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Copper as a Target for Treatment of Neuroblastoma: Molecular and Cellular Mechanisms

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1. Introduction

1.1. Copper and carcinogenesis, a double-edged sword

Copper is a trace metal essential to the catalysis of a wide range of enzymatic activities, including those involved in the process of energy production (cytochrome c oxidase), the cell response to oxidant injuries (Cu,Zn-superoxide dismutase), the catecholamine (dopamine β -monooxygenase) and melanin (tyrosinase) production, the remodelling of extracellular matrix (lysyl oxidase), blood clotting processes (Factors V and VIII) and iron metabolism (ceruloplasmin and hephaestin) [1]. The catalytic properties of copper are linked to its ability to easily assume the oxidized (Cu^{2+}) and reduced (Cu^+) states, but just the metal reactive behaviour can trigger severe cell alterations through the generation of hydroxyl radicals in Fenton-like reactions [2,3]. When the cytosolic copper concentration is above the optimal level, the newly formed reactive oxygen species (ROS) rapidly bind to DNA, thus inducing the breaking of the nucleic acid strands and initiating a series of cascade events that can lead to significant damage to cell structures and function [4].

Considerable intrinsic oxidative stress and enhanced serum and tissue copper levels depict a disease condition that often accompanies the progression of several tumour forms, in turn resulting from a perturbed energy metabolism, mitochondrial dysfunction, release of cytokines and inflammation [5]. Copper is intimately involved in all these cell functions, thus targeting the elevated copper levels would be an ideal therapeutic strategy to effectively counteract the tumour development [5].

This issue is anyway highly debated. In fact, the topical delivery of copper complexes to tumour tissues has been demonstrated to kill the cancer cells through a “therapeutic” induction of oxidative stress [6]. At the same time, especially in the case of solid tumours, as *neuroblas-*

toma, copper is directly involved in the spread of the primary tumour, mainly through the stimulation of tumour angiogenesis [6]. It follows that targeting the tumour copper content to limit the cancer aggressiveness requires a comprehensive knowledge of the cell metal management under the disease state. Here, the multifaceted contribution of copper to the pathophysiology of neuroblastoma will be dissected, with special attention paid to the regulation of membrane copper transporters and their role in sustaining the cancer spread. To make the reader familiar with the main copper transport systems in mammalian cells, a short description has been provided in Box 1.

2. Neuroblastoma and Copper: A complex relationship

Neuroblastoma is the most common pediatric extra-cranial neoplasm [7], whose malignant form accounts for about a 50% of cancer mortality in chemoradiotherapy-treated subjects [8]. The aggressiveness of advanced-staged neuroblastomas is notoriously associated with the *N-myc* oncogene amplification, which translates in a strong expression of a pleiotropic transcription factor, responsible for the rich tumour vasculature, the metastatic behaviour, and the chemotherapy resistance [9-11]. Thus, *N-myc* overexpression is a well-known adverse prognostic factor [12]. Interestingly, the degree of *N-myc* oncogene amplification in neuroblastoma cells has been put in relation to the trace metal cell content (iron, copper, zinc) in both cultured neuroblasts and murine xenografts [13-15]. In particular, the number of *N-myc* oncogene copies has been demonstrated to proportionally correlate with the neuroblastoma copper content. This finding, together with evidences from the literature, lets us suppose that copper accumulation strictly determines the neuroblastoma invasiveness. Plausible mechanisms underlining the copper dependence of neuroblastoma metastasis are both direct/specific, and mediated by the metal-induced accumulation of ROS.

Referring to the latter category of mechanisms, an *in vitro* study in 31 subjects affected by advanced neuroblastoma revealed an elevated activation of specific tissue matrix metalloproteinases (MMP-2 isoform) and the reduced expression of their specific inhibitors (TIMP-2) [16], that can be associated with copper-induced oxidative stress [17]. In this regard, we observe that metalloproteinases are secreted by tumour cells and facilitate the cancer dissemination by the degradation of the extracellular matrix.

The high copper levels detected in neuroblastoma can at least partly confer a growth advantage to the tumour cells by metal specific pathways. Significantly, copper acts as a cofactor for the cytochrome c oxidase enzyme that allows the conversion of cytosolic ferric ion into the ferrous form, subsequently incorporated into ferritin, the most important iron storage protein. Iron-complexed ferritin is then secreted by cancer cells, so enriching the serum protein pool. The importance of this copper/iron antagonism is evident if we consider that neuroblastoma patients with high ferritin levels undergo a bad prognosis [18].

Given the complexity of the copper-neuroblastoma relationship, in order to guide the reader through the text, we observe that the lines of copper intervention in neuroblastoma progression can be substantially subdivided as follows:

- i. cancer energy metabolism
- ii. tumour vascularization

Copper transport systems are gaining growing importance in the studies about the various aspects of the metal role in neuroblastoma, so the peculiar expression pattern will be described before discussing the pathological topics.

3. Copper transport systems in neuroblastoma cells: Regulation and physiopathological implications

Copper critically regulates the degree of neuroblastoma growth and microvascularization, which determines the tumour aggressive phenotype [19,20]. The importance of this metal is emphasized by the strong presence of specific transport proteins in neuroblastoma cells, that testifies to a lively management of tumour copper stores. Highly variegated mechanisms of regulation of copper homeostasis have been specifically reported for neuroblastoma (some of them reviewed here), that make it difficult to establish the nature of copper involvement: is the ion metabolic disruption a cause or an effect?

Copper import. It is widely believed that copper import in neuroblastoma cells is mediated by hCtr1 [21]. However, recent work from our laboratory in an *in vitro* neuroblastoma cell model has enlightened a role for the cellular prion protein PrP^C in mediating the high affinity copper intake, upon normal metal availability [22]. In addition, we demonstrated that copper shortness induces an up-regulation of PrP^C expression in a neuroblastoma cell model, a cell adaptive strategy aimed at restoring the standard copper status [23].

In support of its involvement in tumorigenesis, the PrP^C expression is up-regulated in nervous tissues affected by hypoxia, a condition typically occurring during the growth of a solid tumour [24]. The reader is referred to paragraphs 4.2 and 6.2 for a detailed account of the PrP^C functions in the tumour spread.

Copper efflux. The ATP7A copper ATPase (full length 170 KDa protein) is strongly expressed by neuroblastoma cell lines [21,23] and subjected to an articulated copper-dependent regulation.

In many cell types this efflux pump delivers copper to the secretory compartments and, when copper should accumulate inside the cytosol, it traffics toward the cell periphery to export the ion excess [25]. However, peculiar regulative mechanisms have been documented in neuroblastoma models.

In fact, it has been demonstrated in the M17 neuroblastoma cell line that fluctuating copper levels (excess/starvation) in the cell microenvironment can favour the interaction of ATP7A proteins with clusterins (apolipoprotein J), the last ones targeting the pumps toward degradation through the lysosomal pathway [26]. This copper-regulated clusterin function may have multiple implications, if we consider that a recent study on neuroblastoma cell lines, mouse

models, and human specimens evidenced that this molecular chaperon behaves as a tumour and metastasis suppressor, negatively regulated by *N-myc* in the most aggressive forms [27].

In our opinion, the copper-clusterin link deserves further exploration in the light of the reported elevation in copper neuroblastoma content observed in *N-myc* amplified tumours.

If *N-myc* really down-regulates clusterin (still controversial aspect), one would expect an increase in copper export function and so an overall reduction of the ion cancerogenic action. This evidently contradicts the *N-myc* - tumour malignancy binomial association (where copper should exert a prominent role) and minimizes the contribution of clusterin to the copper-dependent tumour progression. In fact, considering that *N-myc* elevates the neuroblastoma copper content, one can suppose that the cytosolic copper lowering due to a down-regulated expression of clusterin is overridden by other cell mechanisms causing the increase of cancer copper levels.

Copper uptake

Ctrl (Copper transporter 1). High-affinity Cu^+ importer, composed of three main domains: an extracellular N-terminal tail containing multiple copper-binding methionine residues; a trans-membrane segment consisting of three α -helical regions; an intracellular C-terminal domain. Three subunits assemble to form a homo-trimeric channel (9 Å pore diameter) within the plasma membrane (see [118] for a review).

Ctrl2 (Copper transporter 2). Copper permease, whose structure resembles that of Ctrl. Predominantly localized to endosomes and lysosomes, it seems to provide a mechanism of copper recycling from degraded cuproenzymes [119].

PrP^c (Cellular Prion protein). Endogenous copper-binding glycoprotein, mainly expressed in the central nervous system. The protein structure includes an unstructured N-terminal domain and a C-terminal globular region composed of three α -helices and two short beta-strands. When Cu^{2+} ions bind to the N-terminal octapeptide repeats (residues 51–90), the protein undergoes endocytosis, that providing a route for cell copper entry [58,59].

Cytosolic transport

CCS. Metallo chaperone required for copper delivery to Cu,Zn Superoxide dismutases 1; up-regulated in response to copper deficiency [120].

Cox17. Metallo chaperone delivering copper to Sco1 and Cox11 proteins in order to catalyse the cytochrome c oxidase copper loading [121].

Atox1. Metallo chaperone that delivers copper to ATP7A and ATP7B Cu^+ efflux pumps [122].

Metallothioneins. Small cysteine-rich proteins tightly binding copper ions and buffering the ion excess [123].

Copper efflux

ATP7A. Cu^+ -transporting P-type ATPase expressed by all cell types, with the exception of liver. Structural features include eight membrane-spanning domains and six N-terminal cysteine-rich metal binding motifs (MXCXXC) [25].

Box 1. Main proteins involved in cell copper homeostasis

Coming back to main focus of this paragraph, multiple ATP7A spliced variants can be retrieved in human cells, not necessarily related to disease states, with a cell type-specific expression pattern. The expression of a 11.2 KDa splicing product (103 amino acids) has been reported in SY5Y neuroblastoma cells, harbouring a sequence able to bind copper ions [28]. It has been proposed that such spliced product can work as a copper chaperon to direct the cytosolic copper toward the nuclear compartment.

Intracellular copper distribution. Among copper chaperons, the contribution of COMMD1 (Copper Metabolism MURR1 Domain containing 1) to the copper status in neuroblastoma cells is an unexplored issue so far. However, some inputs from the recent scientific literature let us hypothesize an involvement.

Endogenous COMMD1 expression has been reported in the SH-SY5Y neuroblastoma cell line, together with the isoform 3. A punctate cytoplasmic distribution, denser in the perinuclear region, has been shown for COMMD1, while COMMD3 appears more diffused [29]. The role of COMMD1 in neuroblastoma progression is potentially articulated on multiple levels of action, even if direct demonstrations are missing and the following dissertation aims at enlightening some aspects of copper-dependent regulation of the protein fate.

A role in preventing tumour growth and metastasis has been proposed for COMMD1, based on its ability to repress the NF-KB pathway and the HIF1 α / β dimerization and so inhibit the expression of genes involved in tumour angiogenesis [30]. However, as documented in N2a neuroblastoma cell line, upon copper excess, COMMD1 can form a hetero-complex with CCS and SOD1, leading to decreased levels of SOD1 dimers and subsequently reduced anti-oxidant activity [31]. In other words, in the presence of high copper, the COMMD1 cell fate can potentially assume a negative connotation.

COMMD1 is also an interacting partner of ATP7A proteins and, analogously to clusterin, can drive their degradation through a proteasomal pathway [32], this indicating a further contribution of this chaperon to the neuroblastoma copper content. However, knowledge about these aspects is still limited.

The COMMD1 involvement in determining the neuroblastoma copper condition is strictly linked to the protein XIAP (X-linked inhibitor of apoptosis). XIAP protective action is due to the prevention of the activation of a subset of cell death proteases (caspases 3, 7 and 9) [33,34], and inhibitor of Fas- [35] and Bax-induced apoptosis [33].

During the last decade, a role for XIAP in controlling the cell copper homeostasis has been described [36]. In fact, the overexpression of XIAP protein (not transcript) selectively reported in chemotherapy-resistant neuroblastomas, but no other tissues [37], may indicate the occurrence of a particular copper status. XIAP is a copper-binding protein that, in the olo-form, favours the ubiquitination and degradation of COMMD1, that in turn interacts with ATP7A to support copper excretion [36]. Where overexpressed, it is reasonable to presume a subsequent consistent reduction of COMMD1 cytosolic protein levels, and so an increase of the cellular copper content.

However, the binding of copper to XIAP negatively impacts the protein stability, so a negative feedback exists [38]. In the case of chemotherapy-resistant neuroblastomas, the protein overexpression probably overcomes the effects deriving from copper-driven XIAP inactivation.

Preclinical evidences of the importance of XIAP as a target to treat neuroblastoma have been recently collected, all based on the lowering of the threshold for the induction of apoptosis through the depression of XIAP expression. The use of Thymoquinone, a bioactive compound from *nigella sativa*, has been shown to selectively down-regulate XIAP in neuroblastoma cells, but not in normal neuronal cells, with an expected higher copper efflux [39]. Smac (Second mitochondria-derived activator of caspase) mimetics (e.g. LBW242) have been reported to sensitize chemotherapy resistant and XIAP-overexpressing neuroblastomas, by favouring the degradation of XIAP and TNF- α expression [37].

4. Copper-dependence of neuroblastoma metabolic changes

The oxygen partial pressure within a solid tumour ranges from 5-10 mmHg in highly vascularized regions to absence (anoxia) around the necrotic areas [40,41]. Most cancer cells tend to adapt to the intra-tumour hypoxic microenvironment by activating a pro-survival signalling, a pro-angiogenic pattern of gene expression and through the metabolic switching from the oxidative phosphorylation to the glycolytic pathways (Warburg effect) [42].

Currently, there is not a homogeneous view on the causative events, but two major factors are usually indicated as responsible, the Hypoxia-Inducible Factors 1 and 2 (HIF1,2), and p53 transcription factor.

HIF1 and 2 are heterodimeric basic helix-loop-helix-PAS domain transcription factors, composed of a constitutively expressed β subunit and a α regulatory subunit (HIF1 α /2 α), whose expression is induced by hypoxia, cancer-associated mutations, or inflammatory cytokines [43,44].

HIF-1 α and HIF-2 α are major actors in the cell adaptive response to hypoxic conditions and control the expression of distinct, but functionally converging genes [45]. Each cell type exhibits a peculiar profile of HIF-1 α and 2 α expression and their functions may also differ. In the case of *neuroblastoma*, at a careful analysis of the expression pattern, tumour stage, and copper status, it can be observed that copper heavily influences the response to hypoxia and that the tumour progression and the evolution of copper metabolism go hand in hand.

4.1. HIF-1 α

HIF-1 α , but not HIF-2 α , is preferentially expressed and up-regulated by moderate hypoxia in *N-myc* amplified neuroblastoma cell lines and primary tumours, correlating with a poor prognosis [41,46]. In the light of the linear increase of copper neuroblastoma levels with the degree of *N-myc* gene amplification and the proven role of Cu²⁺ ions in stabilizing the structure of the HIF-1 α subunit [47], it can be deduced that copper plays in key role in inducing the neuroblastoma metabolic changes.

Cu^{2+} ions determine the structural stabilization of the HIF-1 α subunit (oxygen-sensitive) through the inhibition of prolyl-4-hydroxylases, which allow the subsequent ubiquitination and degradation of such factor [47].

Interestingly, by this way Cu^{2+} indirectly promotes the synthesis of ceruloplasmin, a plasma and liquor copper chaperon with a ferroxidase activity, whose expression is typically under HIF-1 control [48]. Being ceruloplasmin a major copper vehicle, such mechanism can be interpreted as cancer “self-nourishing”. It must be added that HIF-1 target genes also include VEGF (Vascular Endothelial Growth Factor), a recognized chemotactic and mitogen factor [49], and VEGFR-1 (VEGF Receptor-1) [50], both involved in the positive regulation of the sprouting of blood vessels within the primary tumour.

Further, White et al. (2009) demonstrated that the up-regulation of the hypoxia inducible factor HIF-1 α causes the selective distribution of copper ions to the secretory pathway. They observed in tumour-associated macrophages that the hypoxic stress can influence the intracellular distribution of copper ions, determining an increased ion entry through the high affinity channel Ctr1 and then an elevated efflux through the ATP7A pump [51].

All these experimental evidences underline the prominent role of copper in sustaining the HIF-1 α -dependent adaptation to hypoxia in *N-myc* amplified neuroblastomas, as well as the hypoxia-stimulated activation of copper transport activities.

4.2. HIF-2 α

HIF-2 α , but not HIF-1 α , has been shown to be highly expressed in neuroblastoma vascularized areas, and this pattern seems to be associated with an unfavourable patient outcome, due to the occurrence of distal metastasis [41]. In addition, a small subset of neuroblastoma cells strongly HIF-2 α -positive has been described, which could represent the cancer stem cells [52]. To our knowledge, no precise data are available about the copper-dependent activity/activation of HIF-2 α , however some molecular evidences collected in other cell models strongly point at a potential existence of such a link.

As an example, Menkes copper ATPase (*Atp7a*) gene expression has been demonstrated to be strongly induced by HIF-2 α in mammalian intestine [53]. HIF-2 α has been also demonstrated to induce the expression of DMT1 and Ctr1 (by about 25%) copper importers in human intestinal cells, so determining a parallel increase (fivefold) in the processes of cellular copper uptake [54].

These findings confirm that, independently on the involvement of HIF-1 α or HIF-2 α , tumour hypoxia activates a series of processes functional to distribute copper toward the secretory pathway (enzyme-complexed) or make it available in the extracellular medium. Here, copper may function as a signalling molecule and sustain the angiogenic processes, essential to the neuroblastoma growth.

The scientific literature also suggests that the HIF-2 α prolonged response to hypoxia can be alternatively mediated by a high affinity copper-binding protein, namely the cellular prion protein PrP^C [55] (Box 1). Accordingly, the PrP^C expression degree is elevated in hypoxic

nervous tissues [56], and its overexpression has been shown to confer a highly invasive phenotype to tumour cells [55,57].

By virtue of a direct involvement of PrP^C in the cell copper import [22,58,59], an elevated protein expression under hypoxia could represent a cancer cell strategy to assure the neuroblastoma growth through the enhanced copper intake [23]. In fact, copper stimulates neuroblastoma cell proliferation [60].

Interestingly, although it has been demonstrated that the up-regulation of PrP^C in human colorectal carcinoma cells induces the glucose transporter-1 (Glut-1) expression and a subsequent increase in the glycolytic rate via Fyn-HIF-2 α pathway [55], the transfection of a plasmid expressing wild-type HIF-2 α in *N-myc* amplified neuroblastoma cells has been demonstrated to be marginally involved in the regulation of glycolytic genes [46]. Surprisingly, notwithstanding a rise in Glut-1 expression, the glucose influx was not increased [46].

Conclusively, to reinforce the concept of an autonomous cancerogenic role of copper, it can be observed that elevated HIF levels have been observed even under normoxic conditions, meaning that other factors than hypoxia, e.g. copper, can sustain the aerobic glycolysis and induce the expression of HIF-targeted genes.

4.3. p53

p53 transcription factor is a key tumour suppressor protein, whose functions contribute to prevent cancer progression. Mutated p53 gene products or defects in the integration of proteins with which p53 is connected, are associated with the malignant progression of the majority of human tumours [61]. Neuroblastoma rarely shows mutated p53 at diagnosis, thus therapies result effective at first. However, gene mutations, p53 cytosolic sequestration, or deregulated p53/MDM2 (ubiquitin protein ligase -E3- for p53) pathways have been reported during neuroblastoma relapses or therapies, thus conferring high-level multidrug resistance [62-65].

Loss of p53 function seems to impair the efficiency of mitochondrial respiration by hampering the insertion of copper ions as cofactors into the cytochrome c oxidase enzymatic complex [66]. That would cause the switching from cell respiration to *aerobic glycolysis* (Warburg effect), typical metabolic change observed in cancer cells.

In detail, p53 directly regulates the expression of the SCO2 (Synthesis of Cytochrome c Oxidase) gene, coding for a protein that facilitates the copper delivery to the subunit II of cytochrome c oxidase, determining the assembly of the enzymatic complex [66].

As suggested in [67], given the essential role of copper in determining the Warburg effect in cancer cells, it cannot be excluded that deregulated p53 pathways may affect the expression or function of other proteins involved in cell copper acquisition and utilization.

5. Copper promotes the neuroblastoma survival and growth by sustaining the anti-oxidant enzyme activities

Cutting copper supply can represent a valuable therapeutic strategy for neuroblastoma, as the induced mitochondrial impairment and oxidative stress can make neuroblastoma cells vulnerable. Accordingly, even under unstressed environment, mitochondria in this cell type exhibit a high rate of protein oxidation, this indicating a consistent susceptibility to the oxidative injury [68,69]. The positive connotation of a drop in the neuroblastoma cell copper content has been demonstrated and emphasized by a rich literature showing that copper chelation (triethylene tetramine tetrahydrochloride) can effectively promote the apoptosis of neuroblastoma cells [70,71].

Here follow some argumentations from the literature around the negative impact of copper starvation on neuroblastoma cell survival, extrapolated from *in vitro* preclinical studies.

SH-SY5Y neuroblastoma cells have been widely used as a model to dissect the molecular basis of the tumour sensitivity to copper.

In particular, the continuous exposure (up to three passages) of SH-SY5Y neuroblastoma cells to the copper-chelating agent Trien has been demonstrated to induce the expression of antioxidants and a 40% apoptotic cell loss at the end of the third passage [70]. Copper has been shown to be important in keeping a critical level of ATP. In fact, the relevant Cu,Zn SOD and cytochrome c oxidase activities were reduced by, respectively, 80 and 68% [70]. Another report has confirmed these findings, indicating that copper starvation by Trien impairs the antioxidant defences of neuroblastoma cells, with obvious implications with respect to the therapeutic inhibition of the tumour growth [71].

Arciello et al. (2011) further characterized the effects of Trien treatment in SH-SY5Y neuroblastoma cells [72]. SOD1 (cuproenzyme) expression decline was associated with a reduction of the enzyme activity, mainly due to copper shortness rather than to a decreased protein expression. In fact, copper replenishment was able to reactivate the apo-form of the enzyme, in agreement with previous observations [73]. Copper depletion also favoured the entrance of the SOD1 apo-form (not metallated) into the mitochondria [72], where it was retained due to a partial unfolded and obviously inactive configuration. The authors also observed an increased expression of CCS (Box 1), finalized to optimize the copper intracellular distribution [72].

In the light of these findings, it can be observed that the neuroblastoma commitment to the apoptotic death was not due to an irreversible mitochondrial damage, even considering that the loss of the mitochondria-associated SOD1 was much less evident than observed for the cytosolic one [72]. However, it is plausible that the absence of copper prevented SOD1 from counteracting the oxidative-mediated damage to mitochondrial proteins [74]. Accordingly, it has been shown that brain tissues exhibit a SOD1 localization inside the mitochondrial matrix with an antioxidant function [75].

In our laboratory we analysed the anti-oxidant response to copper starvation in a rat neuroblastoma model (B104), investigating in parallel the expression of copper membrane trans-

porters [23]. A significant increase of caspase-3 activity was detected in copper-starved cells, indicating the activation of a cell death program through the induction of oxidative stress. In agreement, the total Cu,Zn SOD activity resulted half-reduced with respect to normal conditions, as expected in consideration of the role of copper as a cofactor [23]. Interestingly, the cellular prion protein expression in copper-starved neuroblastoma cells was heavily induced. This finding was reconsidered in the light of a rich literature showing that the ^{64}Cu loading and the enzymatic activity of Cu,Zn SOD from the brain of *P-rnp*^{0/0} mice result 10-50% reduced with respect to the wild-type genotype [76-78].

A special attention has been dedicated to the adaptive response actuated by PrP^C, that is physiologically and consistently localized on the outer surface of neurons at synapses and gliocytes [79,80]. Under normal conditions, PrP^C binds copper ions with high specificity and affinity (femto- to nanomolar range), by the repeated sequences present on its N-terminal region. By virtue of this property and the ability to undergo endocytosis upon copper binding, PrP^C is believed to drive the cellular copper intake [22,58,59].

The up-regulation of PrP^C upon copper limitation has been interpreted as a compensatory mechanism to re-establish the standard cell copper status through a direct transport activity. It has been also demonstrated to be responsible for the ability of copper-starved cells to almost completely recover the SOD enzyme function upon re-exposure to standard growth conditions. The authors conclusively demonstrated that the PrP^C neuroprotective action in neuroblastoma cells is due to its ability to translocate copper ions into the cytosol. Here, they can act as cofactors in Cu,Zn SOD activation [23].

6. Critical role of copper transporters in neuroblastoma vascularization and spread

Most pro-angiogenic factors implicated in neuroblastoma progression need copper to properly work or exert their own functions by activating copper-dependent pathways and enzymes.

The best known pro-angiogenic mediator, namely the Vascular Endothelial Growth Factor (VEGF), has been demonstrated to be overexpressed in high-risk neuroblastomas at the time of diagnosis and to be a bad prognostic marker [81]. The elevated copper levels detected in malignant neuroblastoma are expected to heavily sustain the VEGF tumour angiogenesis, since this metal is a potent inducer of VEGF expression and reinforces the stimulating effect exerted by hydrogen peroxide [82].

The growth of neuroblastoma is anyway sustained by multiple pro-angiogenic factors other than VEGF [10], including Platelet Derived Growth Factor-A (PDGF-A), Fibroblast Growth Factor-2 (FGF-2), and Angiopoietin-2 (Ang-2), as documented in 22 neuroblastoma cell lines and 37 tumour samples [10]. Many among these factors share an intimate relationship with copper, known to variously enhance their angiogenic action through direct (physical interaction) or indirect (expression/release) ways.

As an example, the specific binding of copper to angiogenin, a major angiogenic factor, is able to largely increase its efficiency of interaction with endothelial cells [83,84]. This metal is also fundamental for the release of another pro-angiogenic factor involved in angiogenesis, Fibroblast Growth Factor (FGF) 1, as a part of a multiprotein aggregate (FGF1-p40 Syt1-S100A13) [85].

If on one hand high copper levels can facilitate the tumour development, on the other the stimulation of copper uptake and egress has been associated with the sprouting of new blood vessels within solid tumours, this depicting a high complex picture. A prominent role of copper transport systems emerges.

6.1. Potential role of ATP7A and Ctr1 copper transporters

Several experimental evidences point to a crucial role of copper in tumour angiogenesis [86]. Its ability to stimulate the endothelial cell proliferation, migration and sprouting mainly grounds on its role as a powerful inducer/enhancer of the expression of several angiogenic mediators, including VEGF₁₆₅ and interleukins [82,87], and a stabilizer of the angiogenin interaction with its receptor [83]. Surprisingly, well-characterized pro-angiogenic factors as VEGF₁₆₅ and bFGF, if administered to microvascular endothelial cell cultures, have been shown to rapidly promote the relocalization of the intracellular copper stores (about 80-90%) toward the cell periphery, where the ion efflux occurs, presumably by the ATP7A transport activity [88]. Such process may result contradictory in the light of the discussed role of copper as a powerful pro-angiogenic mediator. Nevertheless, this mechanism may be considered "cancer self-sustaining", making copper available in the tumour microenvironment (paracrine loop).

In addition, it must be observed that the vascular remodelling and the stimulation of cell migration depend on the activity of copper-dependent secreted enzymes (Lysyl Oxidase, LOX), so the released metal is probably mostly carried by proteins.

In support of such hypothesis, a report from Ashino et al. (2010) illustrated how the pro-angiogenic Platelet Derived Growth Factor (PDGF) determines in vascular smooth muscle cells the translocation of the ATP7A copper transporter from the Trans Golgi Network toward special membrane domains (lipid rafts), where the pump is essential for the correct release of copper bound pro-LOX [89]. The authors also demonstrated that the membrane recruitment of Rac-1, a GTPase involved in the extension of lamellipodia, is dependent on copper and on the expression of the high affinity importer Ctr1 (Copper Transporter 1), this further confirming the existence of a solid link between the tumour metastasis and copper homeostasis.

6.2. Potential role of the cellular prion protein PrP^C

To our knowledge, a few data are reported in the literature around the prion protein role in defining the neuroblastoma aggressiveness. Nevertheless, the substantial expression level observed within the nervous system, which is further elevated by pathological conditions, testifies to a possible involvement of prion protein in the nervous response to cell injuries. In detail, this particular protein may have major implications in modulating the biological

cascade leading to metastasis in patients with cancer, mainly by virtue of its presumed ability to sustain cell survival and exert a pro-angiogenic action.

A modest literature discusses a likely role of prion proteins in influencing the angiogenic processes, given a large disagreement about its actual expression in endothelial cells. In fact, although prion protein has been detected in the capillaries of the intestinal mucosa and kidney [90], normal endothelial cells derived from the umbilical cord and other vessels in the adults do not show detectable prion protein amounts *in vivo* [91]. However, prion protein seems to be up regulated in some pathological circumstances, such as in advanced carotid plaques, in association with the endothelial marker CD105, increasingly expressed in activated endothelia [92], and in brain tissues affected by ischemia [93,94]. By virtue of the latter studies, prion protein could reasonably play a key role in brain tumour progression, being the related gene responsive to the ischemic/hypoxic injury [94]. Accordingly, a neuroprotective action has been described for prion proteins in this context, based on the following evidences: i. prion protein is bound to caveolin-1 and, by recruiting Fyn tyrosine kinase, it can activate the signalling promoting cell survival and angiogenesis events [95]; ii. prion protein co-localizes with the VEGF receptor 2 (KDR), that indicating that prion protein may have a role in VEGF-driven angiogenesis [96].

7. Anti-angiogenic therapies target the neuroblastoma copper status: two examples

7.1. TNP-470

The administration of angiogenic inhibitors has been introduced as a complement to traditional therapies, in order to hinder the tumour spread.

Several anti-angiogenic therapeutics have been incorporated into clinical trials. Among them, in the '90s, TNP-470, an angiogenesis inhibitor, has emerged as a promising adjuvant in dormancy therapies for high-risk neuroblastoma. In particular, its effectiveness in arresting hepatic metastasis of neuroblastoma has been documented in [97] and [98]. In the light of [99], the anti-angiogenic activity of TNP-470 is reasonably linked to its interference with the hepatic copper metabolism. In fact, the continuous administration of TNP-470 in both normal and tumour-bearing rats has been shown to increase the serum copper levels, as a consequence of a limited hepatic retention [99]. This feature has been associated with a reduced density of hepatic tumour capillaries [99]. Accordingly, when the administration of TNP-470 was interrupted, angiogenesis was activated and at the same time the serum copper levels fell down [99].

7.2. Retinoids target the ATP7A gene expression

Among the most promising possibilities, retinoids (Vitamin A derivatives) may be of help in arresting the cancer growth and delaying the occurrence of recurrences, because of their proven ability to induce cell differentiation and inhibit the VEGF and FGF-2-induced endothelial activation [100]. Interestingly, a recent report from Bohlken et al. (2009) demonstrated

that retinoids are able to starve neuroblastoma cells of copper through a significant increase in the ion efflux processes [60]. In fact, the retinoic acid receptor β (RAR β) up-regulates the expression of ATP7A copper efflux pump in BE(2)-C and SH-SY5Y human neuroblastoma cell models, but not in other cell types.

8. Cell copper transporters modulate the neuroblastoma sensitivity to chemotherapy

Cisplatin-based chemotherapy is commonly employed for neuroblastoma treatment at an advanced stage [101], but the development of resistance to the drug can affect the therapeutic efficacy. Highly diversified mechanisms have been proposed to explain this behaviour, although a definitive understanding has not been achieved. It has been demonstrated that Cisplatin-resistant neuroblastoma cells undergo an increase in the DNA methyltransferase activities that would depress the transcription of specific and widely undefined genes [102]. In fact, it is known that an acute Cisplatin administration can alter the genome methylation status in neuroblastoma cells [103].

Increasing evidences point out a central role of (broad substrate spectrum/specific) drug transporters to explain the onset of Cisplatin resistance. In detail, Haber et al. (1999) observed that malignant neuroblastoma forms, carrying the *N-myc* oncogene amplification, show an up regulation of the Multidrug Resistance-associated Protein (MRP) gene, associated with a poor sensitivity to low affinity substrates, including Cisplatin [104].

Interestingly, it has been widely demonstrated that Cisplatin shares with copper the pathways of cellular efflux and entry [105,106]. In particular, the cellular uptake of cisplatin (water soluble) is mediated by a member of the SLC (Solute Carrier) group, namely the copper transporter 1 (SLC31A1) [105-107], by mechanisms that partially overlap with those copper-specific [105,108]. Candidate Cisplatin-binding sequences have been identified in the extracellular region of hCtr1, this providing further evidence of the Cisplatin transport activity by this channel [109].

Further, the copper efflux transporters, ATP7A and ATP7B, are known to regulate the efflux of cisplatin, and so their expression may be also predictive of drug sensitivity [110].

Neuroblastoma cells are known to express both hCtr1 import and ATP7A export proteins, this suggesting that copper transport systems may participate in determining the development of cisplatin resistance. In support of such hypothesis, a recent study on microRNAs expression pattern in variously *N-myc* amplified and cisplatin resistant neuroblastoma cells, led to the identification of eight microRNAs, each one targeting at least one of the two cited copper transporters [111]. Furthermore, it has been demonstrated that ATP7A expression may be a target to sensitize cancer cells to Cisplatin [112].

In the light of these findings, it has been argued that an increased cisplatin sensitivity may arise from the upregulation of Ctr1 transporter or by downregulation of the copper/cisplatin efflux transporter ATP7A. In this sense, a therapeutic regimen combining a preconditioning

by a copper chelating agent (i.e. Tetrathiomolybdate) and platinum-containing drugs has been proven to enhance the Cisplatin efficacy in a mouse model of cervical cancer, without affecting the integrity of healthy tissues [113].

Another copper-dependent mechanism of resistance to cisplatin involves metallothioneins, a family of low molecular weight copper-binding proteins, whose expression is metal-induced in neuroblastoma cell models [114] and elevated in cisplatin-resistant cell lines [115]. When cisplatin enters a cancer cell, it is vulnerable to metallothionein-inactivation [116]. This mechanism assumes a priority connotation if we consider that *N-myc* amplified neuroblastomas show an increased copper content, that translates in a remarkable induction of metallothioneins and reduced efficacy of Cisplatin-based therapies.

9. Conclusion

Multifaceted pathophysiological features determine the progression of neuroblastoma malignancies. Mainly on the basis of *in vitro* and pre-clinical studies, copper, playing a key role within the human nervous system, is candidate to be the actual target of novel therapies. Accordingly, high copper levels seem to underline the development of tumour malignancies, even if we honestly observe that the scientific literature does not offer so many clear cues about the nature of *in vivo* copper involvement in neuroblastoma. The conclusive impression is that copper interacts with the neuroblastoma microenvironment at various levels, and the effects may be profoundly different, depending on the interested cell type (e.g. endothelial, neuroblast). The overall effects arise from the sum of specific and sometimes discordant copper-driven processes.

If few clinical data are currently available in this regard, the challenge toward the development of a copper-targeting therapy has anyway been launched. On the other hand, recent studies have recognized for neuroblastoma patients the benefits of preconditioning therapies based on the use of copper chelating agents (i.e. tetrathiomolibdate). Such intriguing approach would modulate the expression and/or subcellular localization of copper transport systems, and so both the cancer metal levels and chemoresistance. However, caution is needed in this sense, since the comprehension of copper metabolism in neuroblastoma cancer cells is still preliminary and the routes of copper transport are currently partially known. Significantly, it is only recently that an anion exchanger has been proposed as an additional copper importer in mammalian cells [117].

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