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A review comparing deoxyribonucleoside triphosphate (dNTP) concentrations in the mitochondrial and cytoplasmic compartments of normal and transformed cells

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Abstract

The deoxyribonucleoside triphosphate (dNTP) pools that support the replication of mitochondrial DNA are physically separated from the rest of the cell by the double membrane of the mitochondria. Perturbed homeostasis of mitochondrial dNTP pools is associated with a set of severe diseases collectively termed mitochondrial DNA depletion syndromes. The degree of interaction of the mitochondrial dNTP pools with the corresponding dNTP pools in the cytoplasm is currently not clear. We reviewed the literature on previously reported simultaneous measurements of mitochondrial and cytoplasmic deoxyribonucleoside triphosphate pools to investigate and quantify the extent of the influence of the cytoplasmic nucleotide metabolism on mitochondrial dNTP pools. We converted the reported measurements to concentrations creating a catalog of paired mitochondrial and cytoplasmic dNTP concentration measurements. Over experiments from multiple laboratories, dNTP concentrations in the mitochondria are highly correlated with dNTP concentrations in the cytoplasm in normal cells in culture (Pearson $R = 0.79$, $p = 3 \times 10^{-7}$) but not in transformed cells. For dTTP and dATP there was a strong linear relationship between the cytoplasmic and mitochondrial concentrations in normal cells. From this linear model we hypothesize that the salvage pathway within the mitochondrion is only capable of forming a concentration of approximately 2 μM of dTTP and dATP, and that higher concentrations require transport of deoxyribonucleotides from the cytoplasm.

Keywords

mitochondria; dNTP; deoxyribonucleoside triphosphate; mtDNA; depletion; salvage

Introduction

Mitochondrial DNA depletion syndromes

Mitochondrial DNA depletion syndromes (MDS) are a set of genetic diseases commonly defined as a reduction in the amount of cellular mitochondrial DNA (mtDNA) without other defects such as deletions or point mutations [1]. However, underlying this definition is a clinically and genetically complex set of diseases. Genetic and allelic heterogeneity; variable penetrance, severity of symptoms, variable ages of onset; and gene-dependent tissue-specificity of phenotype are all characteristics of MDS. Another complication in the characterization of MDS is that depletion of mtDNA is not always exclusive of other genomic defects. For example, in mitochondrial neurogastrointestinal encephalomyopathy

(MNGIE) deletions may accompany the depletion of mtDNA [2]. Mutations in the mitochondrial DNA polymerase (*POLG*) may lead to any of a diverse set of disorders, including progressive external ophthalmoplegia (PEO). Also in PEO, mutations, deletions, and depletion of mitochondrial DNA may not necessarily be mutually exclusive [2] and the inheritance may be sporadic, dominant, or recessive [3]. Thus, while the inheritance of MDS is usually deemed to be autosomal recessive [4], features of the MDS phenotype also follow other inheritance patterns. The diseases that make up the MDS family are shown in Table 1, along with their MIM IDs (<http://www.ncbi.nlm.nih.gov/omim>). MDS is nearly always severely debilitating and often lethal in infancy or early childhood for homozygous patients [4]. The prevalence of these diseases is not yet well determined. However, quantification of mtDNA in the liver or muscle tissue of 100 children with unexplained respiratory chain deficiency showed mtDNA depletion to less than 35% of the control values in 50% of these cases, indicating that depletion of mtDNA is a common cause of respiratory chain deficiency in childhood [5]. Mutations in nine nuclear-encoded genes are known to cause MDS (Table 1). Eight of the nine genes code for enzymes that contribute to generating mitochondrial deoxyribonucleoside triphosphates or that participate in mtDNA replication. The function of the ninth gene, *MPV17*, which encodes a mitochondrial inner membrane protein, remains to be determined.

The importance of maintaining mitochondrial dNTP pools

The availability and balance of the intra-mitochondrial dNTP pools are major determinants of the rate and fidelity of mtDNA replication and thus of the integrity of the mitochondrial genome [6]. Besides MDS, mitochondrial dNTP pools are important also in additional human pathologies. Mitochondrial toxicity induced by HIV/AIDS therapy can manifest as severe clinical phenotypes and increased mortality [7, 8]. This toxicity is likely the result of interference with mtDNA replication and intra-mitochondrial nucleotide metabolism brought about by nucleoside analogs [9-12]. To advance our understanding of these numerous human disorders, to elucidate the role of mtDNA mutations in ageing and cancer [13-19], and for devising suitable therapies, it is critical that we understand the formation and regulation of intra-mitochondrial dNTP concentrations.

Generating mitochondrial dNTP pools and maintaining mtDNA

Two genes coding for proteins directly involved in mtDNA replication have been implicated in MDS. The genes are *C10ORF2* (encoding the mitochondrial DNA helicase, *twinkle*) and *POLG* (encoding the catalytic subunit of the mitochondrial DNA polymerase). Upstream of mtDNA replication, defective maintenance of mitochondrial dNTP pools also leads to a similar molecular phenotype, namely depletion of mtDNA. Deoxyribonucleotide metabolism for generating dNTPs within mitochondria occurs through the salvage pathway, though other sources may also occur. There exists one report of a mitochondrial ribonucleotide reductase, thus suggesting the presence also of a mitochondrial *de novo* pathway, but that finding remains uncorroborated [20]. In the mitochondrial salvage pathway, the canonical A, C, G, and T deoxyribonucleosides, after entering the mitochondrion through equilibrative nucleoside transporters, are converted to the corresponding deoxyribonucleoside triphosphates through three successive phosphorylations. This is a complex pathway due to the presence of feedback mechanisms, competition between multiple substrates for some enzymes, and evidence for multiple mitochondrial versions or isoforms of some enzymes such as adenylate kinase [21] and nucleoside diphosphate kinase [22].

The known elements of the salvage pathway are as follows. Deoxyguanosine kinase (DGUOK) and thymidine kinase 2 (TK2) are the purine and pyrimidine deoxyribonucleoside kinases respectively. Apart from these deoxyribonucleoside kinases,

other elements of this pathway are relatively less studied. Mitochondria possess a pyrimidine 5',3'-deoxyribonucleotidase (NT5M) which can dephosphorylate dTMP. The existence of additional deoxyribonucleotidases as well as the extent of NT5M's contribution towards opposing dAMP, dCMP, and dGMP production is not established. For monophosphate kinase activity, candidates include adenylate kinase (AK) isoforms, cytidine monophosphate kinase 2 (CMPK2), and thymidine monophosphate kinase 2 (TMPK2) acting on dAMP, dCMP, and dTMP respectively. It is unclear which AK isoform has the most contribution towards producing mitochondrial dADP. Regarding CMPK2, its kinetics with dUMP as a substrate were far more favorable than the kinetics with dCMP which was its second-most preferred substrate [23]. Additionally, the authors also noted that CMPK2 might be dispensable in dCTP synthesis given that its expression was restricted and was not detected in tissues with high energetic demand such as heart and muscle [23]. Following putative identification, attempts at characterizing the enzyme activity of TMPK2 were unsuccessful both *in vitro* and in cell extracts [24]. No candidates exist for dGMP phosphorylation activity in the mitochondria. Assuming that mitochondria possess a complete salvage pathway, the lack of knowledge of the monophosphate kinases is a fundamental gap in our understanding. NME4 is a candidate for the mitochondrial nucleoside diphosphate kinase activity. Again, knowledge about mitochondrial nucleoside diphosphate kinase activity is scarce and multiple NME isoforms have been reported. The discoveries of the role of a mutated p53-inducible small ribonucleotide reductase subunit (*RRM2B*) [25] and a mutated thymidine phosphorylase (*TYMP*) [26] as causes of MDS highlight the importance of cytoplasmic nucleotide metabolism in maintaining mtDNA. Both *RRM2B* and *TYMP* are cytoplasmic enzymes of nucleotide metabolism. This association between DNA depletion in the mitochondria and defects in cytoplasmic nucleotide metabolism implies that cytoplasmic nucleotides influence and contribute significantly to intra-mitochondrial pools of deoxyribonucleotides. We might speculate that defects in other enzymes of cytoplasmic nucleotide metabolism in addition to *RRM2B* and *TYMP* could also lead to mtDNA depletion. However, even for the putative mitochondrial enzymes there are missing pieces in our understanding and inconsistencies that are rarely addressed. In a recent report, in addition to its presence in mitochondria, a cytosolic form of TK2 was detected in multiple rat tissues [27]. Evidence exists for both AK2 and NME4 that supports their localization to the intermembrane space of mitochondria [28, 29], not to the matrix where the mtDNA is found. It is possible that versions of these enzymes might localize to the mitochondrial matrix. If so, which versions are more crucial to intra-mitochondrial dNTP pools? The intermembrane localization of NME4 is interesting considering the importance of ribonucleotide reduction in the cytoplasm in maintaining mitochondrial DNA. In the presence of active production of deoxyribonucleoside diphosphates (dNDPs) in the cytoplasm, terminal phosphorylation of those dNDPs prior to their entry as dNTPs into the mitochondrial matrix would appear to be a logical benefit of NME4's localization to the intermembrane space. In summary, it is possible that despite intensive study we might currently only know a fraction of the components that participate in the pathways of mitochondrial dNTP pool generation. Nevertheless, it is clear that mitochondrial dNTP pools are not strictly independent of the cytoplasmic pools and have a strong connection with the nucleotide metabolism in the cytoplasm (Figure 1).

Deoxyribonucleotide flow between the cytoplasm and mitochondria

Multiple reports exist to support the transport of deoxyribonucleotides between the cytoplasm and mitochondria [30-34]. dCTP transport activity in proteoliposomes containing the mitochondrial protein fraction of human acute lymphocytic leukemia cells has been observed [30]. Because the orientation of the insertion of proteins in lipid vesicles is not controlled, we cannot conclude whether the dCTP transport was unidirectional or bidirectional [30]. Other ribonucleoside and deoxyribonucleoside triphosphates were able to

inhibit the dCTP transport thus raising the possibility of a more general, non-specific transport function for this protein. A mitochondrial transporter (pyrimidine nucleotide carrier, PNC1) with a preference for UTP has also been described [32]. Again, PNC1 was also able to transport a variety of other molecules including other ribonucleoside and deoxyribonucleoside triphosphates. Transport of dTMP into and out of isolated mitochondria has also been reported [31]. In that experiment, a fraction of dTMP was converted to thymidine, dTDP, and dTTP both in the medium and in the mitochondria. Thus, interpretation of the exact nature of that transport is complicated. Using isotope experiments, Bianchi and colleagues have established the importance of cytoplasmic enzymes towards the maintenance of mitochondrial dTTP and dGTP pools [33, 34]. By following the appearance and dilution of radioactivity, they observed two-way transport of thymidine and deoxyguanosine nucleotides between the cytoplasm and mitochondria and determined that while the cytoplasmic *de novo* pathway is the predominant source of mitochondrial dTTP in proliferating cells, even non-proliferating cells depend on ribonucleotide reduction in the cytoplasm. Details of the transport, such as the identities of the transporter and the transport substrate, and the kinetics of transport remain unresolved. It is also unknown if the behavior of deoxyadenosine and deoxycytidine nucleotides is similar to what has been observed regarding thymidine and deoxyguanosine nucleotides. Thus, even though solid evidence has accumulated to support the transport of deoxyribonucleotides between the cytoplasm and mitochondria, in the end we know little about the molecular mechanisms of this transport process.

Mitochondrial dNTP pools in patient cells

Experiments with patient cells have demonstrated the effects of pathogenic mutations on mitochondrial dNTP pools. In fibroblasts from a patient with DGUOK deficiency where quiescence was induced through serum starvation, reduced mitochondrial dGTP led to an imbalance between the four dNTPs compared to controls [35]. In fibroblasts from patients with TK2 deficiency, mitochondrial dTTP, dCTP, and dATP pools were all decreased while dGTP was slightly increased in one patient and slightly decreased in another patient [36]. The fact that externally supplied deoxyribonucleosides and deoxyribonucleoside monophosphates are able to rescue mtDNA depletion provides further evidence that limited substrate availability can cause mtDNA depletion [35, 37, 38]. Experiments from cellular and animal models have also reported disruptions in mitochondrial dNTP pool homeostasis [39–41]. TK2 H126N (c.378–379CG>AA) knockin mice had unbalanced mitochondrial dNTP pools due to reduced dTTP in brain and reduced dCTP in liver [39]. Culturing HeLa cells in the presence of high levels of thymidine (50 μ M) to mimic the conditions leading to MNGIE, led to an expansion of mitochondrial dTTP and dGTP pools and a reduction of the mitochondrial dCTP pool [41]. In another cellular model of MNGIE, incubation of quiescent fibroblasts in the presence of high levels of thymidine or deoxyuridine led to an expansion of mitochondrial dTTP, but mitochondrial dCTP was unaffected [40]. In conclusion, because mitochondrial dNTP levels are disrupted in MDS, a thorough understanding of the maintenance of mitochondrial dNTPs is a necessary step towards insights on the mechanisms and the potential therapies of MDS.

Results and Discussion

Paired measurements of cytoplasmic and mitochondrial dNTP levels in normal and transformed cells

Although mitochondrial dNTP generation is an important pathway, much about the origin of mitochondrial dNTPs remains unclear. Since the etiology of MDS can be traced to altered mitochondrial dNTP pools, it is critical to understand how the mitochondrial dNTP pools relate to the corresponding cytoplasmic pools. The evidence reviewed above repeatedly

points toward a significant amount of interaction between the mitochondrial and cytoplasmic dNTP pools. So what data exists concerning the relationship between the dNTP concentrations in these two subcellular compartments, and is the existing data consistent with the increasing evidence of communication between the cytoplasm and mitochondria with respect to deoxyribonucleotides? For the remainder of this paper we focus on these questions. We reviewed the available data on simultaneous measurements of cytoplasmic and mitochondrial dNTP pools in wild-type cells [35, 40-46].

Conversion to correct concentration units—With the goal of investigating whether concentrations calculated from previously measured dNTP pools are consistent with the biochemical evidence of deoxyribonucleotide flow between the cytoplasm and mitochondria, we converted the reported data to molar concentrations. This conversion requires the estimation of several parameters. Enzyme activities, kinetics, and the rate and fidelity of mtDNA replication are all influenced by the local dNTP concentrations. These concentrations are maintained by metabolic pathways that function in two subcellular compartments with very different volumes. Although concentrations are indeed the true biochemical driving factors, dNTP data is instead generally reported as amounts per million cells in culture. Per cell amounts can be misleading, since the conversion from these experimentally convenient units to the actual units of concentration in subcellular compartments can vary radically between different cell types. Paradoxically, the justification for using units such as the amount per million cells is to make comparisons possible; but, all the while, the cell lines and culture conditions in different experiments are different in the critical parameters needed to convert these units to actual concentration units. For reporting dNTP pools, the most commonly used units in the literature are picomoles per million cells for both subcellular compartments, or picomoles per milligram mitochondrial protein which is also in use. In the following section we give estimates to convert these units to true concentration units.

To obtain concentrations from data reported as amount dNTP (picomoles) per cell, we must use estimates of cell and subcellular compartment volumes (for details see Table 2). Bestwick *et al.* [42] reported dNTP amounts in subcellular fractions of asynchronously growing HeLa cells. For cellular and mitochondrial volumes of HeLa cells, we referred to a publication from Posakony *et al.* [47] who followed cellular and mitochondrial volume at different times along the cell cycle of HeLa cells. We combined nuclear and cytoplasmic amounts reported by Bestwick *et al.* [42], and converted these to molar concentrations using averaged cellular volume subtracted for mitochondrial volume. We calculated mitochondrial concentrations by using the average mitochondrial volume from different cell cycle phases.

Song *et al.* [41] reported mitochondrial dNTP pools in HeLa cells as the amount dNTP (in picomoles) per milligram mitochondrial protein. To calculate the corresponding concentrations, we used a factor of 0.82 microliters water space per milligram rat heart mitochondrial protein [48]. For converting whole-cell amounts from picomoles per million cells, we first used the calculated mitochondrial concentrations and the mitochondrial volume measure for HeLa cells in G1 phase [47] to estimate the picomole amounts of dNTPs in mitochondria per million HeLa cells. After subtracting mitochondrial amounts and volume from corresponding whole-cell values for HeLa cells in G1 phase [47], we arrived at an approximation of cytoplasmic concentrations of Song *et al.*'s data for confluent HeLa cultures.

Rampazzo *et al.* [45] measured dNTP amounts in exponentially growing human osteosarcoma line (HOS) cells and mouse fibroblasts (3T3) cell lines. Since we did not find a publication that provides the required conversion factors for the HOS cells, as an estimate we used the measurements from HeLa cells. We averaged cellular and mitochondrial

volumes measured in different cell cycle phases of HeLa cells [47] and obtained cytoplasmic and mitochondrial dNTP concentrations. For the cytoplasmic volume, we subtracted the total mitochondrial volume from the cellular volume to account for the fact that Rampazzo *et al.* combined nuclear and cytosolic dNTP pools. We did not find the mitochondrial volume measure for 3T3 cells. As an estimate, we averaged the mitochondrial volumes reported for HeLa and Chinese hamster cells [47, 49] and obtained the cytoplasmic volume after correcting whole-cell volume [50] for the total mitochondrial volume. We then converted dNTP amounts for 3T3 cells to concentrations using these volume measures.

Ferraro *et al.* [44] and Pontarin *et al.* [40] reported dNTP amounts measured in human lung and skin fibroblasts. We used the human skin fibroblast volume reported by Imaizumi *et al.* [51]; and after correcting this whole-cell measure by subtracting mitochondrial volume, we transformed the reported cytoplasmic picomole amounts to cytoplasmic concentrations. We obtained the averaged mitochondrial volume from HeLa cells [47]. We used the cytoplasmic volume of fibroblast cells obtained by subtracting mitochondrial volume from fibroblast cell volume to account for the fact that Ferraro *et al.* [44] and Pontarin *et al.* [40] combined nuclear and cytosolic dNTP pools. The parameters used for these conversions were also used to convert the data from Saada [35] and Frangini *et al.* [46].

Finally, to convert dNTP amounts reported by Desler *et al.* [43] for HeLa cells, we used parameters from HeLa cells [47] for average cellular and mitochondrial volumes. We subtracted mitochondrial amounts and volume from corresponding whole-cell measures and obtained cytoplasmic and mitochondrial concentrations.

The conversion parameter values are listed in Table 2. There exist reports of mitochondrial dNTP pool measurements that are not discussed here [31, 36, 52-54]. In these experiments, because the corresponding cytoplasmic dNTP pools were not also reported, the mitochondrial dNTP measurements were not useful for the purposes of this review. It should be clear from the preceding paragraphs that the conversion of the traditionally used units to the actual units of concentration is not trivial. Ideally, each experiment should take care to measure and report the additional information needed for this conversion to true concentration units. The missing parameter values, which we have had to estimate as described above, may introduce noise into the following analysis potentially obscuring any relationship between the cytoplasmic and mitochondrial pools.

Variation in cytoplasmic and mitochondrial dNTP concentrations—Paired measurements of dNTP levels in mitochondria and cytoplasm, converted to concentration units as described above, are given in Tables 3-5 and shown in Figures 2 and 3. The variation in dNTP concentrations in both compartments is striking. Even for cells within each particular category, the range of dNTP concentrations in both compartments spans an order of magnitude. This argues for measuring dNTP levels in a wide variety of cell lines, including and especially those cell types that are affected in MDS. Also, given these characteristics of the distribution of dNTP concentrations, the usefulness of calculating a mean value across cell types is diminished. Nevertheless, the mean dNTP concentrations that we calculated in both cellular compartments are higher in cycling cells than in quiescent cells (fold difference of ~10 in the cytoplasm and ~4 in the mitochondria). It is interesting that mitochondrial dNTP pools vary proportionally with the cytoplasmic pools and cycling state of cells. This observation is consistent with deoxyribonucleotide flow between the cytoplasm and mitochondria and is indicative of mitochondrial dNTP pools being regulated in a similar manner as the cytoplasmic pools. Mean cytoplasmic dNTP micromolar concentrations in mitotic, postmitotic, and transformed cells were approximately 12, 1, and 21 respectively; and mean mitochondrial micromolar concentrations were about 8, 2, and 10 respectively. These calculated mean concentrations hint that perhaps the equilibrium

between the cytoplasm and mitochondria with respect to dNTP concentrations could be qualitatively and quantitatively different for the three cell categories, since the calculated mean mitochondrial concentration is lower than the mean cytoplasmic concentration in normal mitotic and transformed cells, and higher in postmitotic cells.

A suggested problem with dNTP assays—It is possible that some of the reported dNTP measurements are overestimates. In a recent paper, Ferraro *et al.* [55] concluded that reported dNTP measurements can be contaminated by ribonucleotide triphosphates (rNTPs). This contamination is a consequence of the low discrimination power of the Klenow DNA polymerase fragment that is widely used to measure dNTP amounts by means of a polymerase assay. It was suggested that these assay complications might have resulted in overestimates of dNTPs, including the very high values reported for mitochondrial dGTP levels in rat tissues [53]. When Ferraro *et al.* extracted and measured dNTPs from cycling and confluent human fibroblasts, the original Klenow assay did not result in any overestimation of dTTP. The deoxyribonucleotides dATP, dGTP, and dCTP were all overestimated and the severity of this complication seemed to most affect dGTP and dCTP (to a lesser extent than dGTP). From our interpretation of the Ferraro *et al.* analysis we judged that the dATP measurements analyzed here may not have significant contamination from ATP competition for incorporation. At an ATP/dATP ratio of 350 in cycling cells, the overestimation in Ferraro *et al.*'s results for dATP was about 21% compared to their modified assay and about 26% compared to HPLC. At an ATP/dATP ratio of 1450 in confluent cells, the overestimation in dATP was slightly more than 100% compared to their modified assay. Ferraro *et al.* could not use HPLC for measuring dNTP pools in confluent cells. Since we did not have paired rNTP measurements for our dNTP data and since Ferraro *et al.*'s paper did not provide a spectrum of overestimation effects over a broad enough range of Klenow concentrations and ATP/dATP ratios, we cannot quantitatively determine the true effects of this complication on the dATP concentrations, although at a low ATP/dATP ratio, the observed overestimation was not substantial. Ferraro *et al.* concluded that the Klenow enzyme is unsuitable for measuring dGTP and dCTP pools, but the assay can be used with modifications to measure dTTP and dATP pools. We took these concerns into account by reviewing the data on dTTP and dATP concentrations separately from the data on dGTP and dCTP concentrations. Accordingly, we have split our data review into two groups; the dTTP and dATP group and the dGTP and dCTP group.

Analyzing simultaneous data on cytoplasmic and mitochondrial dNTP concentrations: dTTP and dATP observations—Figures 2 and 3 (and Tables 3-5) show the comparison of paired measurements of dNTP concentrations in the cytoplasm and in the mitochondrial compartments of the cell. Despite the relatively low number of available observations, and the potential for additional noise due to need for some parameter estimations, these data show highly significant correlations. In addition, the pattern of which correlations are highly significant and which are non-significant is interesting. For the combined dTTP and dATP measurements in the mitotic cells (Figure 2A) the mitochondrial and cytoplasmic concentrations had a very highly significant correlation (p -value = 0.0009) while the postmitotic cell measurements alone (Figure 2B) had no significant correlation. However, when the postmitotic cell and mitotic cell measurements were analyzed together (Figure 2C) the correlation became extremely significant ($p = 5 \times 10^{-8}$). The concentrations were also highly correlated ($R^2 = 0.9$ in both cases, Figure 2A and Figure 2C). Such a strong and highly significant correlation over the broad range of concentrations, in data from multiple independent experiments from different laboratories, is extremely compelling evidence for a tight connection between the cytoplasmic and mitochondrial dTTP and dATP pools. Correlations, of course, cannot prove which of these two pools is controlling the other. However, considering the relatively small total cellular volume of the mitochondria

compared to the cytoplasm, it is reasonable to assume that it is the larger cytoplasmic dNTP pool that is controlling the much smaller mitochondrial pool.

We label the combined mitotic and postmitotic cells as “normal” to contrast them with the final category which is transformed cells (Figure 2D) consisting of HeLa, HOS, and 3T3 cells. The transformed cells show no significant correlation between the mitochondrial and cytoplasmic concentrations for dTTP and dATP. This is a strong contrast to the normal cycling cells (Figure 2A) which had a very strong correlation. The statistics are summarized in Table 6.

In Figure 2 the two different deoxyribonucleotides dTTP and dATP are plotted with different symbols. Although the amount of data for each deoxyribonucleotide separately is quite small, both dTTP and dATP appear to be following the same relationship between cytoplasmic and mitochondrial concentrations in mitotic cells and in the combined “normal cell” category. There are enough measurements in the normal cell category (Figure 2C) to allow an analysis of dTTP and dATP separately, and they both are statistically significant separately ($p = 3 \times 10^{-5}$ for dTTP and $p = 0.01$ for dATP). More importantly, the slopes on the linear regressions for dTTP and dATP analyzed separately are consistent with each other (0.42 ± 0.04 and 0.36 ± 0.09 respectively) (Table 6). The simplest interpretation of this is that a common mechanism is acting on both dATP and dTTP.

We fit a linear model of mitochondrial dTTP and dATP concentrations as a function of their cytoplasmic concentrations in normal cells obtaining the following equation:

$$[dNTP]_{mito} = (0.41 \pm 0.04)[dNTP]_{cyto} + (1.8 \pm 0.4)\mu M$$

where dNTP is either dTTP or dATP and concentrations are measured in μM . The R^2 of this model was 0.9 and p-value of the F-statistic was 5×10^{-8} . The model indicates that in normal cells, mitochondrial dTTP and dATP concentrations are slightly less than half the cytoplasmic dNTP concentration plus about 1.8 μM . If we assume that the mitochondrial salvage pathway is independent of the cytoplasm dNTP concentrations, then one simple interpretation of this model is that the slope represents the transport function between these two subcellular compartments and the intercept represents the production of the mitochondrial salvage pathway, independent of the cytoplasmic pathways. Based on this linear model fit to the experimental data, we speculate that the mitochondrial salvage pathway alone is sufficient to support a concentration of roughly 2 μM of dATP and dTTP within the mitochondria. In postmitotic cells, the mitochondrial concentrations are in general higher than the cytoplasmic concentrations (Figure 2B). In these cells, because of low cytoplasmic concentrations the intercept term of the model has increased influence on the mitochondrial concentrations compared to its influence in mitotic cells.

dCTP and dGTP observations—For dGTP and dCTP the connection between the mitochondrial and cytoplasmic concentrations is far less clear (Figure 3). No significant correlations exist for the mitotic and postmitotic cells analyzed separately (Figures 3A and 3B). When these data are combined into the normal cell category (Figure 3C) the correlation is significant ($p = 0.005$). As was seen with dTTP and dATP, dGTP and dCTP were not significantly correlated in the transformed cell data. When dCTP and dGTP were analyzed separately for the normal cell category the dCTP concentrations were significantly correlated ($p = 0.0005$) but the dGTP concentrations were not significant. The slope for the dCTP concentration correlation in normal cells was slightly larger (0.56 ± 0.08) than that obtained for dTTP and dATP (Table 6). The primary difference between the dNTP

concentrations in the mitochondrial and cytoplasmic compartments occurs in dGTP, which is consistent with the concerns raised by Ferraro et al [55], and discussed above, about the validity of measured dGTP levels using current standard methods.

Interpreting the correlations in normal cells—The data show that in normal cells in culture, dTTP, dATP, and dCTP concentrations in the mitochondria are correlated with those in the cytoplasm with very highly significant p-values. While the mitochondrial dNTP concentrations within the quiescent cells when taken alone are not significantly correlated with the cytoplasmic dNTP concentrations, these values fit into the statistically significant linear correlation in the normal cycling cells. The lack of significant correlation within the quiescent cell data may be simply due to the small dynamic range of the concentrations within the quiescent cells, compared to the noise level in the measurements. dNTPs in mitochondria can in principle originate from two routes: import of cytoplasmic deoxynucleosides and subsequent phosphorylation within the mitochondria (the standard salvage pathway), and/or an import of phosphorylated deoxyribonucleotides from the cytoplasm (Figure 1). If the primary source of dNTPs within the mitochondrion was the salvage pathway then one would expect that the mitochondrial and cytoplasmic dNTP concentrations would be independent, since the salvage pathway could produce mitochondrial dNTPs completely independently of the cytoplasmic dNTPs. Our analysis of the available data shows that this is not the case in normal cells and is in agreement with conclusions made by the researchers who produced the data. Our description of the original interpretation of the data must be brief and focused just on those conclusions that relate to our own and we refer the readers to the original papers for the complete interpretations. Rampazzo *et al.* [45] in their study of transformed cells concluded that while dNTP levels were lower in the mitochondrial compartment than in the cytoplasmic compartment the difference might be explained by different patterns of the concentrations in the three phosphorylation states, so that the summed deoxyribonucleotide concentrations may be roughly equivalent in the two compartments. Since the deoxyribonucleotides other than dNTP were not measured in these experiments, their conclusion cannot be directly tested. In that paper Rampazzo *et al.* also reported that the mitochondrial dTTP level in the transformed cells varies with the cell cycle. In the later experiment by the same group Ferraro *et al.* [44] measured dNTP levels in non-transformed cells and concluded that the proportions of mitochondrial to cytoplasmic dNTP levels in non-transformed cells was similar to that in their previous experiment on transformed cells [45]. In contrast to these interpretations, we have chosen to concentrate just on the reported dNTP levels, instead of attempting to extrapolate to the mono-phosphate and di-phosphate levels. Also in contrast to these papers, we have determined that there is a very significant difference between the results from the non-transformed and the transformed cells.

The association between cytoplasmic and mitochondrial dNTP concentrations suggests a strong cytoplasmic control on the pathways that give rise to the mitochondrial dNTPs. The simplest form of this control would be the transport of deoxyribonucleotides from the cytoplasm to the mitochondrion with only a small amount of dNTP production from the mitochondrial salvage pathway. An alternative, but less parsimonious mechanism would involve a coordinated modulation of nucleotide metabolism in the two subcellular compartments. One caveat of our review that the available data is based on cell cultures from a limited range of cell types, primarily lung and skin fibroblasts. Quiescent fibroblasts may not necessarily be a good general model for all non-cycling cells, in particular for neurons and muscle fibers. This data indicates the need to carry out similar experiments simultaneously measuring the cytoplasmic and mitochondrial dNTP concentrations in these post-mitotic cell types that are directly relevant to the genetic diseases involving altered mitochondrial dNTP production. Measuring the compartmental volumes in the same experiment in addition to measuring dNTP levels would allow for a direct comparison of

dNTP concentration measurements from different labs and between cell lines, avoiding the parameter assumptions that were necessary here.

Since the mitochondrial dNTP levels are strongly affected by the cytoplasmic dNTP levels, alterations in many of the other genes of the cytoplasmic deoxyribonucleotide metabolism may also have phenotypes involving failure to properly maintain mitochondrial DNA. The hypothetical transport mechanism that shuttles deoxyribonucleotides into the mitochondria would also be an MDS candidate, and its expression pattern would be a factor in determining the vulnerability of tissues to mutations in the transporter. These results, showing that the mitochondrial and cytoplasmic dNTP levels are tightly correlated, greatly expand the range of proteins that may lead to mitochondrial dNTP imbalances, and thus to problems with mtDNA replication.

Interpreting the lack of correlations in transformed cells—The correlation between the mitochondrial and cytoplasmic dNTP concentrations, which is strong in normal cycling cells, is lost in this data from transformed cells. A comparison of figure 2D (transformed cells) to Figure 2A (normal mitotic cells) shows that the range of values for both the mitochondrial and cytoplasmic dTTP and dATP concentrations is increased by roughly a factor of three in the transformed cells compared to the normal cycling cells. It is possible that the higher percentage of S-phase cells in a transformed cell culture compared to a culture of normal fibroblasts contributes to this increase in dTTP and dATP concentrations. The dNTP metabolism in both pools appears to be altered, though one could argue from the distribution of the data (Figure 2D) that the cytoplasmic values are more systematically increased than are the mitochondrial values. However, there is not enough data currently to make a confident comparison. Certainly, the tight correlation seen in the normal cycling cells is disrupted in the transformed cells by some unknown mechanism. One might reasonably ask whether this disruption of the correlation between the dNTP levels is a minor consequence of the transformation process or is a fundamental (perhaps even necessary) part of the transformation process. The Warburg hypothesis proposes that a shift of energy metabolism from oxidative phosphorylation to glycolysis is intimately involved in the transformation of normal cells to the cancerous state, and not just a side-effect [56]. The Warburg hypothesis has gone in and out of favor over the decades since it was first proposed, though recent experiments support that hypothesis [57]. Diminished transcription or translation of mtDNA-coded components of oxidative phosphorylation would diminish the ATP production in the mitochondria. The correlation of cytoplasmic and mitochondrial dNTP concentrations in normal cells and its disruption in transformed cells indicates that the transformation process alters the cytoplasmic-mitochondrial communication in dNTP levels. By our analysis, decoupling of dNTP concentrations can mostly be attributed to disproportionately higher dNTP concentrations in the cytoplasm, as the mitochondrial concentrations in such cells are generally not drastically elevated compared to normal cycling cells. One speculative mechanism that could account for this disruption of the normally tight correlation in dNTP levels between the two compartments would be a limitation in the transport of deoxyribonucleotides from the cytoplasm into the mitochondrion. This could come about simply through the saturation of the transport mechanism as the cytoplasmic dNTP concentrations rise in the transformed cells, or it could come about through the active suppression of the transport mechanism as part of the cell transformation process. Aside from the potential importance of the loss of this correlation in dNTP levels as a feature of transformed cells, there is a great practical importance to this observation. This data supports the conclusion that transformed cells should not be used in studies of the mitochondrial deoxyribonucleotide metabolism, since something fundamental about that metabolism is altered in transformed cells. This would be inconvenient, since transformed cell lines are generally far easier to use *in vitro* experiments than are primary cell cultures. However, the data in figures 2 and 3 clearly indicate that something critical is

disrupted in the mitochondrial dNTP metabolism pathway, at least in these three transformed cell types. An additional concern is the lack of data on the mitochondrial compartment size in 3T3 and HOS cells, as discussed above. Conceivably, the parameter assumptions needed to convert the reported units to concentrations may introduce enough noise to mask a correlation between the two compartments in the transformed cells. Further work on this question is clearly needed. In particular, an experiment measuring mitochondrial and cytoplasmic dNTP concentrations in both normal cells and their transformed versions would be very valuable.

Our interpretations in the context of the problem with dNTP assays—As described earlier, Ferraro *et al.* [55] recently concluded that the dNTP levels measured by the Klenow polymerase assay could be significantly contaminated by ribonucleotides. Their results indicated that dTTP measurements would have minimal contamination problems. The fact that the dATP correlation slope (Table 6) is consistent with the dTTP correlation slope, and that in general the pattern in the dATP data is indistinguishable from the pattern in the dTTP data (Figure 2) gives us confidence in the dATP measured values. The dCTP measured values also showed significant correlation between the mitochondrial and cytoplasmic concentrations in normal cells, though with a slightly higher slope than the dTTP or dATP correlations (Table 6). This slightly higher slope could reasonably be due to a ribonucleotide contamination in that assay, or it may reflect a slightly different kinetics of the unknown mechanism causing this correlation. Finally the dGTP values in the two compartments showed no correlation and this is consistent with Ferraro *et al.*'s results showing that the dGTP measurements could possibly be strongly contaminated by ribonucleotides, though of course we cannot rule out the possibility that the apparent correlation between cytoplasmic and mitochondrial dNTP concentrations simply does not occur for dGTP.

Conclusion

We have carried out a meta-analysis of the available data on dNTP levels in the cytoplasmic and mitochondrial subcellular compartments. By compiling the data from multiple experiments into a single larger data set, we have been able to make quantitative interpretations and statistically significant tests of the data. Our review of the data showed that in normal cells the concentrations of dTTP and dATP in the mitochondrion are very strongly correlated with that in the cytoplasm, but that this relationship does not occur in transformed cells. Thus, in terms of the subcellular distribution of dNTPs, transformed cells may not be suitable for studying normal *in vivo* regulation of intra-mitochondrial dNTPs.

The meta-analysis that we have carried out here combining the results of multiple independent experiments points the way toward a unified set of experiments carried out under consistent conditions and focused toward testing the hypothesis resulting from this meta-analysis. It would also be valuable to assess whether the extent of the cytoplasmic influence on the mitochondrial levels of dNTPs is different for the individual nucleotides, and if so, how divergent are the origins of the four canonical dNTPs? A suitable next step would be for a research group to simultaneously measure cytoplasmic and mitochondrial dNTPs in a broader set of cell lines including transformed cells, in a controlled, uniform environment. In order for measurement of dNTP levels to be truly useful, the extra effort of converting the experimentally convenient units of amount per million cells to the relevant units of concentration should be done. In many cases in this analysis we had to estimate values for missing information. One side effect of estimating this missing information is the possible introduction of noise into the data. The strong correlations existed despite this unavoidable complication. Fundamental measurements, such as cell volume and total mitochondrial volume, need to be reported in these studies in order to make the data

comparable across different studies. While we understand the difficulty of the extra measurements needed to report dNTP levels in actual concentration units, we would argue that the increased usability of the resulting data makes this effort worthwhile.

Acknowledgments

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Figure 1.

The origin of intra-mitochondrial dNTPs. dN = deoxynucleoside, dNMP, dNDP and dNTP = mono, di, or tri phosphorylated deoxyribonucleotide respectively. The solid arrow denotes the identified deoxyribonucleoside transport mechanism. Dotted arrows represent possible but unidentified deoxyribonucleotide transport mechanisms. The conversions between nucleosides and dNMPs are irreversible and carried out in the forward and reverse direction by separate enzymes.

Figure 2.

Cytoplasmic and mitochondrial concentrations compiled from the literature of dTTP and dATP from (A) mitotic cells data, (B) post-mitotic cell data, (C) mitotic and post-mitotic data combined, and (D) transformed cells. The error bars were calculated by statistically propagating the uncertainties in the original measurements.

Figure 3. Cytoplasmic and mitochondrial concentrations compiled from the literature of dCTP and dGTP from (A) mitotic cells data, (B) post-mitotic cell data, (C) mitotic and post-mitotic data combined, and (D) transformed cells. The error bars were calculated by statistically propagating the uncertainties in the original measurements.

Table 1

Mitochondrial DNA depletion syndromes.

Gene	MIM ID	MIM NAME
<i>TK2</i>	609560	MTDPS2 (myopathic type)
<i>DGUOK</i>	251880	MTDPS3 (hepatocerebral type)
<i>MPV17</i>	256810	MTDPS6 (hepatocerebral type)
<i>C10ORF2</i>	271245	MTDPS7 (hepatocerebral type)
<i>POLG</i>	203700, 613662	MTDPS4A (alpers type), MTDPS4B (mngie type)
<i>SUCLG1</i>	245400	MTDPS9 (encephalomyopathic type with methylmalonic aciduria)
<i>SUCLA2</i>	612073	MTDPS5 (encephalomyopathic with methylmalonic aciduria)
<i>TYMP</i>	603041	MTDPS1 (mngie type)
<i>RRM2B</i>	612075	MTDPS8A (encephalomyopathic type with renal tubulopathy), MTDPS8B (mngie type)

Table 2

Parameter values used to calculate subcellular dNTP concentrations. Not all parameters were available for all cell lines.

Reference	Parameter	Estimate
[47]	Cellular volume per HeLa cell	1766 ± 55 fL
[47]	Cytoplasmic volume per HeLa cell	1611 ± 62 fL
[47]	Number of mitochondria per HeLa cell	545 ± 31
[47]	Volume of a mitochondrion in HeLa cells	0.285 ± 0.005 fL
[47]	Mitochondrial volume per HeLa cell	155 ± 29 fL
[47]	Cellular volume per HeLa cell (G1 phase only)	1353 ± 54 fL
[47]	Cytoplasmic volume per HeLa cell (G1 phase only)	1235 ± 55 fL
[47]	Number of mitochondria per HeLa cell (G1 phase only)	417 ± 39
[47]	Volume of a mitochondrion in HeLa cells (G1 phase only)	0.283 ± 0.006 fL
[47]	Mitochondrial volume per HeLa cell (G1 phase only)	118 ± 11 fL
[48]	Mitochondrial volume per milligram protein	0.82 μ L
[47,49,50]	Cytoplasmic volume per 3T3 cell	2622 ± 67 fL
[47,49]	Mitochondrial volume per 3T3 cell	96 ± 15 fL
[47,51]	Cytoplasmic volume per fibroblast cell	3252 ± 29 fL

Table 3

dNTP concentrations in the cytoplasm and mitochondria of normal mitotic cells.

Cell line (Reference)	dNTP	Cytoplasmic concentration (micromolar)	Error in cytoplasmic concentration (micromolar)	Mitochondrial concentration (micromolar)	Error in mitochondrial concentration (micromolar)
Cycling skin fibroblasts [40]	T	26	0.2	12	2.2
Cycling skin fibroblasts [40]	C	20	0.2	13	2.3
Cycling lung fibroblasts [44]	T	18	2.0	10	1.9
Cycling lung fibroblasts [44]	A	3.9	1.0	1.9	0.3
Cycling lung fibroblasts [44]	C	6	1	4	1
Cycling lung fibroblasts [44]	G	2.2	0.2	1.3	0.3
Cycling skin fibroblasts [44]	T	26	0.2	12	2.2
Cycling skin fibroblasts [44]	A	18	0.2	9.0	1.6
Cycling skin fibroblasts [44]	C	20	0.2	13	2.3
Cycling skin fibroblasts [44]	G	3.7	0.03	2.6	0.5
Cycling skin fibroblasts [35]	T	8.2	1.5	7.2	2.0
Cycling skin fibroblasts [35]	A	3.8	0.5	4.8	1.2
Cycling skin fibroblasts [35]	C	3.7	1.1	7.4	2.1
Cycling skin fibroblasts [35]	G	3.8	1.7	14	3.3

Table 4
dNTP concentrations in the cytoplasm and mitochondria of normal postmitotic cells.

Cell line (Reference)	dNTP	Cytoplasmic concentration (micromolar)	Error in cytoplasmic concentration (micromolar)	Mitochondrial concentration (micromolar)	Error in mitochondrial concentration (micromolar)
Quiescent skin fibroblasts [40]	T	0.30	0.002	1.4	0.3
Quiescent skin fibroblasts [40]	C	0.980	0.008	2.1	0.4
Quiescent lung fibroblasts [44]	T	0.760	0.006	0.8	0.2
Quiescent lung fibroblasts [44]	A	3.5	0.3	1.9	0.3
Quiescent lung fibroblasts [44]	C	2.46	0.61	1.28	0.23
Quiescent lung fibroblasts [44]	G	1.1	0.3	0.25	0.05
Quiescent skin fibroblasts [44]	T	0.92	0.01	0.96	0.17
Quiescent skin fibroblasts [44]	A	1.07	0.01	3.5	0.6
Quiescent skin fibroblasts [44]	C	2.15	0.01	1.4	0.3
Quiescent skin fibroblasts [44]	G	1.23	0.01	0.8	0.1
Quiescent skin fibroblasts [35]	T	0.6	0.3	4.2	1.1
Quiescent skin fibroblasts [35]	A	1.7	0.3	3.1	1.7
Quiescent skin fibroblasts [35]	C	0.3	0.2	2.6	0.8
Quiescent skin fibroblasts [35]	G	0.8	0.3	8.5	1.9
Quiescent skin fibroblasts [46]	T	0.5	0.1	0.7	0.2

Table 5

dNTP concentrations in the cytoplasm and mitochondria of transformed cells.

Cell line (Reference)	dNTP	Cytoplasmic concentration (micromolar)	Error in cytoplasmic concentration (micromolar)	Mitochondrial concentration (micromolar)	Error in mitochondrial concentration (micromolar)
HOS [45]	T	27	5.5	4.4	0.8
HOS [45]	A	6.8	0.3	2.9	0.5
HOS [45]	C	12	0.4	4.8	0.9
HOS [45]	G	3.6	0.1	1.7	0.3
3T3 [45]	T	7.2	0.2	5.4	0.8
3T3 [45]	A	4.2	0.1	4.7	0.7
3T3 [45]	C	7.6	0.2	5.8	0.9
3T3 [45]	G	2.09	0.05	4.0	0.6
HeLa [41]	T	26	5.3	35	6.1
HeLa [41]	A	23	2.3	15	0.3
HeLa [41]	C	11	0.6	7.9	1.2
HeLa [41]	G	3.3	0.9	29	1.2
HeLa [43]	T	57	9.6	5.5	2.7
HeLa [43]	A	28	6.2	6.4	2.2
HeLa [43]	C	17	2.8	14	2.8
HeLa [43]	G	20	2.0	7.1	3.3
HeLa [42]	T	79	3.0	17	3.1
HeLa [42]	A	74	2.8	11	2.0
HeLa [42]	C	14	0.5	15	2.8
HeLa [42]	G	6.1	0.2	6.4	1.2

Table 6

Statistics for correlations between mitochondrial and cytoplasmic dNTP concentrations. NS = not significant.

Cell Type	dNTP Type	Number of Observations	Pearson R ²	p-value	Slope
Mitotic	dTTP and dATP	7	0.90	9×10^{-4}	0.37 ± 0.05
Postmitotic	dTTP and dATP	8	0.01	0.77	NS
Normal (Mitotic + postmitotic)	dTTP and dATP	15	0.90	5×10^{-8}	0.41 ± 0.04
Normal (Mitotic + postmitotic)	dTTP	9	0.92	3×10^{-5}	0.42 ± 0.04
Normal (Mitotic + postmitotic)	dATP	6	0.77	0.01	0.36 ± 0.09
Transformed	dTTP and dATP	10	0.05	0.53	NS
Mitotic	dCTP and dGTP	7	0.42	0.11	NS
Postmitotic	dCTP and dGTP	7	0.14	0.39	NS
Normal (Mitotic + postmitotic)	dCTP and dGTP	14	0.49	0.005	0.53 ± 0.15
Normal (Mitotic + postmitotic)	dCTP	8	0.88	0.0005	0.56 ± 0.08
Normal (Mitotic + postmitotic)	dGTP	6	0.16	0.42	NS
Transformed	dCTP and dGTP	10	1×10^{-5}	0.99	NS
Normal (Mitotic + postmitotic)	All dNTPs	29	0.62	3×10^{-7}	0.43 ± 0.06
Transformed	All dNTPs	20	0.03	0.45	NS