eoDNA expression. In order to assess the release of DNA over time, a single cell culture could not be used, as such a procedure would cause certain interference with eoDNA concentrations, even if the process is simply governed only by the flux of DNA release and independent of other factors. Additionally the sudden decreases in the volume of culture medium may very well have an influence on the eoDNA turnover, if there is a homeostatic feedback mechanism involved. Multiple cultures are therefore used to avoid interfering with eoDNA levels. These cultures have to be of similar confluence to assure that eoDNA release is not influenced by cell cycle phases, as may very well be the case (see Section 2.1). In accordance to the results in

Chapter Four, culture media is harvested at 80% confluence. This ensures that the near maximum number of cells is present in each well while assuring the cells are not influenced by a state of stagnant growth or possible mechanisms that side step eoDNA to be freely released into the medium.

Wells are marked 0h, 1h, 3h, 7h and 15h respectively. These different times should give a pertinent idea of how eoDNA concentration changes over periods of time. Marking the start of the series of extractions is the zero hour time mark. At this point the medium from all five wells are replaced and is directly followed by harvesting the cells and culture medium from the 0h well. Following this, one hour later is the harvesting of cells and medium from the 1h well and so forth, until the last well has been harvested at a time of 15 hours.

After the growth medium is extracted from a particular well, the cells remaining intact and bound to the well surface is trypsinated and removed. A fraction (1 ml of the 2 ml growth medium sample) of this cell volume is treated with trypan blue dye (15 µl PBS, 10 µl culture medium, 25 µl Trypan Blue), in order to determine a percentage of cell death, under magnification by microscope. The remaining volume of cells in trypsin suspension is pelleted at 700g/5min. The pellet is washed, using PBS (e.g. resuspension in PBS, after which the cells were once again pelleted. Repeated twice.), removing extracellular protein debris. Results are determined from two separate growth medium extractions. Each of these extracted DNA samples are quantified twice using RQ-PCR. Protein content is determined by rupturing the total cellular content and thus performed only once per well. Protein quantification is performed by Pico Green assay using three dissimilar sample dilutions and two repeats of each.

5.3.2 Subjection of heat shock

In order to assess the impact of heat shock treatment, various lines of 143B cells are cultured in 400mg/L glucose, 10% FBS, until an 80% confluence is reached. The cells were then subjected to heat shock at a temperature of 43˚C for one hour (Schliess et al., 1999:1557.). At the hour's end, growth medium from all wells were replaced and thus marked the zero

hour incubation time, at which the first sample was extracted. Subsequent extractions, from respective wells, are then performed at times 1h, 3h, 7h and 15h.

5.3.3 Subjection of nutritional differences

In order to assess whether differences in nutrition has an influence on eoDNA release patterns, 143B2 cells (empty vector) are incubated in growth media, containing 183 mg/L galactose content, instead of the standard glucose (4500mg/L). Additionally glucose growth medium contains 4mM L Glutamine and Pyruvate. Galactose growth media contains DMEM and L-Glutamine, with no D-Glucose or Sodium Pyruvate.Cells incubated in galactose growth medium is initially grown in the standard glucose medium used in previous experiments, until the cells reach a confluence of 70%. At this point the glucose-containing growth medium is replaced with the galactose-containing growth medium. The cells are now grown to a confluence of 80% in the galactose-containing growth medium. This is done because the switch in nutrition stunts the growth of the cells. It is also necessary for the cells to metabolically adapt to the nutritional change. According to Dr. Oksana Levenets, who has worked on the 143B cells in experiments involving forced shifts in metabolism, the 143B knockdown cells require a period of at least 48 hours, to adequately adapt to the metabolic switch. Even though this experiment is performed on 143B2 cell, which are not knockdown cells, the same amount of time is allowed for the cells to equilibrate. The 48h period is also just about the amount of time required for the cells to reach 80% confluence and at this time the cells are ready for the initial start of the experiment.

Protein- and eoDNA extraction- and quantification protocols are described in Chapter Three.

5.3.4 Assessing eoDNA breakdown in culture

At an incubation time of 7 hours, an additional sample growth medium from a standard 143B culture is separated from culture medium. A fraction thereof subject to eoDNA extraction and RQ-PCR, while the remaining fraction is incubated, apart from culture cells, in a 2ml microtube at 37˚C, up to the 15h time mark. At this time the DNA content was extracted and

quantified as well. The results for this procedure are given in Figure 8 of the results section in this chapter.

In addition to the previous experiment on turnover, the decrease in eoDNA of the transfected 143B cell lines is investigated separately and somewhat trivially, to establish simply whether eoDNA release in these cultures are also opposed by hydrolyzing effects. In this experiment, the 143B2, 143B3 and 143B5 cell lines were cultured for a time of 24h after growth medium replacement. At the 24h mark the growth medium was removed from the wells and one fraction of each medium sample subjected to eoDNA extraction and quantification. The remaining medium fraction was then incubated (separate of the cells themselves) at 37˚C for a period of 24h, before being subjected to eoDNA extraction and quantification. Results for this experiment are shown in Figure 9 of the results section in this chapter.

5.4. Results

5.4.1 Genetic manipulation

Firstly, it should be noted that eoDNA levels at the zero hour time is remarkably higher than what is expected from culture medium that was allowed virtually no incubation time while in contact with the cell, prior to being removed for extraction purposes.

Due to the very large scale of variation in the levels of the eoDNA, observed in the various cultures over time, it is necessary to express the values by means of logarythmic charts. In assuring that the y-axis of these logarythmic charts are expressed as a positive value, the units selected for eoDNA are very small (μg or ng) in comparison to the unit for cellular protein (g). It may also be worth mentioning that, in these log charts, the standard deviations appear smaller as average values increase, due to the nature of expression by means of logarythmic scales.

The release of eoDNA over time for the various 143B cell lines are shown in Figure 6. Figure 6A shows the values of eoDNA release in the standard, untransfected 143B cell line. The values remain virtually constant, with a rather large ten fold increases measured, only at 15 hours of incubation. All the values in Figure 6A seem rather insignificant with regard to

the maxima observed in the media of the transfected 143B cells and also shows a completely different eoDNA release pattern.

Note that the transfected cell cultures (Fig.6 B, -C and –D) resemble the same patterns of release with regard to one another, in which eoDNA reaches peak levels at the 3h incubation point and decline thereafter, Fig.6D is the exception, wherin eoDNA levels reach its peak value at 3h, but remain high up to the 7h incubation time, after which a decline is once again observed. Visually, very few differences can be seen in the release patterns of the respective, transfected 143B cells. Differences only seem to present themselves as a shift in the entire series of eoDNA expression, thereby being higher or lower, with regard to other transfected 143B eoDNA patterns. Even so, the values retain in the same relative increases and decreases of eoDNA over time. The cells, tranfected with an empty vector, show exponetially higher levels of eoDNA with regard to all of the corresponding extraction times, in some cases, exceeding other cell lines's eoDNA levels of corresponding incubation times by more than a hundred fold.

5.4.2 Heat Shock

Figure 6 E and F, represents the release patterns of eoDNA from the 143B2 (Figure 6 E) and 143B5 (Figure 6 F) cell lines after heat shock treatment had been administered. Judging from figure 6 E, it is clear that the 143B2 culture patterns are seriously affected by heat-shock administration and deviate massively from that of 143B2 control. Heat-shock exposure resulted in eoDNA levels remaining virtually constant in the early hours (zero-, one- and three hours) of the experiment, after which (at seven hours incubation) a rather dramatic and sudden exponential increase, that surpasses the maximum of the eoDNA released from the control cell line, is seen as a spike in the release flux. At the 15 hour time line one can see the eoDNA values lowered in much the same way, as can be seen in the previous release patterns measured from transfected 143B cell lines.

In Figure 6 F, the heat-shock treated 143B5 cell line, containing the complex III knockdown, shows eoDNA levels that are visually a bit more on par with the 143B5 control, than is the 143B2 heat shock and control eoDNA release patterns. A slight increase in eoDNA is observable over the first three hours of incubation, after which and once again at the seven

hour incubation time, the eoDNA levels of the heat-shock treated 143B5 cell line spikes with a 10 fold increase and greatly surpasses the eoDNA levels of the 143B5 controll. At the 15 hour incubation time, the eoDNA levels, once again, drop dramatically.

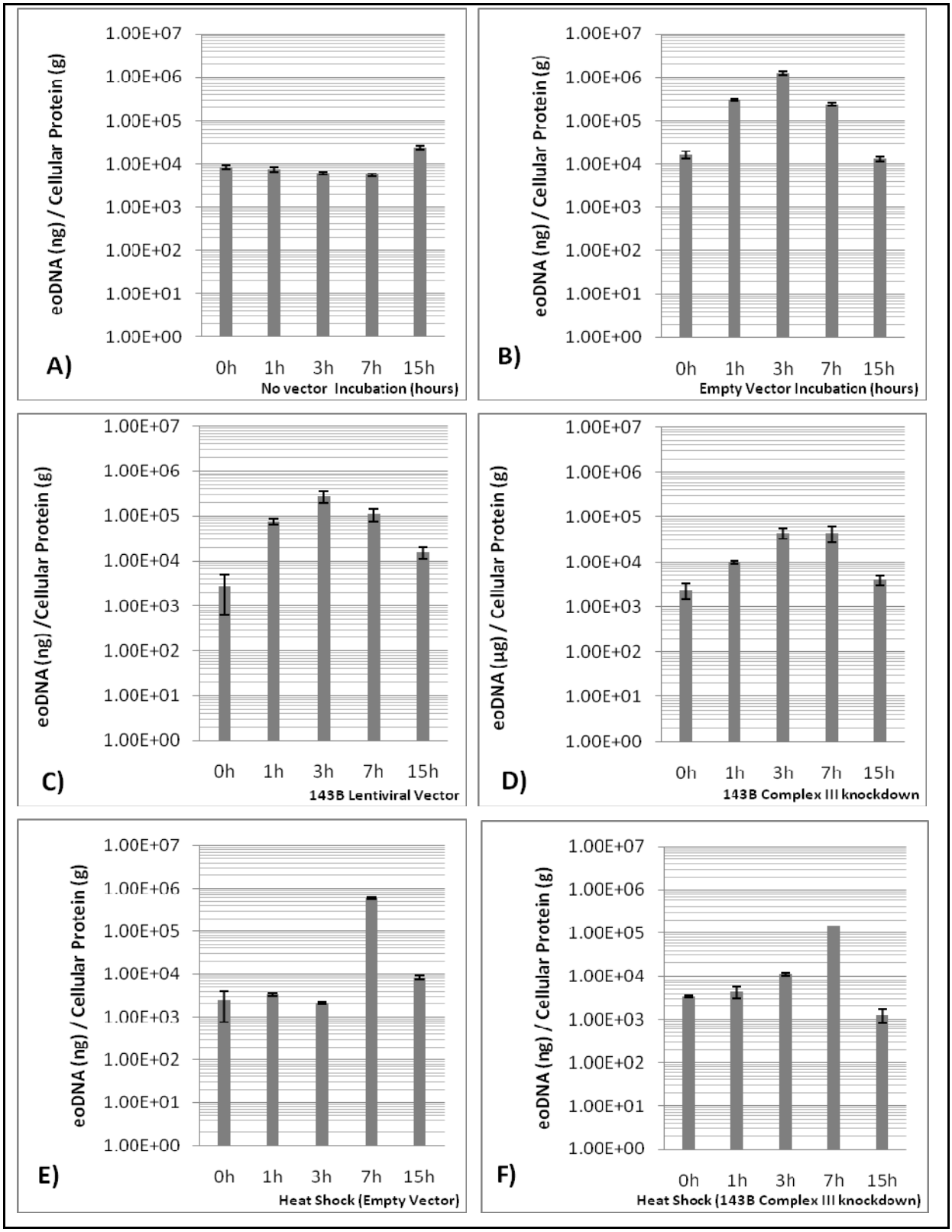

Figure 6. The release pattern ratios of eoDNA with regard to cellular protein of various cell cultures are shown in respective logarithmic scales. Graph A represents untransfected 143B cells. Graphs B, C and D represents three manipulated 143B cell lines, while Graphs E and F represents heat shock treated cells. Error bars represent standard deviation.

5.4.3 Nutrition

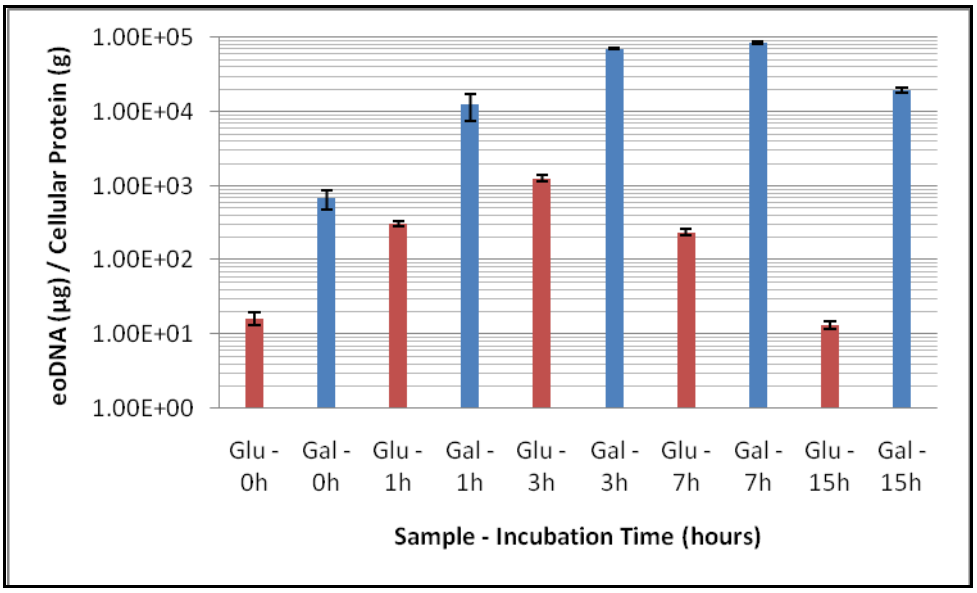

Figure 7. Cultured 143B cells containing an empty transfection vector incubated in glucose-containing growth medium is indicated by'Glu' on the x-axis, while 'Gal' indicatesthe cells grown in culture media containing only galactose. The results clearly show an exponential increase in eoDNA under the stress conditions posed by a change in the nutritional energy source. Error bars represent standard deviation.

The results in Figure 7 show that the cells incubated in galactose retain similar trends as that seen in glucose incubated cells, with a gradual increase notable in eoDNA, reaching its peak levels after three hours of incubation. Apart from the fact that the galactose incubated cells show much greater values, even from the start, a notable difference in the release trends become apparent at the seven hour incubation time. At this point (seven hour incubation), the glucose grown cell are seen to have decreased levels of eoDNA present in the culture media, whereas the levels of eoDNA in the galactose incubated cells have slightly increased. Also, the decrease at the 15 hour incubation point is much more prominent in the glucose containing culture than in the galactose grown culture.

5.4.4 The nature of turnover in culture

When referring to Figure 8, growth medium removed from culture at 7h incubation and placed in isolated incubation at 37˚C up to the 15h incubation time line ('clean incubation' sample), before DNA extraction was performed, showed very low levels of eoDNA, that had decreased dramatically since the original 7h 'in culture' incubation. In contrary, the levels of eoDNA, as measured in the 'in culture' sample is seen to have increased greatly.

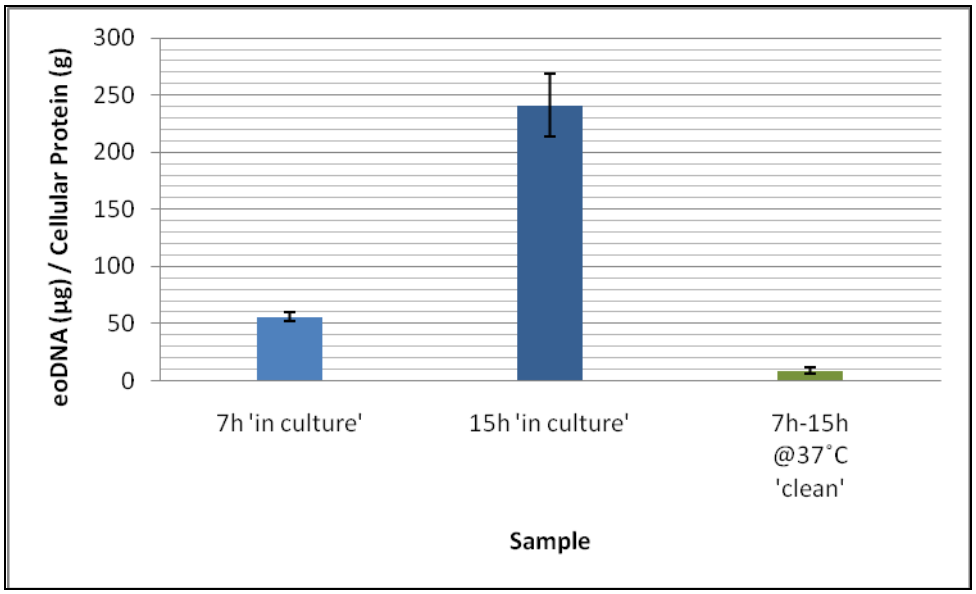

Figure 8. 7h 'in sample column indicates the eoDNA: Cellular Protein ratio for the eoDNA obtained after a 7h incubation period, where the cells are present in the medium and the medium is thus subject to the cellular release of eoDNA. The same applies for the 15 h 'in culture' sample, while the 7h-15h 'clean' incubation refers to growth medium removed from cells in culture at 7h and is incubated to 15h at 37˚C. Error bars represent standard deviation.

When comparring the concentrations of eoDNA observed in the media of the transfected 143B cell lines in Fig.9, the same reductions are observable. Here the cellular protein content is not brought into account, as the measurements in this part of the experiment are only to clarify that the same decreasses in eoDNA concentration do, in fact occur in culture media of the transfected 143B cells, as well. The results clearly show the dramatic decrease of DNA in the 'clean' incubated medium, with β-globin barely amplifying, if at all.

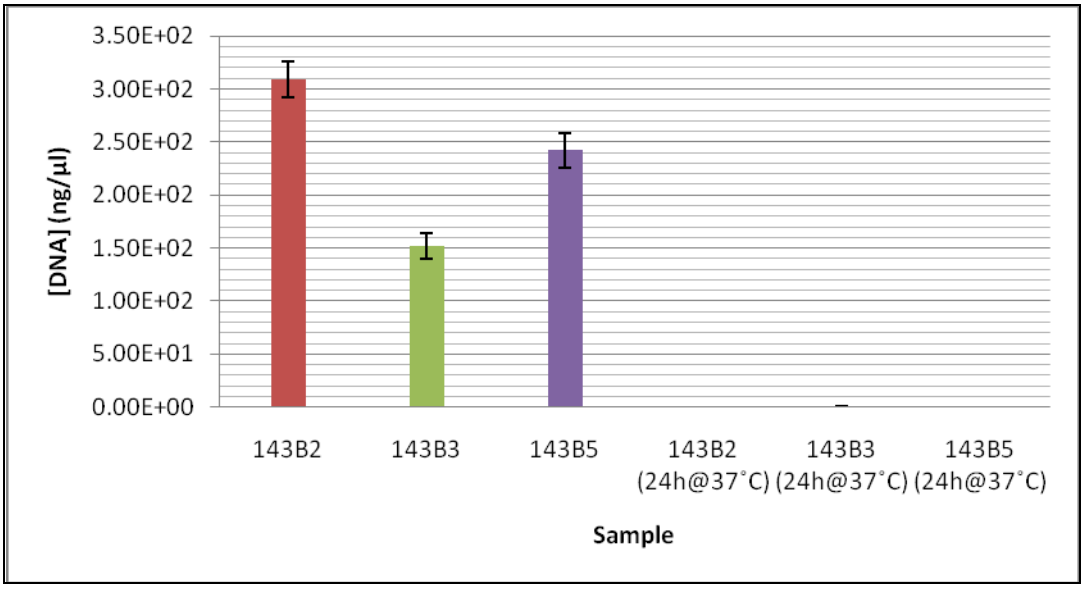

Figure 9. Effects of ' clean incubation' on 143B2, 143B3 and 143B5 medium samples. The eoDNA concentrations in culture of the three genetically manipulatred cell lines, after 12h of incubation are shown in this figure. The respective eoDNA concentrations in the growth media that had been separated from the cells eoDNA from the cells and subject only to breakdown by ezymes of nucleatic nature. Error bars represent standard deviation.

5.5 Comprehensive discussion

Before continuing with an in depth consideration of the resulting release patterns, it must be resolved, why the zero hour time incubation media showed such high levels of eoDNA being present. A possible explanation for this may be the following: Recall that the zero hour incubation time is marked by replacement of growth medium with fresh media. After the first well (which is also to be the zero hour well) is drained of growth medium, an amount of time is required for consecutive wells to be drained, prior to having fresh medium replacements added to each well, once again individually. The replacement of medium marks the initial start of the experiment and the zero hour incubation time. When the growth medium from the zero hour well had actually been drawn however, a short, but substantial amount of time had already passed, that allows eoDNA to be released into the fresh medium added to the zero hour- and indeed the remaining wells. Fortunately, as eoDNA values are seen to increase greatly after the zero hour time and as all the experiments regarding release patterns, featured in this dissertation are subject to the same handling protocols, the results remain relative and therefore adequate deductions can still be made.

As the cellular protein mass remains high or increases over time and cell death remains at a minimum (lower than 3%) throughout all the experiments performed in this study, cell death can be eliminated as a factor that would carry an effect on lowering eoDNA concentrations at the later incubation times. This eliminates apoptosis and necrosis as possible sources of eoDNA and supports the notion of eoDNA appearance through active release.

The results illustrated in Figure 6 indicate clear differences observable in the release patterns of the transfected 143B cells and the standard 143B cell line. As all factors, beside the variation of the genes cloned into the transfection vectors were controlled and identical, one may expect these changes in release patterns to be specifically associated with the presence of the transfection vectors.

The results also suggest that variation in the products, resulting from dissimilar transfection vectors, did not cause any dramatic differences in the likeness of the eoDNA release patterns and show only minor deviations from the general trend in Figure 6D. The eoDNA levels are however notably higher in the culture media obtained from the 143B cells containing empty vectors. If the products of the transfection vectors are somehow coupled to differences in the release patterns (which is evidently the case), it may be possible that the higher levels of eoDNA released from the 143B cells containing empty vectors, is due to subsequent expression of empty vectors that would hardly be capable of activating a feedback system, if indeed such feedback systems are indeed in place. Cells that express vectors of which the products that show higher molecularly activity, subsequently has graspable molecular features, that may activate such a feedback system.

Considering the immense complexity of protein and genetic interaction one may expect such feedback to be indirect with many precursors that need to take place before the feedback event may take effect. The answer to such a riddle may lie in epigenetic changes, which are known to be responsible for the activation or deactivation of certain genes by means of control mechanisms like the state of DNA methylation.

The standard 143B cell line shows patterns of eoDNA that remain virtually constant and relatively low, throughout the early hours of the experiment. This may be an indication of a homeostatic feedback system at work. An increase in eoDNA is only seen at the 15 hour incubation point.

The results from the 'clean' incubation of 7h samples to 15h in isolation, shown in Figure 8, suggest that the breakdown of eoDNA in culture medium is rather significant. These results, in addition to the results displayed in Figure 9, show that the breakdown of eoDNA is very extensive in the extracellular space, but that the rate of breakdown is greatly exceeded by the rate of eoDNA release in many of the cultures used in this study.

The decline in eoDNA at the 15h incubation times, observable in the media of the transfected 143B cells, may likely be due to the systematic release of nucleatic enzymes. Strangely, neither heating the samples to 95º C to denature protein nor incubation of with Proteinase K could eleviate the degradation of DNA in the samples. Non the less, it is clear that the rate of breakdown exceeds the rate of release. Additionally, as the central dogma explains, the synthesis of proteins occur after the transcription of RNA, that codes for the production of the proteins, has taken place. It is simple enough to imagine that metabolic DNA would appear in the extracellular space prior to the proteins, that the metabolic DNA itself had coded for. In such cases eoDNA levels may elevate in the period of time that is required for the production, assembly and transport of the protein complexes that would eventually be released into the extracellular space to finally degrade them. This does however not explain the increase in eoDNA observed at the 15h extraction point of the standard 143B cells. It may be possible that the following argument explains the observation:

These resulting eoDNA release patterns of the 143B2 cell incubated in growth medium containing only galactose, shown in Figure 7, keeps the very basic trend of increase prior to decrease, but the release patterns of galactose incubated cells are evidently different from the glucose –incubated 143B cells. It may be possible that the effect of multiple influential factors summate to lead to even greater amounts of eoDNA. Whether this may be the case or not, it would appear that differences in nutritional content of a growth medium, does in fact have a visible effect on the pattern and the levels of eoDNA released. As the catabolism of glucose and galactose cause variation in eoDNA levels over time, we find that fluctuations in eoDNA are very easily provoked by differences in nutrition.

The nutrients in culture medium (Refer to Figure 6 A) can be expected to have notably declined after an incubation period of 10 hours, while metabolites can be expected to have accumulated. The combination of these events may require notable changes to occur in the molecular environment of the cells, required for maintaining metabolic homeostasis and lead

to the amplification of certain housekeeping genes (involved in maintaining proper cell function under changing environmental conditions). The transcription of metabolic DNA that leads to elevated levels of eoDNA would, according to the studies done by (McIntosh et al., 1985:147.), be observable in the extracellular space after five hours following transcription and so be observable at the 15h incubation time of this experiment, Therefore possibly explaining the increase in eoDNA. This cannot be said with certainty however, and requires further investigation.

If the above model is correct, we may have hoped to see a similar release pattern, as observed in the transfected cells beginning at or around the 15h incubation time. The changes in nutrition and accumulation of metabolic waste in the medium (which continues to increase) may however cause the release patterns to deviate completely. A possible solution may therefore be to repeat the experiment and determine the eoDNA levels at more frequent intervals, as it may be possible that the effects are observable prior to the 15h incubation time.

Heat shock administration has a debilitating effect on the molecular machinery of cell. This causes normal cell function to be stunted, until the cell can respond with a recovery process. Under conditions of stress, researchers have found cells to express proteins that aid in the correct folding of other proteins that may be hampered by stressful condition(s). These proteins have been named heat-shock proteins, after the stress state with which their expression was first provoked. According to the model of eogenics proposed in Section 2.2, the cells should respond to the heat-shock by epigenetically activating the cistrons coding for these genes. This would be done to maximise the rate of expression of the proteins that are now essential for recovery and the survival of the cell. As should additionally be expected, the metabolic DNA is produced in great amounts to counter the shock treatment as swiftly as possible and is then released after about five hours of its initial replication as eoDNA, that would still be visible at the seven hour incubation period.

The slightly different behaviour in the heat-shock treated 143B2 and 143B5 cells (where 143B2 cell shows a much more prominent response to the heat-shock treatment as does the 143B5 cell line) may be explained in the following way, while still referring the eogenics model: The biological influence and implications of the complex III knockdown in the 143B5 cell line is rather significant, when considering a major energy production pathway must now be bypassed. These cells may therefore, not be capable of responding to other

stressors in what would be their normal, full capacity. This lack of response may be caused by dominant states of methylation or other epigenetic switches that are in place in order to produce adequate amounts of energy, essential for function. This causes competition between the mechanisms required for overcoming the stressors that are in place, and therefore leads to less prominent responses, observed in their eoDNA release patterns.

As an alternative explanation, it is possible that the release of eoDNA is dependent on membrane proteins, which are damaged during the heat-shock process. eoDNA release may thus be stunted until the damaged protein had been repaired or replaced. If the DNA had not been cleared in the intracellular space and had built up prior to release, it may explain the sudden spike in release after a seven hour incubation period. This seems very unlikely and of course, in Chapter Two, it is also explained that eoDNA may possibly pass directly over cell membranes by means of the histone- and membrane interactions.

It is clear that genetic alteration had a notable influence on eoDNA release patterns, with regard to standard 143B cells. Expected results from the standard 143B cell line had however been that, since the cells were under no forms of biological stress, eoDNA release patterns would show no deviations over time and that the levels would remain constantly low. This would have amply supported the notion that a homeostatic system is at work, regulating eoDNA levels in the extracellular space.

The deviations in eoDNA observed after the addition of stressors deters from the notion that eoDNA is created and released, as the result of mitotic DNA replication. It seems highly unlikely that the increased eoDNA levels are due to mitotic replication of DNA, since cells placed under such conditions of stress would be stunted in growth, rather than thriving.

Chapter Six

Reaching a common perspective

6.1 The nature of eoDNA fluctuation over time

The results from Chapter Five show that none of the 143B cell lines investigated, succeeded in showing a true homeostatic mechanism that oversees the maintenance of constant eoDNA levels. One must however bear in mind that the *in vitro* model differs remarkably from *in vivo* models, in that there is not a constant clearance of waste products by a process such as circulation. The time frame at which results are judged may also be too small, and there may possibly be a form of homeostasis over longer periods of time, providing that nutritional access and metabolic waste removal remains constant. The rise in eoDNA may very well be due to a homeostatic system for maintaining metabolic and genetic homeostasis. As a 3% maximum cell death cut-off value is applied to all experiments, the influence of necrotic and apoptotic residue can be expected to have had minimal influence. Changes in genetic makeup, by the addition of expression vectors clearly had an influence, although the reason for this remains unclear and requires more attention, it may be possible that transfection has an influence on methylation and thus amplification of genes.

The main advantages of using eukaryotic cell cultures as a model for the study of eoDNA, is that the biological conditions wherein the cell function, are under strict control, allowing the researcher to elucidate the specific effects of individual factors on eoDNA, that are under investigation. The main drawback from the use of these cell cultures, is that it does not represent the same responses as would be seen *in vivo*. One of the primary factors in this, is that unlike *in vivo,* where there is a constant removal of waste products and a constant renewal of nutrition, cell cultures show a build-up of waste products and a decline in nutrion.

eoDNA levels in culture with no environmental stress shows very low levels of eoDNA, as the nucleases released by cells are capable of breakdown of lower levels of eoDNA. When, however, a stressor is added, the rate of release is increased. At this point in time the extracellular nucleic acids are incapable of removing the increased amounts of eoDNA, which increases their window of opportunity, to be taken up by adjacent cells and thus be incorporated and/or expressed. This would ensure proper expression of the genes required

for maintaining metabolic homeostasis throughout an entire collection of cells in a tissue or organ, subjected to a particular stressor. The conclusion from this observation is that eoDNA release is provoked by changes in the cellular environment, possibly indicating that a genetic response is intended to achieve homeostasis and that the deviation seen in eoDNA is the genetic by-product thereof.

Judging from the results obtained, that clearly show notable differences in the values of various eoDNA concentrations among the various samples investigated, it is quite clear that eukaryotic cell culture methods prove to be valuable in research encompassing characteristics of eoDNA. More importantly however, the results all seem to suggest the same thing, that any changes in genetic makeup of the cultured 143B cells, or environmental conditions, caused notable changes in the release of eoDNA into the culture medium. Once again, referring to the fact that *in vivo*, elevated levels of circulating DNA is associated with pathological onset (van der Vaart et al., 2008:18.), it may be no coincidence that this occurs.

Studies using frog heart auricles indicate that very similar amounts of DNA is released in 4h incubation sessions (Stroun M et al., 1977:229.). This is described as confirming proof that DNA is released in a homeostatic manner. The experiments done in this study show eoDNA release over periods of time and at intervals, without replacing the growth medium between incubations, thus providing a real-time consideration of eoDNA release.

The expression of eoDNA release with incremental increases of incubation time poses as a effective way to investigate the turnover events that occur within the *in vitro* extracellular space. This approach seems to be a more appropriate model than the initial studies done in this field during the 1970s. It should also be said that the progress in techniques and equipment make the study of such low concentrations of nucleic acids a real possibility and so we may finally embark on a new journey of discovery that leads us to new frontiers in understanding the infinite complexity that is life.

6.2 The validity of the eogenics model

The eogenetics model seems to have fitted into place with all the results from the experiments done in this study, since cells under no stress revealed eoDNA levels that remain relatively low and constant, while cells under stress showed great fluctuations and increases in eoDNA. The only real way to prove the notion however, is through sequencing of eoDNA, in similar experiments, to assert that the findings are true for not only β-globin, to approve or disprove other possible mechanisms at work and also, to examine the relationship between genes expressed in this way. If the model is to be correct, there would be an over expression observable in certain genes, being the ones required for the maintenance of cellular homeostasis, with relation to genes that are not involved in retaining homeostasis under the specific stresses applied. Further investigation will also be required to assert the nature of incorporation of sequences following horizontal gene transfer.

6.3 The final conclusion to the primary hypothesis

The use of 143B human osteosarcoma cells as a model of eukaryotic cells, provided reproducible and comparable results in the light of a mechanistic model that characterized eoDNA. Even though the use of eukaryotic cell cultures does not resemble the *in vivo* system, cell cultures do provide information on specific influential factors and possibly hold information on the specific genes expressed under controlled conditions. This makes eoDNA research, using eukaryotic cell cultures an invaluable asset to the cause, of understanding genetic behaviour.

Chapter Seven

Insights and future prospects

Upon the addition of a stressor, or any form of environmental change, a particular cell must conform by altering its cellular machinery in such a way that homeostasis may be maintained. Even though many proteins, enzymes and hence metabolic pathways easily achieve such a state of equilibrium, in that they have a certain degree of buffer capacity, it seems possible that special or demanding conditions may require the cell to increase the production of certain specialized proteins in order to maintain metabolic homeostasis. In order to achieve this, the cell is required to increase the number of cistrons operating to transcribe the particular proteins of interest. By doing so, the number of transcribed RNA molecules can, in its turn, be increased and ultimately leads to the production of the required proteins in a time effective manner, that allows the cell to survive the stressful environmental changes.

When looking at possible biological functions that may be associated with the release of the metabolic DNA sequences, one must look at the target destination of the DNA. As was discussed in the CCA review article, it becomes clear that the fraction of DNA released by cells, have the ability to act as biologically active complexes, in this instance, that they may taken up and expressed by other cells. If a stressor is thus added to a colony of cells, that thereafter undergo a form of gene amplification to produce the required amount of cistrons necessary to maintain homeostasis, the cells then release this DNA as an ‘instruction’ to the surrounding or reachable cells. Cells that receive the ‘instruction’ then express the gene sequence and thus also conform to the stressful changes. It may therefore be seen a positive feedback system, that amplifies itself in all of the cells in the direct or reachable environment.

Once again, healthy individuals show very small amounts of circulating DNA in their blood, just as the untranfected 143B cells released very small amounts of eoDNA into their surrounding growth medium. Also shown in this study is that a great deal of eoDNA in culture is broken down by nucleases. Maintaining an elevated concentration of eoDNA thus requires constant and increased release.

As the epithelial cells lining blood capillaries do, under normal circumstances, not allow passage of large macro-molecular structures such as proteins, one is lead to assume that when eoDNA complexes are transferred across this barrier it is due to some mechanism that has not

yet been observed, but must in fact be a regulated process, as it should otherwise be impossible.

If the model is correct, it would imply that any change a cell experiences are met by the appropriate changes in genetic expression, in order to adapt to the new conditions. The eoDNA released by the cells then reflect these changes. If this is true, it may have tremendous implications for the way in which modern medicine is practised. eoDNA would then pose as the interface by which an individual's genome may communicate to an observer, equipped with the necessary sequencing platform, the genetic processes are currently

The use of RQ-PCR in the study of eoDNA is useful and due to this method's high sensitivity seems to be reliable and accurate, but limited and virtually obsolete, as only single sequences can be investigated at a time. If one would for example repeat the experiment done in Chapter Six, using modern sequencing techniques, one would be able to investigate whether eoDNA of genes required for normal metabolism are also affected and in what way this occurs. Studies like this would shed a whole new perspective on the genetic behaviour and how the genome functions to maintain homeostasis. Another important aspect is to determine how the incorporation of eoDNA takes place, following uptake of eoDNA by accepting cells.

The critical review article featured in this dissertation, published in Clinica Chimica Acta, stresses the importance to of which fraction of genomic DNA is represented by the blood nucleome and also states that eoDNA is to be considered a cornerstone in epigenetic variation. If indeed eoDNA sequences can be specifically incorporated into appropriate sections of an accepting cell's genome, it suggests that one of the biological meanings of extracellular DNA in the higher organism is to keep the current genomic activity inside the body cells 'up to date' in short time frames. An additional value of nucleic degradation in this light may be to keep messenger sequences from circulating outside the area effected by the stressor. Such a model would bring about interesting concepts concerning the formation of organs. An organ is comprised of specialized cells, all serving to achieve the higher functions of the organ itself. An important characteristic of an organ is that it is confined from dissimilar tissues by a membrane that normally encloses the organ as a whole. If a higher organism ingests a foreign food source posing as a stressor, for example, the organ required to break down possibly unfamiliar metabolites would probably be the liver. As the tissues and cells in this 'target' organ responds to the stressor, they produce additional copies of the cistrons

necessary for detoxification purposes. The release of eoDNA sequences, thus easily translocates between the cells via the interstitial space and so creates a positive feedback system, since the released sequences may also be taken up by adjacent cells. This quickly has the organ produce great amounts of the proteins required for oppose the stressor. This constant increased expression of certain genes in the organ may actually be the mechanism for specialization and proper formation.

References

ADAMS, D.H., DIAZ, N. & GAHAN, P.B. 1997. In vitro stimulation by tumour cell media of [3H]-thymidine incorporation by mouse spleen lymphocytes. *Cell biochemistry and function,* 15(2):119-126.

ANKER, P., JACHERTZ, D., STROUN, M., BROGGER, R., LEDERREY, C., HENRI, J. & MAURICE, P.A. 1980. The role of extracellular DNA in the transfer of information from T to B human lymphocytes in the course of an immune response. *Journal of immunogenetics,* 7(6):475-481.

ANKER, P., LYAUTEY, J., LEFORT, F., LEDERREY, C. & STROUN, M. 1994. Transformation de cellules NIH/3T3 et cellules SW 480 porteuses d'un mutation K-ras. *CR acad sci de la vie,* (317):869-874.

ANKER, P. & STROUN, M. 2006. Immunological aspects of circulating DNA. *Annals of the new york academy of sciences,* 107534-39.

GAHAN, P.B. 2006. Circulating DNA: Intracellular and intraorgan messenger? *Annals of the new york academy of sciences,* 107521-33.

GAHAN, P.B., ANKER, P. & STROUN, M. 2008. Metabolic DNA as the origin of spontaneously released DNA? *Annals of the new york academy of sciences,* 11377-17.

GAHAN, P.B. & STROUN, M. 2010. The virtosome-a novel cytosolic informative entity and intercellular messenger. *Cell biochemistry and function,* 28(7):529-538.

GARCIA-OLMO, D., GARCIA-OLMO, D.C., ONTANON, J. & MARTINEZ, E. 2000. Horizontal transfer of DNA and the "genometastasis hypothesis". *Blood,* 95(2):724-725.

GARCIA-OLMO, D., GARCIA-OLMO, D.C., ONTANON, J., MARTINEZ, E. & VALLEJO, M. 1999. Tumor DNA circulating in the plasma might play a role in metastasis. the hypothesis of the genometastasis. *Histology and histopathology,* 14(4):1159-1164.

GARCIA-OLMO, D.C., DOMINGUEZ, C., GARCIA-ARRANZ, M., ANKER, P., STROUN, M., GARCIA-VERDUGO, J.M. & GARCIA-OLMO, D. 2010. Cell-free nucleic acids circulating in the plasma of colorectal cancer patients induce the oncogenic transformation of susceptible cultured cells. *Cancer research,* 70(2):560-567.

GARCIA-OLMO, D.C., RUIZ-PIQUERAS, R. & GARCIA-OLMO, D. 2004. Circulating nucleic acids in plasma and serum (CNAPS) and its relation to stem cells and cancer metastasis: State of the issue. *Histology and histopathology,* 19(2):575-583.

HOURY, W.A. 2001. Chaperone-assisted protein folding in the cell cytoplasm. *Current protein & peptide science,* 2(3):227-244.

LEVANETS, O., REINECKE, F., LOUW, R., PRETORIUS, P.J., DU PLESSIS, L.H., NIJTMANS, L., SMEITINK, J.A. & VAN DER WESTHUIZEN, F.H. 2011. Mitochondrial

DNA replication and OXPHOS gene transcription show varied responsiveness to rieske protein knockdown in 143B cells. *Biochimie,* 93(4):758-765.

LO, Y.M., ZHANG, J., LEUNG, T.N., LAU, T.K., CHANG, A.M. & HJELM, N.M. 1999. Rapid clearance of fetal DNA from maternal plasma. *American journal of human genetics,* 64(1):218-224.

MCINTOSH, A.A. & ADAMS, D.H. 1985. Further studies on the extrusion of cytosol macromolecules by cultured chick embryo fibroblast cells. *The international journal of biochemistry,* 17(2):147-153.

PELC, S.R. 1972. Metabolic DNA in ciliated protozoa, salivary gland chromosomes, and mammalian cells. *International review of cytology,* 32327-355.

PELC, S.R. 1968. Turnover of DNA and function. *Nature,* 219(5150):162-163.

PETERS, D.L. & PRETORIUS, P.J. 2011. Origin, translocation and destination of extracellular occurring DNA - A new paradigm in genetic behaviour. *Clinica chimica acta; international journal of clinical chemistry,* 412(11-12):806-811.

PRINCE, W.S., BAKER, D.L., DODGE, A.H., AHMED, A.E., CHESTNUT, R.W. & SINICROPI, D.V. 1998. Pharmacodynamics of recombinant human DNase I in serum. *Clinical and experimental immunology,* 113(2):289-296.

PROKOP, A. & TECHNAU, G.M. 1994. BrdU incorporation reveals DNA replication in non dividing glial cells in larval abdominal CNS of *drosophila*. *Development genes and evolution,* 204(1):54-61.

RUMORE, P.M. & STEINMAN, C.R. 1990. Endogenous circulating DNA in systemic lupus erythematosus. occurrence as multimeric complexes bound to histone. *The journal of clinical investigation,* 86(1):69-74.

RUSTOM, A., SAFFRICH, R., MARKOVIC, I., WALTHER, P. & GERDES, H.H. 2004. Nanotubular highways for intercellular organelle transport. *Science (new york, N.Y.),* 303(5660):1007-1010.

SCHLIESS, F., WIESE, S. & HAUSSINGER, D. 1999. Osmotic regulation of the heat shock response in H4IIE rat hepatoma cells. *The FASEB journal : Official publication of the federation of american societies for experimental biology,* 13(12):1557-1564.

STROUN M, ANKER P, GAHAN PB & HENRI J. 1977. Spontaneous release of newly synthesised DNA from frog auricles. *Arch sci genève,* 30229-241.

SU, K.Y. & PISETSKY, D.S. 2009. The role of extracellular DNA in autoimmunity in SLE. *Scandinavian journal of immunology,* 70(3):175-183.

VABULAS, R.M., RAYCHAUDHURI, S., HAYER-HARTL, M. & HARTL, F.U. 2010. Protein folding in the cytoplasm and the heat shock response. *Cold spring harbor perspectives in biology,* 2(12):a004390.

VAN DER VAART, M. 2006.
Characterization of circulating DNA as a
biomarker for genetic aberrations in humans. Potchefstroom: NORTH-WEST UNIVERSITY. (M.Sc.) 1-85p.

VAN DER VAART, M. & PRETORIUS, P.J. 2008. Circulating DNA. its origin and fluctuation. *Annals of the new york academy of sciences,* 113718-26.

WATERHOUSE, M., THEMELI, M., METAXAS, Y., LAGADINOU, E.D., FINKE, J. & SPYRIDONIDIS, A. 2009. Horizontal DNA and mRNA transfer between donor and recipient cells after allogeneic hematopoietic cell transplantation? *Frontiers in bioscience : A journal and virtual library,* 142704-2713.

Addendum

Invitation to submit and article addenda to Mobile Genetic Elements

List of Figures

Dear Dr Peters 25th February 2011

In early 2011 we plan to launch ***Mobile Genetic Elements***, the first international peer-reviewed journal of its kind to focus exclusively on all aspects of mobile genetic elements and horizontal gene transfer throughout life (http://www.landesbioscience.com/journals/mge). We are now open for submissions.

I read with great interest your Clinica Chimica Acta paper today on extracellular occurring DNA and would like to invite you to contribute an article addendum or review article on the aspects related to horizontal gene transfer to ***Mobile Genetic Elements*** as your research falls squarely within its remit and it is exceptionally interesting.

Addenda are essentially an auto-commentary. We invite authors of the most significant recent and forthcoming papers, published elsewhere, to provide a short summary with additional insights, new interpretations or speculation on the relevant topic. These manuscripts may include data or models, which due to space limitations were not included or discussed in the original paper. In other words, the authors may provide biased and uncensored points of views, complementing their article. As with other papers published in ***Mobile Genetic Elements***, Addenda will appear online and in print. Addenda will appear simultaneously, or very soon after, publication of the original paper. The typical length of an addendum will be approximately 1,000 – 3,000 words and may include up to 30 references. There will be no page charges for Article Addenda and you are encouraged to include figures. Alternatively, or indeed in addition, I would be extremely interested in a research article on this subject in the future.

I look forward to hearing from you and congratulations on an excellent manuscript.

Best wishes

Adam P. Roberts

University College London

List of figures

Chapter 1

Chapter 3

Chapter 4

Chapter 5