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Review

FROM PHYSIOLOGICAL TO NEOPLASTIC TRANSFORMATION: THE CRITICAL ROLES OF CONNEXINS AND WT1

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ABSTRACT

Glioblastoma and soft tissue sarcomas are tumors characterized by poor prognosis, low overall survival and critical hallmarks that allow such tumors to evade homeostatic controlling mechanisms. Herein, we revised literature on connexins, the core gap junction forming proteins, and WT1, a transcription factor involved tumor progression, to highlight the close connection between intercellular communication deregulation and aberrant intracellular signalling. Both connexins and WT1 have been widely studied during physiological conditions and development and they hold a critical role in recapitulating such phenomena during tumorigenesis in glioblastoma and soft tissue sarcomas.

1. Introduction

Physiological processes regulating ontogeny and physiological development are finely guided by molecular mechanisms involving multiple genes. Alteration of these processes leads to severe disfunction and morphological aberrations and, in adult life, they may trigger pathophysiological mechanisms resulting in the promotion of diseases such as cancer.

Intercellular communication disfunction and Wilms Tumor 1 (WT1) are ideal biomarkers involved in ontogenetic processes whose deregulation is associated with tumorigenesis. Several studies have been performed to understand their physiological and pathophysiological role, but their correlation has not been clarified yet.

Cell-to-cell communication is essential for the maintenance of cell homeostasis, allowing molecular exchanges between extracellular, intracellular and intercellular networks (1). In this context, gap junctions (GJs) exert a key role in the development and maintenance of cells physiological conditions, by forming intercellular channels of communication that allow direct cellular interactions (2).

Disruption of the balance mediated by GJs allows cells to evade homeostatic controlling mechanisms, leading to a number of degenerations and even cancer promotion (3). Connexins (Cxs), GJs, and hemichannels (HCs) in the central nervous system (CNS) are critical for their roles in homeostatic glia/neuron activities and for their alteration in different neurological disorders (4). Alterations of Cxs based communication are involved in the development of neurodegenerative diseases, such as amyotrophic lateral sclerosis (ALS), a multi-system disease, leading to motoneuronal loss (5), Alzheimer's Disease (AD), the most common cause of dementia, especially in women (6, 7) and Parkinson's Disease (PD), a progressive disorder characterized by loss of dopaminergic neurons (8). In addition to neurogenerative diseases and neuroinflammation, Cxs have been found deregulated in many tumors, in which they may either promote cancer progression and metastasis or act as tumor suppressors, in relation to Cx isoform, cancer type and progression stage (9). Indeed, intercellular crosstalk mediated by GJs regulates cancer development and progression in different types of tumors, including glioblastoma (GBM), bone and soft tissue sarcomas (8). GBM is considered the most aggressive brain tumor, with a poor prognosis and a median overall survival of 15-18 months.

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The GJs-forming protein connexin 43 (Cx43) plays a critical roles in the genesis, progression and proliferation of malignant gliomas, capable of conferring chemotherapeutic resistance to GBM cells (10). GJs are also involved in both primary and secondary bone and soft tissue sarcomas, a rare and heterogeneous family of cancers derived from mesenchymal lineage (11-14). Particularly, Cx43 has been related with Epithelial Mesenchymal Transition (EMT) induction, implicated in carcinogenesis, invasion and metastasis processes (12). Furthermore, in this context Cx43 has been identified as a tumor suppressor in osteosarcoma and Ewing sarcoma, given its involvement in proliferation and progression of cell cycle. Indeed, upregulation of Cx43 leads to a repression of proliferation, cell cycle blockage *in vitro* and tumor growth reduction in preclinical mouse models. Vice versa, the ablation of Cx43 is related to promotion of proliferation *in vitro* (15, 16).

A number of evidences confirm that tumorigenesis recapitulates developmental processes. On this aspect, transcription factor WT1, a zinc-finger transcription factor, which regulates the expression of several genes involved in cell proliferation, differentiation and apoptotic processes, has been described during neurogenesis and foetus development and holds a controversial role in tumor context. Embryonic progenitor and stem cells regulate their differentiation in response to specific stimuli coordinating the development of organs (17). Several findings identified WT1 as a key biomarker involved in both physiological differentiation and tumorigenesis (18).

Being correlated in hematopoietic progenitors differentiation, WT1 deregulation has been strongly connected to hematologic malignancies including various types of leukemia (19).

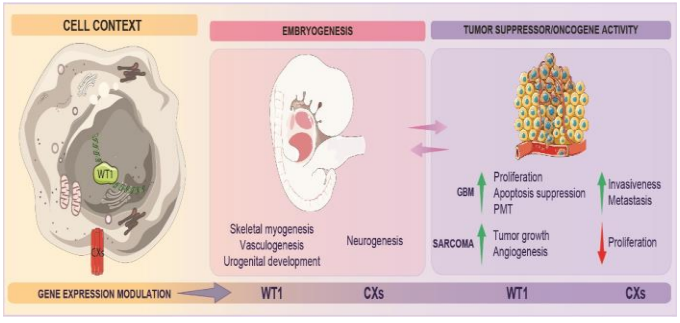

Figure 1. In physiological conditions, WT1 represents an essential transcription factor involved in embryonic development and tissue differentiation. In particular, WT1 plays a crucial function in skeletal myogenesis, vasculogenesis and urogenital development. Cxs exert a critical role in cell-to-cell and extracellular communication. Focusing on embryogenesis, Cxs represent a key element in neurogenesis and CNS differentiation. These performed functions are altered in a neoplastic scenario. WT1 induces whether an increase of proliferation or apoptosis suppression and promotes pro-neural to mesenchymal transition in GBM. Moreover, WT1 enhances tumor progression and angiogenesis in bone and soft tissue sarcomas. Upregulated Cxs act as a suppressor of proliferation in sarcomas, instead promote invasiveness and metastasis formation in GBM.

As per Cxs, also WT1 has been found highly deregulated in brain tumors, including GBM, and in a number of sarcomas subtypes (20). The 70% of soft tissue sarcomas are WT1 immuno-positive, which is mainly localized in the cytoplasm.

Such a mislocalization might also have prognostic relevance in high grade soft tissue sarcomas (21, 22). Herein we propose a systematic review of current knowledge on Cxs and WT1 role in physiological condition and in tumorigenesis and whether an interplay between Cxs and WT1 may represents a biological substrate for cell fate regulation and disease progression (Figure 1).

2. Gap junctions, hemichannels and connexins

Intercellular communication is largely mediated by GJs, which are group of intercellular channels that directly connect the cytoplasm of neighbouring cells, coupling them electrically and/or metabolically (23). A number of aspects ranging from cell growth to differentiation, signalling and death, are driven by GJs-transferred molecules (24). GJs form cell-to-cell junctions enabling the exchanges of small hydrophilic molecules, such as glucose, glutamate, lactate, and several second intracellular messengers including adenosine triphosphate (ATP), cyclic adenosine monophosphate (cAMP), inositol triphosphate (IP3) and ions, such as calcium, sodium and potassium (24-26).

GJs are composed of two docked HCs, also known as connexons, comprise of 6 subunits of Cxs, forming an annular structure which surrounds the aqueous central pore between adjacent cells. They typically arranged in arrays, known as plaques, in the sites of cell-cell contact where intercellular communication occurs (27).

Cxs structures are composed by 4 trans-membranes domains, exposing both amino- and carboxy-terminal domains to the cytoplasm and connecting them with two extracellular and one intracellular loops (28, 29). Cxs are encoded by 21 genes in human and named according to their molecular weight, ranging from 26 to 56 kDa (3). The structural features of HCs allow the discrimination between the homotypic GJs, consisting of two HCs that contain the same type of Cxs, and the heterotypic GJs, consisting of two HCs that have different Cxs. In contrast, heteromeric HCs are composed by different subunits of Cxs within the same HC (30, 31). Cxs intracellular domains can interact with protein components of the cytoskeleton (such as tubulin) and proteins involved in cell signalling pathways (such as E-cyclin, β-catenin). Thus, Cxs can modulate cell growth, differentiation, migration, and other cellular processes also in a channel-independent ways via protein-to-protein interactions (32). Among the Cx family members, the cytoplasmic tail length and amino acid sequence are variable. In many Cxs, this domain is frequently phosphorylated, and it is implicated in the bindings of Cxs interactome (33).

All Cxs contain a short (≤ 24 amino acids) cytoplasmic N-terminal domain, which plays a role in regulating the channel opening (34), while the HC docking between neighbouring cells is regulated by the two conserved disulfide-linked extracellular loops (35). The cytoplasmic loop size differs significantly amongst Cxs subtypes and has been demonstrated to participate in pH-sensitive gating of the channel (29). As such, Cxs composition defines permeability and signalling properties of GJs and HCs (3, 28).

GJs are widely expressed in the CNS cell populations (36, 37). Cx43, which is the most represented Cx in mammals, is also widely expressed in glial cells in all the stages of neurogenesis and in adult brain, with a great impact in homeostasis maintenance (38). Cx43 allows to create an intercellular network in CNS, in fact Cx43-based GJs allows astrocytes coupling but also mediates contact between astrocytes and microglial cells. Reactive astrocytes express higher levels of Cx43, and it has been observed that in acute and chronic degenerative diseases Cx43-mediated intercellular communication is increased (24). Indeed, Cx43 altered expression has been correlated with neurodegenerative processes (3). In experimental models of motoneuronal depletion, it has been observed a modified glial activity with reactive Cx43 expression (39, 40); motoneuronal loss and alterations of glial cells are critical hallmarks of ALS, underlining the important role of Cxs either in neurodegenerative diseases or in compensatory mechanism (5). In the spinal cord, Cx43 expression is related to inflammation and apoptotic signalling, indeed it has been found to be increased in acute and chronic injuries. In particular, Cx43-based channels inhibition reduces secondary damages in acute and chronic disorders, whether Cx43 upregulation in spinal cord astrocytes supports the late-phase neuropathic pain (41). Several areas of the CNS highly express Cx43, including the deep mesencephalic nucleus, interpeduncular nucleus, central gray dorsal nuclei, and pontine nuclei (42), particularly in the pontine nuclei basilar and in the reticular nucleus tegmenti pontis, which are at the origin of the afferent input to the mossy fiber to the cerebellum (43, 44). Besides its role in the formation and development of the cerebellum (45), Cx43 is also highly expressed in Bergmann glial cells (BGCs), but Cx43-mediated GJs are not needed for the neuron-glia connections involved in cerebellum-dependent motor coordination and motor learning (46). In adults, Cx57 is also highly expressed in the cerebellum, particularly in Purkinje cells and cerebellar nuclei, but also in pre-cerebellar nuclei, such as the lateral olivary and reticular nuclei, including the lateral reticular nucleus, that is one of the main pre-cerebellar nuclei (47, 48). Cx36-formed GJs are typical of neuronal coupling and have not been observed between glial cells. Other Cxs have also been reported in neurons, such as Cx45 and Cx62, but they have lower levels and less importance as compared to Cx36 (49, 50).

3. Gap junctions, hemichannels and connexins in glioblastoma

Cxs, in addition to brain disorders and neurodegenerative diseases, have been also linked to tumorigenesis with context-dependent roles. Indeed, it has been shown that Cxs are able to suppress the growth of tumors, but they can also increase tumorigenicity by stimulating the growth, migration and invasiveness of tumoral cells (9, 51).

Recent studies demonstrated that the expression of Cxs promotes malignancy of tumors in specific conditions. Some Cxs could promote invasion and metastasis of cancers in advanced stages. Indeed, Cxs in tumors can be involved in the alteration of intracellular communication, modifying signalling pathways or modulating cells via autocrine and paracrine mechanisms (52).

Moreover, it has been showed that metastatic brain tumor cells form GJs with astrocytes, promoting tumor growth and resistance to chemotherapy (53).

Given the importance of GJs-mediated intercellular communication in homeostasis maintenance, inherited or acquired alterations in Cxs-based channels has been linked to tumorigenesis and cancer progression, including GBM (41, 54).

GBM is the most aggressive and common brain cancer, primarily affecting the adult population, and classified as a grade IV glioma according to the World and Health Organization (WHO) (55). GBM accounts for about 15% of all brain tumors, with 17’000 new diagnoses every year, and a higher prevalence in men than in women. A higher incidence of GBM has been reported in Asia, followed by European population (56). Despite recent advances in prognostic and predictive biomarkers, GBM survival remains low: few patients survive for 2.5 years, and less than 5% survive for 5 years after diagnosis (56, 57). Although genetic and environmental factors have been studied, the aetiology of GBM is not completely clear (58). According to the WHO classification, GBM can be distinguished in relation to the status of isocitrate dehydrogenase gene (IDH) in GBM, IDH-wild type (approximately 90% of all cases) and GBM, IDH-mutant (59).

Necrotic areas with surrounding cellular pseudopalisades and microvascular hyperplasia are pathological features of GBM that are thought to play a major role in the rapid growth and invasion of GBM. Low tumor oxygenation, also known as hypoxia, is a major issue for GBM patients promoting tumor cell invasion into healthy brain tissue to escape this hostile environment (59, 60).

Despite recent evidence expanding the current knowledge of GBM, the therapeutic approaches for newly diagnosed cases remain limited to surgical resection followed by standard protocols of chemotherapy (i.e., temozolomide, TMZ), and radiotherapy (61). However, severe side effects highlight the need to develop new adjuvant treatments aiming at reducing off-target damages.

Intercellular communication in GBM represents an active research field: healthy and cancerous cells need to communicate with each other in order to proliferate and drive tumor growth.

Controlling Cxs expression and activity means induce changes in microenvironment composition and intercellular signalling, which are responsible for stress resistance and tumor progression. Some evidence suggests that reduced expression of Cxs, which has been hypothesized to play a regulatory role in GBM development, may facilitate the acquisition of a malignant GBM phenotype (62). Cxs profile, glioma subtype, cancer stem cells, differentiation and tumor malignancy are all factors that influence Cxs activity in glial cells during neoplastic disease (63). The role of Cx43, the most abundant astrocytic GJ protein, in GBM is controversial, making difficult the development of Cx43-based therapies. Cx43 could prevent glioma formation and, therefore, it could be considered a tumor suppressor.

Cx43 was discovered to have a tumor suppressive function in a rat C6 malignant glioma model, where over-expression of Cx43 in C6 cells enhanced GJs function and significantly suppressed cell growth and tumor formation in rats (64, 65).

Indeed, the reduction of GJ-mediated intercellular communication represents a hallmark of cancer (66). However, Cx43 can increase the migration of some tumors depending on the cell type, instead of blocking their proliferation (67).

Cx43 overexpression has been shown to promote migration in malignant gliomas in a channel-dependent manner, particularly in the presence of normal stromal cells such as astrocytes.

Indeed, it has recently been demonstrated that Cx43 forms a channel between tumor cells and astrocytes that allows the exchange of microRNAs (miRNAs), small, non-coding RNA molecules that frequently regulate multiple protein targets, reprogramming normal stromal cells. Indeed, miR-5096 can increase the invasiveness of malignant glioma cells in a Cx43-dependent manner by reprogramming astrocytes to alter the tumor microenvironment.

According to recent research, astrocytic Cx43 may also contribute to TMZ resistance, enhancing GBM cell proliferation and migration (10, 68). There are numerous controversial theories regarding the role of Cxs and Cx43 in cancer. More in-depth and large-scale studies are needed to overcome these contradictions. In any case, it is critical to determine whether Cxs targeting could be used to treat cancer, and thus GBM (69).

4. Gap junctions, hemichannels and connexins in soft tissue sarcoma

Sarcomas are mesenchymal-derived tumors, accounting for less than 1% of adult malignant tumors (70). Sarcomas are ranked into more than 100 different malignant subtypes, with many histopathological differences (71). The heterogeneous characteristics of sarcomas are due to the undefined origin of the disease. Indeed, sarcomas develop from tissue of mesenchymal lineage, including muscle, blood vessels, nervous and connective tissue, such as bone, adipose and cartilage tissues (11, 13, 14). The onset age is also very different among the various subtypes. Chondrosarcoma, characterized by hyaline cartilaginous neoplastic tissue, shows a predominance in adults, while the rhabdomyosarcoma, a muscle derived sarcoma, is typical of pediatric age (72, 73). The rhabdomyosarcoma represents approximately the 7% of the pediatric sarcomas and shows a marked aggressivity, invasiveness and a prominent inclination to form metastasis (74-76). Instead, Ewing sarcoma, a bone-derived tumor presents important chromosomal translocations, leading to the formation of fused oncogenes and aberrant epigenetic regulation (14, 71). Osteosarcoma is the most common primary bone tumor, whose onset is related to p53 and Rb1 mutations in osteoblasts (77-79).

The gold standard for the treatment of localized soft tissue sarcomas includes surgical resection associated with radio and chemotherapy (72, 80). The chemotherapy approach has improved the outcome of sarcomas, increasing the five-year survival rate to 60-80%, against the 20-40% of the past (81).

In the recent years, some studies have been carried out to improve the current available therapies. Among them, promising results were found in osteosarcoma cell line treated with Bortezomib, a proteasome inhibitor molecule emerged for the treatment of multiple myeloma (77, 82, 83). However, Bortezomib showed minimal activity in the treatment of metastatic sarcomas (77).

On the contrary, better results have achieved with Ixazomib, a second-generation proteasome inhibitor, that has been investigated for its suppressor ability and promotion of osteoclastogenesis and osteoblastogenesis, respectively. Ixazomib, already accepted for the multiple myeloma treatment, reports an enhanced penetration in osteosarcoma cells in vitro, compared to Bortezomib (77, 84). Although more studies are necessary to improve the in vivo data, Ixazomib shows a marked anticancer activity also in osteosarcoma metastases (77).

Investigating the genesis of metastases, Gjs emerge for their important role in EMT.

Indeed, EMT consists in molecular and cellular alterations, including the reduction of intercellular junction constituents, which results in loss of cell-to-cell communication (12). The implication in cell-cell interactions make Gjs alterations relevant for many other processes. In co-coltures of bone marrow-derived cells, it has been observed the involvement of Cx43 in osteoclasting-supporting activity of osteoblasts. Indeed, the Cx43 ablation shows an increase of osteoclastogenesis, led by prostaglandin E2 (PGE2) (85). In particular, in a Cx43-null mouse model, Cx43 plays a crucial role in signalling transmission among osteoblasts and osteocytes, regulating the modelling, remodelling, development and differentiation of the tissue (16, 86). Moreover, the involvement of Cx43 in pre-osteoblasts terminal differentiation into mature osteoblasts makes the Cx43 a potential prognostic biomarker in different bone sarcomas (16, 86). Several studies have been carried out to examine the connection between Cx43 and carcinogenesis in bone and soft tissues sarcomas. As reported by Fukuda et al., GJs play an essential role in primary and secondary bone tumors growth and soft tissue sarcomas (12, 16), where GJs signalling is implicated in cell proliferation and apoptosis in chemo resistance (87).

Regarding primary bone tumors, Cx43 plays a tumor suppressor function in Osteosarcoma and Ewing Sarcoma, two of the most frequent sarcomas studied in children and young adults (16, 78). In Osteosarcoma, Cx43 expression is involved in the regulation of proliferation; in human U2OS cell lines, upregulation of Cx43 shows a repression in cell proliferation, by means of a lower rate of G1/S transition of the cell cycle. On the contrary, following the ablation of Cx43, the activation of Wnt/b-catenin signalling pathway was observed, resulting in the promotion of proliferation and inhibition of apoptosis in U2OS cell line (16).

Even in Ewing sarcoma studies the tumor suppressor role of Cx43 was investigated. In preclinical mouse model of Ewing sarcoma, Cx43 over expression showed a relevant tumor growth reduction, resulting in a significant gain of animal survival. Moreover, high levels of Cx43 are correlated to the increase of p27 level and a marked dwindle of Rb phosphorylation, following by the blockade of the cell cycle in G0/G1 phase. Besides, over expression of Cx43 in Ewing Sarcoma cells modifies the connection between tumor cells and osteoclasts (15, 16).

Recently, innovative studies have investigated the role of Cx43 also in other sarcomas, including Kaposi sarcoma, derived from vascular mesenchymal cells. However, more studies are necessary to improve the knowledge about the involvement of Cxs in these tumors (88).

5. WT1 functions and role in physiological conditions

The physiological embryogenic processes require the involvement of several genes and molecular mechanisms that regulate the normal functions for organs development (89). Among them, WT1 can be considered a master regulator gene for human tissues and organs ontogenesis (90). As suggested by its name, WT1 gene was originally discovered by Max Wilms in 1899, in a young patient, as responsible for the generation of nephroblastoma (91). From that time, the knowledge of the role of WT1 in development processes has steadily increased. Indeed, it has been included in several studies aimed at identifying its genome and cellular localization related with several physiological functions (92).

From genetic evaluation, WT1 has been recognized as a tumor suppressor gene located at chromosome 11p13 and encoding for a zinc finger transcription factor with different post-transcriptional modifications associated to alternative splicing that produces more than 30 isoforms (93).

The transcriptional regulatory function of WT1 is associated with the N-terminal domain whereas the C-terminal domain allows DNA, RNA and protein interactions (94). Genes involved in cellular growth, extracellular matrix components, growth factors and other transcription factors are the main targeted under WT1 regulation (95). WT1 knock-out mice have revealed the critical role of WT1 in kidney development and embryonic death associated to defects in kidneys, gonads and mesothelial tissues development (96). The involvement of WT1 in kidneys organogenesis is linked to a primary stage regarding the epithelial transition of metanephric mesenchyme which condensates around the ureteric bud tips; at later stages of renal development, the role of WT1 is addressed to the formation of S-shaped bodies, inhibiting mesenchymal cell proliferation and leading to the mature nephrons derived from S-shaped bodies elongation and connection to the branching collecting duct tree (97). Moreover, the physiological function of kidney is regulated by expression of WT1 at the glomerular podocytes, where it activates the transcription of podocalyxin gene and additional signalling pathways (98). However, although its nuclear localization has been confirmed in the urogenital system developments, many efforts have been made to detect its temporal and spatial distribution in other developing human tissues. Cytoplasmatic WT1 occurrence has been detected in developing skeletal, cardiac muscle cells and endothelial cells from epithelial and mesenchymal developing human tissues, examined between 7 and 24 gestational weeks (75). Moreover, a common neural crest-derived cell precursor has been discovered for ganglion and chromaffin cells differentiation mediated by cytoplasmatic WT1 expression, that is maintained during the morphologic differentiation of sympathetic neuroblasts but lacked in both ganglion and chromaffin cells from weeks 8 to 28 gestational age (99). The role WT1 during embryogenic development has been elucidated thanks to several mouse models, which also represent some main related syndromes, such as Wilms' tumor, aniridia, genitourinary anomalies, and mental retardation (WAGR), Denys–Drash Syndrome (DDS) and Frasier Syndrome (100). Embryonic neurogenesis mediated by WT1 has been demonstrated in $Wt1^{cKO}$ mice, which exhibit depressive-like behaviours (101). Synaptic plasticity regulation is also linked to WT1 expression that induces long-term potentiation and long-term memory in hippocampal CA1 neurons. In particular, WT1 induces IGF-2 receptor activation for *de novo* protein synthesis due to lysosomal degradation, determining structural changes, involved in cellular plasticity, and leading to learning improvement as downstream effects (102).

More recently, systems for permanent WT1 lineage tracing, such as Cre/loxP system within the embryo/tissue context, make possible Wt1-expressing cell lineage analysis and the spatiotemporal expression pattern of WT1 for studying its function during the developmental fate in many organs (103). Indeed, WT1-based Cre alleles has been reported to be useful for genetic lineage tracing of epicardial cells and mesothelium of other organs (104). WT1 expression is also detected for heart embryogenesis and development since it has been involved in promoting the proliferation of cardiac myocytes (97).

In addition, fat loss in WT1 knockout mice demonstrates the role of WT1 in the stromal vascular fraction of preadipocytes, but not in mature adipocytes for development and maintenance of visceral fat depots (105). Moreover, it has been demonstrated that WT1 represses brown adipose tissue signatures in visceral white adipose tissue, preventing a thermogenic gene expression program (106). Summarizing, WT1 has a pivotal role during foetal development and regulating adult tissue homeostasis; further studies for spatial and temporal expression during development will help to clarify better ontogenesis processes with fundamental implications in several related fields.

6. WT1 in glioblastoma

Initiation, progression and maintenance of pathophysiological process lies in the occurrence of accumulated alterations on genes that are normally involved in the human tissues and organs developments (107). WT1 is an ideal candidate to link the loss of physiological functions and genes (oncogenes or oncosuppressors) deregulation (108). The oncogenic role of WT1 is indicated in both hematologic malignancies and solid tumors, such as leukemia, breast cancer, ovarian cancer, brain tumors and soft tissue sarcoma (109). Mechanisms of altered WT1 expression in tumors have been associated with both overexpression and loss of expression; while there is controversy in defining the significance of WT1 overexpression, somatic deletion and point mutation are mainly accepted to explain WT1 downregulation (110). Proliferation, apoptosis and cell cycle regulation are the main biological processes that are controlled by WT1 target genes, including E-cyclin, p21 and bcl-2 (110). Interestingly, changes on expression levels of WT1 have been associated with different grades and types of gliomas, distinguishing signature profiles in ependymomas, medulloblastoma, grade II/III astrocytoma and high-grade gliomas (111, 112). GBM tumorigenesis from genetic mutations of stem cells like remains an unsolved topic, although recent studies have revealed the involvement of genes linked to growth factors, growth factor receptors and signalling proteins, including second messengers as key factors for the malignant transformation of a normal tissue stem cell (113). A deeper understanding of biomarkers that initiates tumors could allow the promotion of efficient therapeutic druggable targets for such an aggressive disease. However, the knowledge that GBM stem cells (GSCs) mimic the ontogenesis processes of healthy stem cells, displaying regenerative potential and the capacity to self-renew, it makes appropriate the investigation of specific genes, such as WT1, which are physiological involved in organogenesis (114). The role of WT1 in GBM proliferation and apoptosis has been demonstrated in both *in vitro* and in xenograft mouse models with WT1 shRNA transfected GBM cells (111). WT1 expression has been found upregulated in mesenchymal GSCs, showing higher proliferation, radioresistance and worse prognosis than pro-neural GSCs (115). Recently, it has been reported that in GSCs CD133+ the inhibition activity of the WT1 by WT1-associating protein (WTAP) is reversed by the low expression of miR-29a, which allows the promotion of Quaking gene isoform 6 (QKI-6), leading to the pro-survival target genes transcription, such as EGFR and PI3K pathway (116).

WT1 expression is also involved in pro-neural-mesenchymal transition of GSCs, in response to stress mechanisms such as by CDK4/6 inhibition and miR-17~92 cluster expression, that regulates WT1 levels in mesenchymal GSCs (117). Moreover, gene expression analysis from microarray explains that GBM invasion is associated to CD97 upregulation by WT1 expression (118). In addition, WT1 has been linked with epigenetic processes in brain tumors, being involved in enzymatic oxidation of 5-methylcytosine (5mC) catalysed by Ten-Eleven Translocase (TET) proteins interaction (119). The strong involvement of WT1 in the pathogenesis of GBM has contributed to the therapeutic approach's promotion aimed at its targeting. Immunotherapy, including the vaccines, chimeric antigen receptor T (CAR T) cells, oncolytic viruses and immune checkpoint inhibitors, represent a promising strategy for the treatment of GBM (120). Predictive biomarkers in GBM patients have been investigated and it has been demonstrated that Syndecan-4 (SDC-4) mRNA expression levels from peripheral blood mononuclear cells distinguish between the long- and short-term survivors in WT1 peptide vaccinated patients. Indeed SDC-4, negatively correlates with overall survival of recurrent or conventional therapy-resistant GBM treated with WT1 peptide vaccine. The predictive role of SDC-4 is associated not only to proliferation and differentiation but also to regulation of responses mediated by immune cells system (121). WT1 peptide vaccine has also been tested in combination with cell death-1 (anti-PD-1) antibody in preclinical model of GBM, revealing that the synergistic treatment is better than each monotherapy, driving a larger immunocompetent infiltration and reducing the immunosuppression mechanisms (122). WT1 immune therapies for GBM have been subsequently evaluated in the clinical phase, and it has been observed the increase of IgG antibody levels against the WT1 in association with a better prognostic marker for long-term overall survival (123).

7. WT1 in soft tissue sarcoma

WT1 plays an essential role in many stages of development, in which, depending on the tissue and context, may have whether anti- or pro-functions (124). In this scenario, these controversial and opposite functions of WT1 are reflected in their critical role in proliferation/apoptosis and also in tumor suppressor/oncogene activity, explaining the multiple WT1 protein facets. Moreover, the extremely variability of WT1 is attested also by tissue-specific expression profile at nuclear, cytoplasmic or concurrently nucleo-cytoplasmic level, that are potentially related to the dual role in cancer as a tumour suppressor or potential oncogene in others (125).

High WT1 protein expression is observed in the developing skeletal muscle tissue: in the early phases of human skeletal myogenesis, WT1 represents a key cytoplasmatic regulatory protein that mediates transcriptional and translational patterns involved. A really abundant and diffuse WT1 cytoplasmic expression is found in foetal myotubes, while it decreases especially in late phases of human myogenesis and in healthy adult skeletal muscle tissues (75). Nevertheless, this dynamic and mutable expression profile of WT1 in human developing was also observed in normal and neoplastic adult skeletal muscle tissues.

In particular, a considerable and diffuse cytoplasmic re-expression of WT1 was detected in paediatric rhabdomyosarcoma, a malignant sarcoma which shows a multitude of undifferentiated cells exhibiting a different degree of skeletal muscle differentiation. The predominant cytoplasmic localization of WT1 in human rhabdomyosarcomas and in many vascular tumors strongly suggests an oncofoetal expression of this protein (75, 125). MiRNAs also acting as crucial regulators of skeletal muscle cell fate determination. It was seen that are deregulated in rhabdomyosarcoma: miR-9; miR-183; miR-206 are up-regulated in this kind of paediatric tumor and act to sustain neoplastic growth and tumorigenesis through the modulation of different genes including cyclin D1, cMET and EGR1. Rhabdomyosarcoma could be distinguished into two main histological subtypes: embryonal and alveolar. The distinct and specific miRNA expression profile would seem to be a promising strategy for discriminating specific variants among rhabdomyosarcoma subsets that, all together, accounts for approximately 7% of pediatric tumors . WT1 is involved in vasculogenesis process with a large variety of genes and secreted growth factors. WT1 role in blood vessels formation is well documented not only in de novo expression (74) related to reparative neoangiogensis, such as in response to myocardial ischemia, but also in tumor vascularization and angiogenic processes, that influence cancer growth and metastasis (125). Katuri V. et al. demonstrated that WT1 is involved in Ewing sarcoma angiogenesis. This tumour represents one of the most malignant bone tumor in young population that frequently occurs in axial skeleton (126). *In vitro* analysis demonstrated that WT1 is upregulated by hypoxia in Ewing sarcoma cells and regulates various pro-angiogenic genes, such as angiopoietin-1 (Ang-1) and its receptor, matrix metallopeptidase 9 (MMP-9) and vascular endothelial growth factor (VEGF) (127). Evaluating miRNA expression profile of paediatric tumours, Ewing sarcoma is characterized by the expression of miR-214-3p, miR-214-5p and miR-92b-3p, condition that discerns Ewing tumors from osteosarcomas and rhabdomyosarcomas (74). These findings suggest that WT1 represents a novel member of the family of proteins that manage the genetic program concerning the new blood vessels formation in tumor angiogenesis process (125). WT1 expression is also confirmed in a large amount of Scwhann cells of nerve fibers as early as 7–8 weeks of gestational age, and the relative overexpression was observed in various type of cancers, such as Malignant Peripheral Nerve Sheath Tumors (MPNST), also known as malignant schwannomas. In fact, WT1 is expressed weaker in the cytoplasm but in a predominant way in nuclear and perinuclear areas of MPNST cells. A critical interaction with Signal transducers and activators of transcription 3 (STAT3) was evaluated, which is overexpressed or constitutively activated in a several human malignancies. The concomitant overexpression of WT1 and STAT3 in tumor development increases the expression level of STAT3-dependent target genes, including cyclin D1 and Bcl-xL, resulting in an advantage of cell proliferation and neoplastic progression. In MPNST, at least *in vitro*, WT1 acts as an oncogene rather than a tumor suppressor (128). WT1 related mRNA is absent in normal bone but detected in osteosarcomas, in which underlying poor survival of patients with high-grade osteosarcoma, especially in the context of metastasis (129). Osteosarcoma is a primary malignant cancer of bone affecting predominant children and young population.

A strong WT1 expression level is described in various types of human bone and soft-tissue sarcomas, including osteosarcoma. Graziano A.C.E. et al., by *in vitro* analysis on MG-63 cell lines, evaluated those high levels of WT1 expression is related to the high grade of cancer cells proliferation in assessed sarcoma tissue samples. In particular, the authors found a predominant expression level in perinuclear area and a lower distribution in nuclear space. WT1 targeting siRNA suppresses cell proliferation, inducing cell cycle arrest and activates apoptosis in MG-63 cell line. WT1 is expressed in 50% of human osteosarcoma cases, suggesting that WT1 expression profile may be dynamically and quickly modulated according to the functional and phase-specific contexts of tumorigenesis in which WT1 acts. Furthermore, WT1 is associated with very poor survival of patients with osteosarcoma metastasis (130). To date, it is not well known if WT1 is expressed during development of the tissues in which neoplastic masses will originate. The related expression observed in tumours might reflect both de-differentiation of mature cells and the cancer stem cell origin of the tumour, suggesting that WT1 might not be limited to transcriptional regulation and RNA metabolism (21).

8. Concluding remarks

Different studies have highlighted the efficacy of drugs that target Cxs-based channels, finding new approaches for the treatment of a broad range of diseases. It is important to understand the complexity and mechanisms that link cancer biology with connexins, deepening intercellular communication, in order to find new therapeutic approaches by modulating the expression of Cxs. There are numerous controversial theories regarding the role of Cxs in cancer, especially Cx43. Cx43 represents a suitable candidate of prognostic biomarker for bone and soft tissue sarcomas, since its tumor suppressor role in osteosarcoma and Ewing sarcoma (131). Pharmacological targeting of Cx43 has also emerged as a mean of preventing tumor invasion and recurrence following tumor resection in GBM, as well as overcoming intrinsic or acquired resistance to TMZ.

In addition, the close connections between WT1 ontogenesis and its deregulation for GBM tumorigenesis has deeply contributed to the gain additional insights. Many efforts have been made to understand how the physiological role of WT1 may change acquiring a pathophysiological role in tumor promotion, as in malignant sarcomas. In this context WT1, influencing pAKT/Cyclin D1 pathway, might be tested as a potential element useful to gene targeted therapy in a very large number of cancers, including bone and soft tissue sarcoma. WT1-target therapy may be considered as innovative treatment, especially in rhabdomyosarcoma and MPNST (22, 128), becoming a valid therapeutic alternative for malignant tumour therapy in the near future (21).

Further studies may be carried out in order to find innovative treatments, also for metastatic secondary bone tissue, including lungs, thyroid, prostate, breasts, and kidneys, the most common carcinomas that metastasize to bone, which have not any effective therapy (16, 132). In this scenario, even the regenerative medicine may be an interesting field to explore, since bone and cartilage regeneration has already been investigated as potential solution for arthritis and degenerative diseases and it may be applied also for the treatment of bone and soft tissue sarcomas in the future (133, 134).

Given the involvement of Cxs and WT1 in physiological and pathophysiological processes, such as embryonic development and tumorigenesis, it would be interesting to study their potential interplay in molecular and cellular mechanisms in the future.

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