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Review

Learning from oncocytic tumors: Why choose inefficient mitochondria?☆

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ABSTRACT

A prominent role for mitochondrial genes and metabolism has been recently characterized in oncocytic transformation of cancer cells. From mitochondrial ultrastructure alterations to respiratory complexes disruption and mutations within mitochondrial genes, oncocytic tumors present with a plethora of features that have helped understand the role that these organelles and their fundamental metabolic functions may play in cancer development. The history of this under-diagnosed subset of tumors and the bioenergetic implications of their mitochondrial derangement are discussed in this review along with the opportunities that oncocytic tumors offer to draw general conclusions on the involvement of mitochondria in cancer. This article is part of a Special Issue entitled: Bioenergetics of Cancer.

1. Introduction—clinical, histopathological and ultrastructural features of oncocytic tumors

Oncocytic neoplasms are tumors composed of cells characterized by an aberrant amount of mitochondria that is responsible for their "swollen" (i.e. oncocytic) appearance [1]. They are of epithelial origin and mainly occur in endocrine and exocrine tissues such as the thyroid, parathyroids, kidney, salivary and pituitary gland (for a review see [1]). Nevertheless oncocytic transformation has been seldom observed in organs such as the lungs [2], endometrium [3], colorectum [4–6], liver [7,8], breast [9–11], ocular adnexa (for a review see [12,13]) and even in melanocytic nevi [14]. Both oncocytic adenomas and carcinomas have been reported, particularly in the thyroid, where they have been most frequently described. Unlike in other organs, thyroid tumors are defined as oncocytic when at least 75% of neoplastic cells display the typical mitochondrial hyperplasia. Kidney and salivary gland neoplasms (Warthin tumor and parotid oncocytoma) require instead stricter criteria in order to be classified as oncocytic and hence represent a more homogeneous neoplastic tissue than in the thyroid [1]. Heterogeneous tumors have been described in which oncocytic foci and/or mitochondria-rich cells have been reported, such as in pituitary adenoma or breast cancer [15–17]. The majority of oncocytic neoplasms is considered to be benign and displays low invasiveness. Prognosis of kidney oncocytoma, Warthin tumor and oncocytic pituitary adenoma is in fact usually favourable. In the thyroid the best indicator for prognosis remains the degree of differentiation of neoplastic cells which defines adenoma or carcinoma, regardless of the occurrence of oncocytic transformation. Hence, the conventional criteria of vascular and capsular invasion may be applied to predict malignant behaviour [1], and the reported increase in mortality of oncocytic compared to non-oncocytic carcinoma may be due to a reduced competence in iodine-131 uptake [18] rather than to the occurrence of a specific oncocytic phenotype. This observation is in agreement with the findings that oncocytic metaplasia is not uncommon in non-neoplastic epithelia with high metabolic activity and may be associated with inflammatory conditions such as Hashimoto's thyroiditis [1].

Diagnosis of oncocytic tumors is generally carried out on tissue sections since cytoplasmic eosin staining is particularly intense (hence the term of oxyphilic or eosinophilic tumors). However, since such staining is not always specific, antibodies against subunits of the respiratory chain have been introduced in diagnostic procedures as markers of mitochondrial hyperplasia [1]. Nonetheless, the most striking feature of oncocytic tumors is appreciated through ultrastructural analysis, which displays cells packed with enlarged globular or ovate mitochondria with a stack of lamelliform, tubular or flat cristae and occupying up to 60% of the cytoplasm [1,19,20]. The heterogeneity in mitochondrial morphology observed in oncocytes is suggestive of a functional, along with a structural alteration of these organelles. Nevertheless, the evidence of a dysfunction of

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mitochondria in oncocytes has been collected only recently in the attempt to verify the hypothesis that the mitochondrial hyperplasia typical of these cells may be due to a compensatory effect.

2. Why do oncocytic tumors present with mitochondrial hyperplasia?—The compensatory effect hypothesis

The presence of morphologically altered mitochondria in oncocytic tumors has led to hypothesize that a compensatory effect may be triggered in presence of a retrograde signalling from the organelles to the nucleus. Such signalling has been widely investigated and several mechanisms have been described in which a mitochondrial stress response is activated upon the occurrence of a variety of stimuli, i.e. loss of mitochondrial function caused by collapse of electrochemical potential, impaired respiratory chain activity or by the accumulation of unfolded proteins in the organelle (for a review see [21]) and a decrease in oxygen tension (at least in the initial tumor stages before neovascularization). A metabolic stress may be envisioned in tumor cells, which induces a nuclear response leading to the activation of mitochondrial biogenesis pathways with the intent to restore a defective respiration. In the attempt to reject the compensatory effect hypothesis, Ebner et al. measured the activity of the four respiratory complexes and mitochondrial DNA (mtDNA) copy number in nine oncocytic thyroid tumors, showing an increase of all complexes activity and of mtDNA copies that did not display molecular alterations upon restriction pattern analysis [22]. It has to be underlined that few samples were analyzed by Ebner et al., and that thyroid oncocytic tumors often display a heterogenous composition that may mask a specific phenotype. In fact, the compensatory effect hypothesis has later been re-proposed and reinforced. Several studies have investigated the increase in mitochondrial biogenesis in oncocytic tumors as well as in the only actually existing model of oncocytic cancer, the XTC.UC1 cell line. The authors reported a defective mitochondrial ATP synthesis and overexpression of uncoupling proteins such as UCP2 in thyroid oncocytic tumors [23–25], which may explain the observed increase in the expression of the biogenesis regulator PGC-1-related coactivator (PRC) [26] as well as that of a large number of mitochondrial- and nuclear-encoded mitochondrial proteins [27]. The group of Savagner, therefore, concluded that the defective ATP synthesis may explain the mitochondrial hyperplasia and may hence justify the compensatory effect and this may be due to an oxidative phosphorylation coupling defect whose causes, though, remain to date unknown [24]. Noticeably, these mechanisms were investigated only in thyroid and may not be necessarily involved in other types of oncocytic tumors. Tissue specificity, tumor stage and homogeneity and the presence of an inflammatory infiltrate may affect both the metabolic status of developing tumor cells and gene expression. Before a coupling defect is defined as the main responsible of oncocytic transformation, analyses should therefore be performed in other oncocytomas. Overall, different studies confirm the up-regulation of mitochondrial proteins both in thyroid and kidney oncocytomas, despite the clinical and phenotypical differences of the two types of neoplasms [27–29]. Particularly, the increase both in protein content and activity of respiratory complexes II, III, IV and V as well as of citrate synthase, a marker of mitochondrial mass, seems to be a common feature of kidney oncocytomas in correlation with their benign behaviour [30]. In apparent contrast, deficiency of cytochrome *c* oxidase has been observed both in terms of activity and of subunits histochemical staining patterns, in oncocytes of both normal and hyperfunctional parathyroids [31–34]. However, in the same tissues, Muller-Hocker did not observe a decrease in the mitochondrial transcription factor TFAM, nor in the gamma polymerase (POLG), whereas mtDNA content was higher in oncocytic cells [34], suggesting a compensatory effect due to a complex IV deficiency [33].

With respect to the decrease in oxygen tension observed during the initial exponential growth of the tumor, it has been proposed that the respiratory chain produces increased levels of reactive oxygen species (ROS) when cells experience hypoxia [35]. Whether these ROS may represent the trigger for a retrograde signalling to the nucleus underlying an early-stage oncocytic modification, either through the induction of a mitochondrial DNA damage or through a proliferative stimulus, remains yet to be demonstrated. More likely appears the involvement of nitric oxide (NO) in contributing to oncocytic change. NO levels seem to increase as oxygen tension decreases in a pH-dependent fashion since catabolism by the cytochrome *c* oxidase may not occur when the enzyme is prevalently in a reduced state (for a review see [36]). Since NO has been implicated in the regulation of mitochondrial biogenesis [37], this gas may constitute a stimulus for the mitochondrial modifications typical of oncocytic tumors. Caution is however warranted, since it seems unlikely that in shortage of oxygen, when the respiratory chain may not be utilized for ATP production, cells may be predisposed to activate mitochondrial biogenesis pathways.

3. Does the mitochondrial energetic impairment in oncocytomas trigger the compensatory mechanism?

Until 2003 the compensatory effect in oncocytic tumors was described as a predominant feature but the trigger for such a mechanism was not investigated. On the track of the embryonal suggestion by Muller-Hocker that a respiratory deficiency may underlie oncocytic transformation in normal parathyroid cells, likely in correlation with an aging process [33], the group of Godinot demonstrated that deficiency of complex I in renal oncocytomas might be the early event causing the increased mitochondrial biogenesis, attempting to compensate a respiratory dysfunction [38]. In addition they reported complex I enzyme activity to be moderately decreased in the proximity of the oncocytic tumor, when compared with normal tissue adjacent to the other more aggressive renal tumors analyzed. This led them to speculate that oncocytomas may be the result of two consecutive alterations of the mitochondrial respiratory chain [38]. Following these *in vivo* studies on renal oncocytoma our group fully characterized the bioenergetic competence of the only existing cell model for thyroid oncocytic cancer, namely the XTC.UC1 cell line [39]. We reported a mitochondrial energetic impairment due to a decrease in both complex I and III activity, and, in parallel, an increased level of most respiratory chain complexes subunits, whereas complex I NDUFA9 and mitochondria-coded ND6 were specifically reduced. Moreover, we observed that complex I mitochondria-coded ND1 subunit was absent [40]. In the same paper we also demonstrated that the energetic dysfunction was transferred along with mitochondria in a trans-mitochondrial cell hybrid (cybrid) model and hence concluded that the cause for this dysfunction resided within these organelles. A similar decrease in complex I subunits content was reported by our group in a rare case of nasopharynx oncocytoma, along with a mtDNA copy number increase [41]. Noticeably, a decrease in the activity of complexes I and IV was recently reported *in vivo* in a peculiar case of Warthin tumor, thus extending the feature of a bioenergetic defect to a plethora of oncocytic tumor types [42]. All the data collected so far point at a dysfunction of at least respiratory complex I as the main trigger for the subsequent increase in other mitochondrial proteins and, hence, for the compensatory effect in oncocytic cells.

4. Is there a genetic signature of oncocytic tumors?—Familial forms and genetic predisposition

Cancer is a genetically determined disease. Oncocytic neoplasms are no exception, although no oncogene is known to date to be univocally responsible for oncocytic tumor genesis. The occurrence of oncocytic cancer in familial forms has been extensively reported, although exclusively in the thyroid and in kidney. In thyroid, a locus predisposing to a familial form of thyroid carcinoma with cell oxyphilia (TCO) has been mapped on chromosome 19p13, although

the disease gene has not yet been identified. Molecular aspects of TCO have been recently discussed by our group elsewhere [43] and are not within the aims of the present review. It has to be underlined that oncocytic thyroid tumors also occur within the frame of genetic syndromes whose causative genes are well characterized, such as Cowden syndrome, that derives from *PTEN* inactivation [44]. Overall, two genes encoding mitochondrial proteins have been proposed as candidates for thyroid oncocytic tumorigenesis, namely the mitochondrial inner membrane translocase *TIMM44* and the complex I assembly subunit *GRIM-19* (*NDUFA13*). Both genes were proposed independently by our group [45] and by Maximo et al. [46] respectively, on the basis of their physical position within the TCO locus that had been previously genetically mapped by our group on chromosome 19 [47]. Although the position of *GRIM-19* was subsequently corrected (19p rather than 19q), Maximo et al. reported occurrence of somatic mutations in *GRIM-19* in three out of 20 sporadic, but not in the 6 cases of familial thyroid oncocytic tumors analyzed, thus ruling out a role for this gene in familial predisposition to TCO [46]. *TIMM44* remains a potential candidate as germ-line mutations were shown to segregate with the TCO phenotype, although functional studies of such mutations did not indicate a clear deranging effect on the protein function [45]. These studies suggest that nuclear genes coding for mitochondrial proteins should not be overlooked in the search for candidate genes predisposing to oncocytic tumorigenesis, although mutations in such genes may simply be responsible for the mitochondrial hyperplasia, rather than cell transformation. Caution is therefore warranted to distinguish genes implicated in oncocytic transformation from those predisposing to familial cancers.

Renal oncocytomas have been found at higher frequency in family pedigrees and this familial oncocytosis has been associated with Birt–Hogg–Dubé syndrome (BHD), a rare autosomal dominant disorder first described in 1977 [48]. The syndrome affects the skin inducing benign proliferation of the hair follicles (fibrofolliculomas) at 20–30 years of age. It also increases the risk of certain types of neoplasia, particularly kidney tumors. The genetic alteration for BHD has been mapped to chromosome 17p11.2 and the gene which is likely responsible for BHD has been characterized [49]. This gene codes for the protein folliculin (FLCN) that seems to act as tumor suppressor. Although the function of FLCN is still unknown, it has been suggested that this protein might have a role in energy/nutrient-sensing signalling pathways [50]. Different types of renal tumors were noted in patients affected with the BHD syndrome including bilateral oncocytic neoplasms. Microscopic oncocytosis has been found in many patients and these lesions may be precursors of oncocytic tumors [51]. Noticeably, more than one case of parotid oncocytoma in association with BHD syndrome has been documented [52].

Familiarity of oncocytic tumors suggests that a common genetic event, at least underlying thyroid, kidney and parotid oncocytic cancer, may be the main determinant of the disease, regardless of the organ in which it occurs. Moreover, even in sporadic renal oncocytoma, unlike in thyroid, a fairly large number of cases may be grouped according to specific chromosomal aberrations. The first subgroup of oncocytomas shows allelic loss at chromosome arm 1p along with loss of material from one sex chromosome (Y in men, X in women), a reason why the involvement of genes in the pseudo-autosomal region was suggested [53]. The second subgroup of renal oncocytomas shows a characteristic chromosomal anomaly in the 11q12–13 region and several case reports suggested that this region is prone to rearrangements in renal oncocytoma [54,55]. All these data point to the involvement of specific oncogenes whose identity to date remains unknown. Because of the peculiar mitochondrial hyperplasia, though, it does not seem unlikely that such oncogenes may derange, among others, mitochondrial biogenesis or metabolism triggering the compensatory effect discussed above. It is noteworthy that several genes for mitochondrial proteins map on 11q13 [56–58], hence a role for mitochondrial proteins has been hypothesized [59]. This is further supported by studies on gene expression in renal tumors [28] in which over-expression of mitochondrial proteins encoding genes such as nicotinamide nucleotide transhydrogenase, fumarate hydratase (*FH*) and solute carrier family 25 members 4 and 5 was demonstrated in oncocytoma and clear-cell renal cell carcinoma along with over-expression of the stem cell factor receptor *KIT* which is implicated in several neoplasms [28]. Both Higgins et al. and Fuzesi et al. suggested that a link may exist between defects in mitochondrial proteins and the characteristic oncocytic phenotype of mitochondrial hyperplasia [28,54], although mutations in succinate dehydrogenase subunit B (*SDHB*), a gene coding for a subunit of complex II of the mitochondrial respiratory chain, were not found in 4 samples of oncocytoma analyzed by Morris et al. [60].

The importance of specific nuclear genes in causing the common oncocytic phenotype of certain syndrome-associated neoplasms should not be underestimated. Candidates for further investigation may be suggested by gene expression studies. For instance, over-expression of both nuclear respiratory factor 1 (NRF1) and nitric oxide synthase 3 (NOS3) in oncocytic thyroid cancer indicates a role for these genes in determining, or at least contributing to, oncocytic transformation, as they may be responsible for regulating the mitochondrial biogenesis so obviously deranged in oncocytic tumors [27]. Nitric oxide in fact has been shown to regulate biogenesis by inducing PGC1α [61]. Similarly, over-expression of PRC [26] as a consequence of nutrients and growth factors abundance (for a review see [62]) may provide clues on the nuclear mechanisms responsible for a common oncocytic transformation among different tumors. The involvement of PRC may in fact explain the delay between tumor genesis and the appearance of the oncocytic phenotype, which would hence occur once the tumor becomes well supplied through neoangiogenesis. Noticeably, over-expression of PRC was also observed by our group in parotid oncocytoma and Warthin tumors (unpublished results). Since PRC appears to be a ubiquitous regulator, his role in determining oncocytic transformation will likely gain importance.

5. Where mitochondrial genetics fit within oncocytic transformation

5.1. The mitochondrial DNA

The most striking biological feature of oncocytic tumors is undoubtedly their marked mitochondrial hyperplasia. Human mitochondria possess a multicopy, circular chromosome encoding 13 essential polypeptides which become assembled within four of the five oxidative phosphorylation complexes. The physiological polyploidy of the mtDNA gives rise to a peculiar pattern of inheritance, regulated by the phenomena of homo- and heteroplasmy, the latter being the coexistence in a cell, or a tissue, of two or more different mitochondrial genotypes. Because of this phenomenon, a single copy of a mutated mitochondrial chromosome may be selected against or shift to increase the mutation load until detrimental effects take over to generate a pathological phenotype. The threshold level may vary according to the mutation type and it has been shown in neurological diseases that the penetrance or the severity of a condition may depend on the level of heteroplasmy of the mutation [63,64]. Features that render the mtDNA more prone to the accumulation of mutations than the nuclear DNA, include (i) the proximity to reactive oxygen species production sites, (ii) a less efficient DNA repairing system, (iii) the lack of protective histones, (iv) the highly compact structure which lacks buffering sequences such as introns. Besides coding sequences, the mtDNA contains a regulatory region called the displacement loop (d-loop), which presides to both transcription and replication upon binding of a set of nuclear encoded mitochondrial specific proteins such as DNA-polymerase gamma (POLG), mitochondrial transcription factor (TFAM) and the helicase Twinkle.

The d-loop is about 1300 bp and specific functional regions have been identified along with hypervariable segments. A long list of variants and mutations is to date available for the d-loop region in public databases, although functional studies to define the effects of such genetic changes are very scarce in the literature. The d-loop is essential for producing the polycistron that is then transformed into separate transcripts, upon excision of the tRNA sequences interspersed within the mtDNA [65]. Hence, mutations impairing functional regions of the d-loop may determine a more severe pathologic phenotype than those within coding segments, since they may affect transcription of all mitochondria-coded genes. The same paucity of mechanistic studies also concerns the role of the latter type of mutations in tumor genesis or development.

5.2. MtDNA alterations in oncocytic tumors

The genetic cause of the mitochondrial energetic impairment reported in oncocytic tumors is a recent discovery and resides, unsurprisingly, within the mitochondrial genome. The presence of aberrant mtDNA in oncocytic cancer was at first investigated by restriction pattern analysis and PCR, leading initially to the wrong conclusion that no alterations of the mitochondrial genome were present in oncocytic thyroid tumors. Nonetheless, later on, Tallini et al. detected the common deletion, a frequent alteration of the mitochondrial genome reported in cancer and aging and comprising 4977 bp, in a panel of thyroid oncocytic tumors. The same alteration was also found, though, in perilesional thyroid tissue [66]. Maximo et al. confirmed the occurrence of the common deletion in thyroid cancer, although not in association with the oncocytic features of the neoplasm, and occurring as a somatic mutation at variance with the study by Tallini et al. [67,68]. It has to be underlined that a biological significance of this clearly pathogenic mutation may not be evident if one considers that 16% was the highest heteroplasmy level detected in samples [67]. It is plausible to reckon that the threshold should be higher even for such a large mitochondrial chromosomal aberration in order for a defective phenotype to appear, as it is shown for other mutations [69].

As sequencing of the entire mtDNA has become a more feasible approach allowing detection of even low heteroplasmy levels, an increasing number of mtDNA mutations has been described in oncocytic tumors. Maximo et al. reported a high prevalence of somatic mitochondrial variants in 79 thyroid cancer samples, 43 of which presented oncocytic features [70]. Direct automated sequencing was used to screen up to 70% of the mitochondrial genome, covering however only protein-coding segments. It is hence plausible to think that the actual number of somatic variants might have been underestimated. D-loop instability was reported to occur in many of the oncocytic samples, with the homopolymeric C and CA stretches varying in length with respect to the tumor-adjacent normal tissue, especially around positions 303 and 514 [70]. However, these repetitive stretches are hot-spots for DNA fragment length variation, which represents a common polymorphism of still undeciphered biological significance [71]. Few of the 57 somatic changes reported by Maximo et al. were clearly damaging, being frameshift or nonsense changes in coding sequences and good candidates for the respiratory defects observed in oncocytic tumors, opening the way to subsequent studies by our and other groups. The pathogenic potential of non-disruptive mutations was not addressed, nor was the heteroplasmic status, a necessary issue when dealing with heterogeneous tumors such as in the thyroid. In the same study, the authors underline that no significant difference was found between the occurrence of mtDNA variants in oncocytic and non-oncocytic tumors, except for those occurring in the *ATP6* complex V gene. Such changes were hence suggested to be hallmarks of the oncocytic transformation [70], although *in silico* prediction of the pathogenic potential of these mutations fails to provide a striking indication of their role in the energetic impairment.

On the basis of our observation of a specific decrease in complex I subunits expression and activity in the only existing thyroid oncocytic tumor cell model, our group demonstrated that the mitochondrial energetic failure was dependent on two mtDNA mutations affecting complexes I and III of the respiratory chain [37]. One of these mutations was a single base pair homoplasmic insertion in a C-homopolymer in the *ND1* complex I gene, causing a premature stop codon, which explained the absence of the protein. The second was a non-conservative heteroplasmic missense mutation in the *CYTB* complex III gene. These two mutations were sufficient to explain the defective ATP synthesis already reported in the same cell line [23,24]. We hence demonstrated that clearly pathogenic, truncating mutations impairing complex I were univocal genetic markers of thyroid oncocytic tumors, upon analysis of a panel of 45 samples against 52 non-oncocytic control tumors. In the same study we ruled out an age-dependent accumulation of such somatic mutations. The same lack of correlation was hence reported with respect to tumor aggressive/malignant behaviour [17]. Even more strikingly, 100% of kidney oncocytomas, analyzed in a study by Simonnet's group in collaboration with ours, presented the same type of disruptive mutations, substantially explaining the corresponding defective respiration paralleled by an increase in citrate synthase activity [72]. Independent observations by Mayr et al. confirmed the same findings in renal oncocytoma [73] and, later on, in thyroid oncocytic tumors [74]. The same high frequency of disruptive mutations was reported also in parathyroid oncocytic neoplasms [75]. Recently our analysis was extended to a panel of oncocytic tumors of the pituitary and salivary glands and the significant association of a high frequency of pathogenic mtDNA mutations with the oncocytic phenotype was confirmed, ruling out a tissue-specificity for mutations occurrence [42]. Most mutations reported so far by our group were both disruptive and homoplasmic, indicating that a shift towards a full mutant load (100%) in cancer cells occurs and may not be due simply to random genetic drift, sustained by transformed cells despite the apparent detrimental biochemical effects.

To date 152 entire mtDNA sequences have been obtained from oncocytic tumor samples so that some statistical analysis is possible (Table 1). In order to assess whether a hotspot mitochondrial gene does exist, we have collected all the somatic (cancer specific) variants reported in the five papers where at least all the coding mtDNA sequences have been analyzed (Table 1). Assuming that all mtDNA regions should be equally susceptible to mutations, we have then calculated the ratio between the frequency of reported and expected mutations *per* mitochondrial gene after normalizing on the gene length. Fig. 1A clearly shows that *ND1* is indeed a hotspot for somatic changes with a ratio of over 3, meaning that mutations occur in this gene at a frequency more than three times higher than expected on the basis of the gene length. Similarly, although less strikingly, *ND4*, *ND5* and *ATP6* also display a tendency to accumulate somatic changes (Fig. 1A). On the contrary, *COI* and *ATP8* appear to be more protected from mutational events. We have further extended this type of analysis by taking into consideration only those changes for which a high pathogenic potential could be predicted. Overall, 101 changes were predicted to be pathogenic (i.e. were predicted to be possibly or probably damaging by PolyPhen). In this frame, *ND1* still had a ratio above 3, indicating that this subunit may more frequently face a mutational damage. Similarly, *ND3*, *ND4*, *ND4L* and *ND5* also displayed a ratio greater than 1.5. Interestingly, all complex IV and V genes (*COI*, *COII* and *COIII*, *ATP6* and *ATP8*) seemed to be preserved from the occurrence of damaging mutations (Fig. 1B). Several hypotheses may be formulated to speculate on these differences in mitochondrial susceptibility to damaging mutations. First, it has been shown that different regions of the mammalian mtDNA display a different degree of conservation, with *ND1*, *ND3*, *ND4*, *ND4L* and *ND5* being less evolutionary conserved than *COI*, *COII* and *COIII* [76]. In this context the mentioned ND subunits may better sustain the occurrence of non-conservative missense variants. Second, a different threshold for a

pathogenic phenotype has been reported for mutations in *COI* with respect to *ND1*. In fact, whereas for *COI* 40% of mutated DNA is sufficient to generate a defective respiration [69], over 90% of mutated *ND1* copies are necessary to obtain the same effect [42,69]. Finally, it is interesting to note that *ND4* and *ND5* are the richest in homopolymeric stretches (here arbitrarily defined as polyA, C, G and T longer or equal to 5 bases), with 11 and 12 stretches respectively. *ND2*, *ND1* and *ND6* are also rich in homopolymers (9, 7 and 7, respectively). Instability of homopolymeric stretches is suspected to predispose to accumulation of somatic mutations, similarly to what is observed in the polyC stretches of the d-loop region, where somatic variations in the length of the homopolymer have been extensively reported in cancer and particularly in thyroid oncocytic tumors [77]. Abundance of such unstable regions is in agreement with the higher susceptibility of ND subunits to truncating mutations, which very often occur in homopolymers [17,42,72].

5.3. MtDNA mutations as a cause or a consequence of the oncocytic phenotype—The egg or the chicken metaphor

Oncocytic transformation has been strongly associated with a compensatory effect due to a mitochondrial dysfunction leading to an increase of mitochondrial mass. Nonetheless where and how mtDNA mutations fall within this process is yet unclear and scarcely investigated. It is worth noting that the mtDNA mutations underlying the oncocytic phenotype may not be the primary hit in tumorigenesis, as previously suggested [17]. We reported a peculiar case of a patient that presented three tumor nodules, only one of which displayed an oncocytic phenotype and, along with it, a nonsense mutation in a complex I subunit. The exclusive occurrence of the mutation in the oncocytic nodule led us to hypothesize that this should be a secondary event in tumorigenesis responsible for the mitochondrial hyperplasia and hence for the oncocytic transformation [17]. To further support this hypothesis, we described a disruptive frameshift mtDNA mutation affecting the ND5 subunit of complex I present in homoplasmy in a nasopharyngeal oncocytic tumor. This mutation was inherited at low degree of heteroplasmy through the germline in the patient family but became homoplasmic in the tumor of the patient only. The mutation was hence present in the patient tissues prior to the tumor occurrence and, although it might be predisposing to oncocytic transformation through a shift to homoplasmy, it is

Table 1
Somatic and potentially pathogenic changes reported in oncocytic tumors.

Gene (length, bp)	Gasparre et al. [17]	Gasparre et al. [72]	Porcelli et al. [42]	Maximo et al. [70]	Costa-Guda et al. [75]	Total
(A) Somatic changes reported in oncocytic tumors						
ND1 (955)	10	5	6	1	3	25
CYTB (1140)	5	1	1	2	0	9
ND6 (524)	0	1	0	2	0	3
ND4 (1377)	7	1	8	2	1	19
ND5 (1811)	7	4	10	2	3	26
ND4L (296)	1	0	0	1	1	3
ND3 (345)	0	0	1	3	0	4
COIII (783)	0	0	0	5	0	5
ATP6 (680)	3	0	0	7	0	10
ATP8 (206)	0	0	0	0	0	0
COII (683)	0	0	0	5	0	5
COI (1541)	0	1	2	2	2	7
ND2 (1041)	4	0	3	2	1	10
(B) Potentially pathogenic changes reported in oncocytic tumors						
ND1 (955)	10	5	6	0	3	24
CYTB (1140)	5	1	1	2	0	9
ND6 (524)	0	1	0	2	0	3
ND4 (1377)	7	1	8	0	1	17
ND5 (1811)	7	4	10	0	2	23
ND4L (296)	1	0	0	1	1	3
ND3 (345)	0	0	1	3	0	4
COIII (783)	0	0	0	2	0	2
ATP6 (680)	3	0	0	0	0	3
ATP8 (206)	0	0	0	0	0	0
COII (683)	0	0	0	2	0	2
COI (1541)	0	1	2	0	0	3
ND2 (1041)	4	0	3	0	1	8

The table reports the number of somatic (tumor-specific; Table 1A) and tumor-specific potentially pathogenic changes (Table 1B) occurring in protein-coding mtDNA genes. Values have been extrapolated from the five papers in which at least the entire protein-coding mtDNA sequence was obtained. All missense changes reported have been subjected to *in silico* prediction of pathogenic potential by PolyPhen and probably/possibly pathogenic changes only have been included in Table 1B.

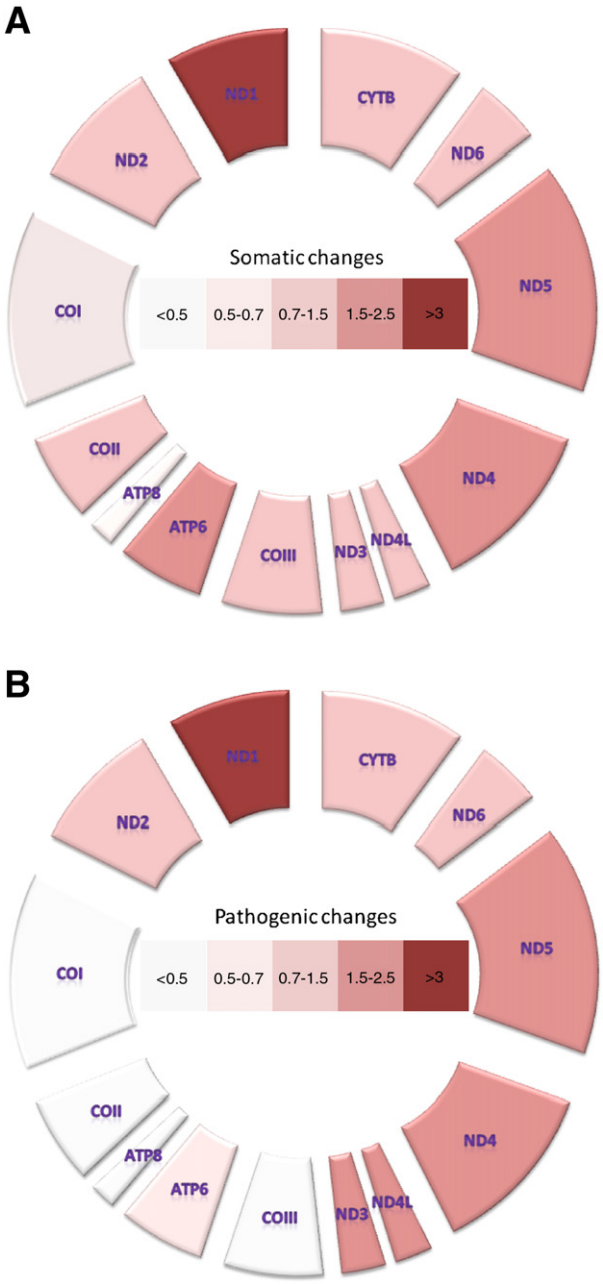

Fig. 1. Frequency ratio of mtDNA mutations occurring in protein-coding genes. (A) Somatic changes. (B) Potentially pathogenic changes as predicted by PolyPhen. Different shades of colour indicate different susceptibility to mutations occurrence. Scale value from 0 to above 3 was calculated as ratio of mutation frequencies (obtained dividing the single values reported in Tab.1A and B *per* gene by the overall number of mutations) over the percentage of mtDNA occupied by the gene. Genes with values below 0.7 have been arbitrarily defined as "preserved from the occurrence of mutations", whereas genes with values above 1.5 have been defined as "mutational hotspots".

unlikely that it may have been the primary tumor hit [41]. Moreover, in different oncocytic areas of the same tumor of the reported case, a damaging homoplasmic *ND1* mutation was described where the *ND5* germline mutation was still heteroplasmic, suggesting a common nuclear tumorigenic hit and at least two subsequent and independent mitochondrial mutations [41].

In this context, oncocytic transformation ought to be conceptually and temporally separated from oncogenic transformation, which should occur first. The question remains open of whether mtDNA mutations actively contribute to the oncocytic transformation or occur and become selected in the context of a deregulated mitochondrial biogenesis and hyperplasia. The first hypothesis fits better with a compensatory process in which the mtDNA mutation is the event causing the mitochondrial dysfunction that triggers a retrograde signalling to the nucleus. The response to such signalling is an increase in mitochondrial biogenesis in the attempt to rescue the defective phenotype (Fig. 2A). It has been reported that nonsense mtDNA mutations in *ND5* and *COI* specifically trigger the up-regulation of *PGC1α/β* and the over-expression of mitochondrial proteins so that cell respiration is somehow rescued. Although this was observed in a non-oncocytic tumor cell line, the authors suggested a retrograde mechanism calcium-mediated at the basis of the observed increase in biogenesis. Nevertheless the occurrence of an oncocytic-like phenotype in these cells was not investigated at all [78]. According to the second hypothesis, mitochondrial biogenesis may be triggered in response to an increased influx of nutrients or growth factors (as discussed in paragraph 4), or by the activation of the same oncogene responsible for the primary cell transformation, such as, for instance, *H-RAS*. Such a mechanism has been demonstrated in primary fibroblasts infected with this oncogene, upon which up-regulation of several genes involved in mitochondrial biogenesis occurred. Interestingly, up-regulation of biogenesis appears to be dependent on genes involved in the DNA damage repair and regulating oncogene-induced senescence such as *TP53* and *Rb*, although the mechanistic links are still lacking [79]. In this context, the oncogene-dependent biogenesis may facilitate the occurrence and accumulation of mtDNA mutations (Fig. 2B).

The relationship between oncocytic transformation and the mitochondrial involvement in oncogene-induced senescence warrants further investigation and will be decisive in placing the mtDNA mutation within tumor progression.

6. Structural and functional consequences of mtDNA mutations on oncocytic cancer cells

6.1. The impact of mtDNA mutations on respiratory complexes assembly

Altogether, all data collected thus far in oncocytic tumors point to respiratory complex I as the major hotspot for the occurrence of damaging mtDNA mutations. Once again the XTC.UC1 cell model of oncocytic neoplasia has been very useful in clarifying the effects of the homoplasmic truncating mtDNA mutations on the energetic function and, in particular, on the respiratory complex content and assembly [80]. In fact, the 3571insC *ND1* mutation was shown to ablate ND1 content as well as to reduce the levels of other complex I subunits such as the nuclear-coded NDUFA9 and the mitochondria-coded ND6. No other complexes subunits appeared to be affected among those analyzed [40]. On these bases, our group showed that the complex I subunits are lost *in vivo* in oncocytic tumors exclusively in the presence of homoplasmic truncating ND mutations [41]. Immunohistochemical staining for the ND6 subunit has proven to be a useful tool to identify at least partial complex I disassembly so that it may easily be utilized with a predictive potential of the occurrence of disruptive mtDNA mutations. The peculiarity of ND6 is indeed that its expression is influenced by that of other ND subunits, because of its pivotal role in the ND-arm assembly [81]. Hofhaus and Attardi had in fact demonstrated that a disruptive mutation in the ND5 subunit was associated with under-expression of ND6 [82]. In agreement with these findings, we reported that nuclear-coded NDUFB6 subunit showed a positive staining even in the absence of ND6. Although lack of ND6 is strongly suggestive of a disassembly of the γ subcomplex of the complex I hydrophobic arm, positive staining of NDUFB6 *in vivo* does not provide indication on whether the proteins are synthesized and retained in the cytosol or they are imported and assembled within abortive β subcomplex of the complex I hydrophobic arm [83]. Interestingly, the hydrophilic λ-arm of complex I was also reported to be lacking in oncocytic thyroid tumors upon occurrence of mtDNA mutations. The same authors reported an increased staining of subunits belonging to the other four complexes and the structural protein porin [74]. Similar results were recently reported by our group in a large panel of pituitary, head-and-neck, and thyroid oncocytic tumors, in correlation with homoplasmic mtDNA truncating mutations, where intense complex V staining strongly indicated the oncocytic phenotype [42]. An example of a thyroid oncocytic tumor in which lack of ND6 and NDUFB6 is evident, along with over-expression of complex V subunit ATP5B, is reported in Fig. 3. The alteration of mitochondria cristae, likely following respiratory complexes disruption, is also shown.

6.2. Do ROS have a role in oncocytic tumorigenesis?

The occurrence of mtDNA mutations implying an incomplete or partial assembly of complex I raises the question of whether this may contribute to ROS generation. Zimmermann et al. speculated that the observed lack of complex I staining in oncocytic thyroid may have a role in tumor formation through the increase in ROS production and, simultaneously, a downstream inhibition of pro-apoptotic pathways, although the nuclear-coded complex I subunits called into play are not apoptotic effectors, but rather caspases targets [74]. Lack of data on the role of ROS in oncocytic tumors is mainly due to the fact that studies on tumor biopsies are not feasible and that cell models for *in vitro* studies are very scarce. Investigation of ROS production in the XTC.UC1 cells showed that the different degree of heteroplasmy of the 3571insC *ND1* mutation does not influence ROS amounts. This was explained with a differential expression of ROS detoxifying enzymes such as manganese superoxide dismutase and catalase in presence/absence of complex I [42]. Similarly, peroxiredoxin I, an oxygen peroxide scavenging enzyme, was shown to be overexpressed in oncocytic tumors of the salivary glands, which led the authors to suggest that detoxifying mechanisms may be up-regulated in these neoplasms likely carrying mitochondrial dysfunctions and electrons leakage [84].

Nonetheless, further studies are warranted to dissect the role of ROS in oncocytic tumors *in vivo*, where most of the mutations reported are indeed homoplasmic and complex I appears to be disassembled, to understand whether ROS may influence the proliferative potential and the accumulation of mutations in these tumors.

6.3. Apoptosis in oncocytic tumors

It is widely accepted that the growth advantage in tumors may derive from increased survival due to prevention of apoptosis. The intrinsic pathway of apoptotic cell death is also called the mitochondrial pathway, owing to the essential role played by these organelles. In fact, at the level of the mitochondrial outer membrane, interaction among anti-apoptotic and pro-apoptotic proteins of the Bcl-2 family can occur, thus determining the cell fate. Furthermore, some pro-apoptotic proteins (cytochrome *c*, AIF, Smac/DIABLO, OMI/Htr2A, endonuclease G, etc.) are sequestered within the intermembrane space, from where they can be released into the cytosol following permeabilization of the outer membrane. Cytochrome *c* in the

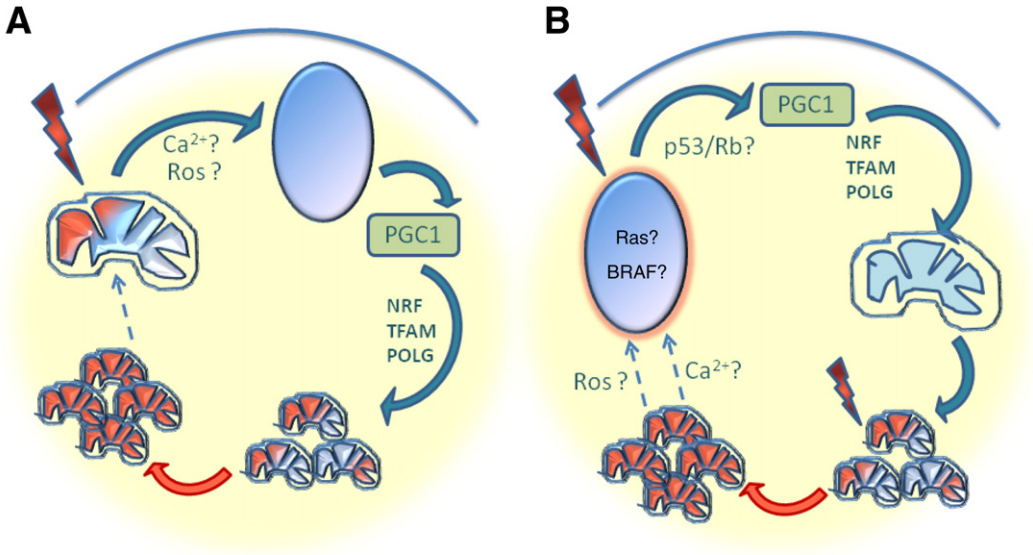

Fig. 2. Scheme of the genesis of the oncocytic phenotype: the egg or the chicken metaphor. (A) MtDNA mutation (red lightning arrow) causes the mitochondrial dysfunction (shaded mitochondrion) that triggers a retrograde signalling to the nucleus mediated by alterations of calcium homeostasis. The response to such signal is the up-regulation of *PGC1α/β*, which in turn triggers activation of downstream effectors (as NRF, TFAM and POLG) leading to mitochondrial biogenesis with increase in mtDNA copy number in the attempt to rescue the defective energetic phenotype. In this context accumulation of mutations may occur, in a feed-forward loop that would further stimulate the retrograde signalling. (B) A nuclear (pre)-oncogenic stimulus triggers the DNA-repair response pathways such as those mediated by p53 and Rb, leading to the activation of mitochondrial biogenesis regulators and effectors through still unknown mechanisms. The increase in mtDNA copy number subsequent to PGC1 up-regulation may then determine the occurrence of the homoplasmic shift of mtDNA mutations, which in turn may contribute to trigger a retrograde signalling and accumulation of nuclear damage, i.e. through an increased oxidative stress.

presence of ATP has been shown to be required for caspase activation [85], whereas the other proteins are differently involved in promotion of other types of cell death mechanisms (for a review see [86]). Owing to these findings, it has been hypothesized that mtDNA mutations impairing mitochondrial energetic function may protect cells from apoptosis, thus promoting growth advantage. In this regard, two different homoplasmic pathogenic *ATP6* mutations were previously reported to lower the frequency of apoptosis both in cybrid cells and in tumors derived from cybrid transplantation [87]. Furthermore, a subsequent comparison of resistance to oxidative stress-induced

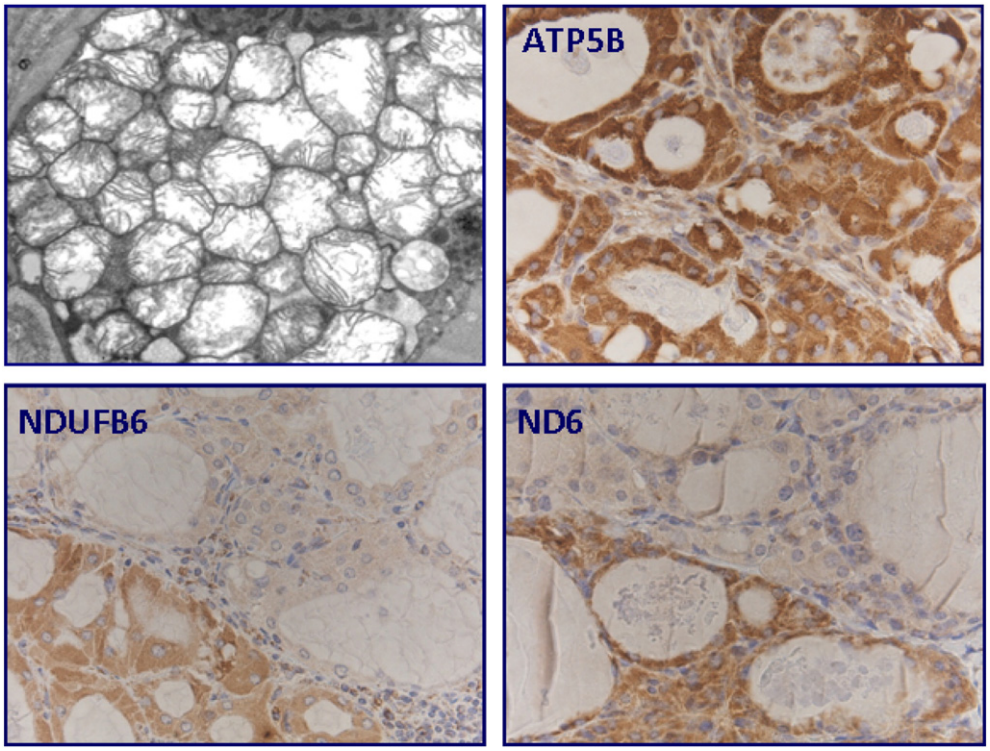

Fig. 3. Impact of mtDNA mutations on respiratory complexes assembly. Representative electron microscopy and IHC images of an oncocytic hyperplastic thyroid nodule. In the upper left panel, ultrastructural features of mitochondria in an oncocytic tumor cell, displaying the dramatic alteration of the cristae organization (image courtesy of Dr. C. Betts). In the upper right panel, staining for complex V subunit ATP5B marks the oncocytic phenotype. Negative staining for nuclear-coded NDUFB6 complex I subunit and mitochondria-coded ND6 complex I subunit is apparent in the more intense complex V stained, oncocytic part of the nodule (upper side of NDUFB6 and ND6 panels) (Images courtesy of Dr. C. Ceccarelli).

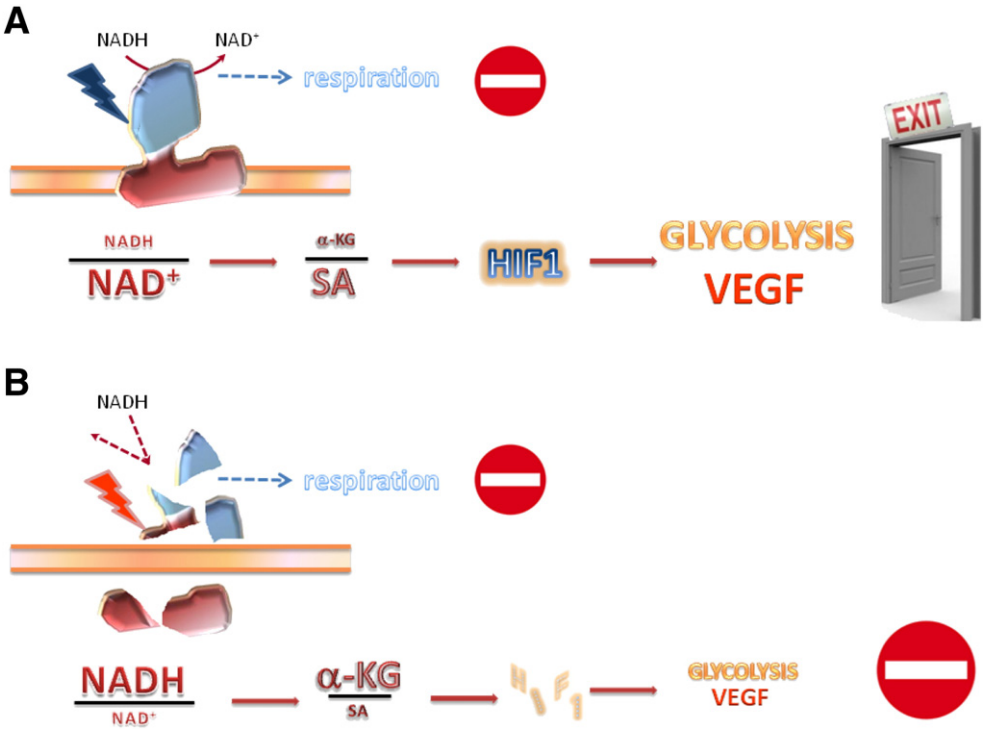

Fig. 4. The blind alley of oncocytic tumors: a non-exit strategy? (A) A mtDNA mutation impairing complex I function but not its assembly may still allow and/or increase NADH consumption, keeping a low NADH/NAD$^+$ ratio and a consequent low α-ketoglutarate (α-KG)/succinate (SA) ratio. This in turn may not prevent HIF1α stabilization under hypoxic conditions, allowing the up-regulation of glycolytic and pro-tumorigenic genes (i.e. *GLUT1*, *LDHA* and *VEGF*), finally permitting the metabolic adaptation of cells and hence tumor progression (exit door). This would occur despite lack of mitochondrial respiration due to the mtDNA mutation (Warburg effect). (B) A mtDNA mutation disassembling complex I, such as those typical of the oncocytic phenotype, may not allow NADH consumption, shifting the NADH/NAD$^+$ ratio towards NADH and consequent α-ketoglutarate (α-KG) accumulation. This Krebs cycle alteration may induce activation of prolyl-hydroxylase and HIF1α degradation even in low oxygen tension conditions, preventing the up-regulation of glycolysis needed to compensate for the defective mitochondrial respiration and leading the tumor into a blind alley (no access sign) without a way out to metabolic adaptation.

death in cells bearing a ND5 mutation showed that inhibition of apoptosis was indeed more pronounced in heteroplasmic than in homoplasmic cells. This result was explained by the very limited production of ATP by cells with homoplasmic ND5 mutation, that would compromise cytochrome *c*-dependent caspase 9 activation [88]. Whether mtDNA mutations prevent apoptosis also in oncocytic tumors is still poorly defined. So far, a microarray analysis showed significant down-regulation of caspase 3 expression in thyroid oncocytic tumors, indicating a mechanism for inhibition of cell death [89]. Once again studies in progress with the XTC.UC1 cell model will provide useful information on this topic.

6.4. Consequences of mtDNA mutations on Krebs cycle and beyond

In the recent past, several observations have linked respiratory complexes dysfunctions to alteration of upstream mitochondrial metabolism, i.e. the Krebs cycle, in cancer. The best example is given by hereditary paragangliomas, leiomyomas and renal carcinomas in which an aetiological role for complex II mutations has been demonstrated. In fact, mutations in the B, C and D subunits of *SDH* have been shown to lead to accumulation of the Krebs metabolite succinate at the expenses of α-ketoglutarate. Similarly, mutations in *FH* lead to accumulation of fumarate, slowing down the cycle [90,91]. These hereditary tumors are characterized by a condition of chronic pseudohypoxia. In fact the accumulation of Krebs cycle metabolites succinate and fumarate contributes to the inhibition of prolyl-hydroxylases (PHD), key activators of the hypoxia inducible factor-1α (HIF1α) degradation [92]. This factor has been long known to be a critical player in mediating the metabolic adaptation needed by cancer cell to progress from a benign to a malignant state [93].

The complex I dysfunction described in oncocytic tumors raises the question of whether a parallel may exist between these neoplasms and *SDH*/*FH* mutated cancers. To shed light on this issue we recently attempted to define the levels of HIF1α-regulating Krebs cycle metabolites in the XTC.UC1 cells bearing the homoplasmic truncating *ND1* mutation. We demonstrated that the ratio between succinate and α-ketoglutarate is opposite to what reported in SDH and FH mutated tumors, with increase of α-ketoglutarate at the expenses of succinate [42]. It is likely that the absence of a functional complex I in these cells leads to accumulation of NADH, the main substrate of complex I. Previous inhibitor affinity studies in mitochondria harboring different mtDNA mutations in complex I have clearly demonstrated that the hydrophobic portion of the ubiquinone binding site involves ND1, ND5, and ND6 subunits [94–96]. It is therefore likely that in the context of oncocytic transformation, ubiquinone may not act as acceptor for NADH-derived electrons. The consequent NADH accumulation may, in turn, inhibit α-ketoglutarate dehydrogenase and prevent succinate production with a consequent α-ketoglutarate increase. Since α-ketoglutarate is the main feeding reagent of PHD, HIF1α should undergo a chronic destabilization, even in the tumor hypoxic environment. We have indeed shown this to be the case in oncocytic tumors harboring homoplasmic disruptive mtDNA mutations causing disassembly of complex I [42].

7. The blind alley of oncocytic cells: A strategy lesson on tumor development?

These data raise the issue of how oncocytic cells can survive and proliferate in the adverse tumor microenvironment, having a deranged respiratory metabolism and being unable to stabilize HIF1α, which powerfully regulates the expression of glycolytic genes such as the glucose transporter GLUT1 [93]. HIF1α is also the main inducer of the vascular endothelial growth factor (VEGF), a key player regulating the generation of novel vasculature in the hypoxic

environment of the tumor mass. We observed in fact that most oncocytic tumors displaying HIF1α destabilization do not present features of neovascularization. Although HIF1α is expected to be stabilized in central tumor areas lacking vessels, this may be explained by the deregulation of VEGF. This observation led us to propose that HIF1α destabilization in oncocytic cells should be an event occurring after the homoplasmic shift of the mtDNA mutation and before neovascularization in tumor progression [42]. This consideration is in agreement with the observation that the large majority of oncocytic tumors harboring mtDNA mutations are indeed benign, non-aggressive and low proliferating lesions [1,17,42]. The functional correlation between homoplasmy of mtDNA mutations and a benign behavior of oncocytic tumors is supported by similar findings in non-oncocytic cancer models, such as those recently described by Park et al. These authors reported a lower tumorigenic potential of a homoplasmic truncating ND5 mutation with respect to the same mutation in heteroplasmy [88]. Their study comes in a context where a positive association between mtDNA mutations and tumorigenic, pro-metastatic potential is investigated, also in terms of increase of HIF1α stabilization [87,97], which is clearly not the case in oncocytic tumors. When all these data on mtDNA mutations in tumor development are gathered and critically analyzed, they indicate that both the degree of heteroplasmy and the type of mutations (missense versus truncating) must be carefully taken into account since they may determine a completely opposite tumor phenotype.

Overall, the molecular marker of oncocytic tumors, namely the homoplasmy of disassembling mutations, drives the oncocytic cell in a blind alley: the mitochondrial energetic impairment on one side and the inability to compensate with an increased glycolytic metabolism, along with the inability to induce neovascularization, should lead the cell to proliferative arrest (Fig. 4). What specific molecular and cellular pathways may be actively involved to regulate this inhibition of growth is still an unexplored field of research, which we are currently investigating. Both the induction of senescence and autophagy may represent a smart strategy for the cell to counteract the deregulated tumor proliferation. These processes may be synergistic with the homoplasmic shift of mtDNA mutations. The demonstration of whether this derives from a random event or from a controlled induction, through the activation of mitochondrial biogenesis mechanisms, represents the most exciting challenge in the current research on the role of mitochondrial metabolism in oncocytic tumors.

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