Wnt Pathway: A Tailored Target

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Cancer represents one of the greatest public health challenges. One of the most cancer-driving events embodies the dysregulation of both the canonical and the non-canonical Wnt/β-catenin pathway. The impact of the Wnt/β-catenin pathway has been widely reviewed in colorectal, breast, and ovarian cancers.

Genetic and epigenetic alterations are commonly detected in colorectal cancers (CRCs). As a matter of fact, 70% of CRCs are connoted by the APC mutations and almost all patients display an overactive Wnt/β-catenin pathway also mediated by oncogenic miRNAs. Therefore, miRNAs have been proposed as anti-cancer and/or diagnostic/prognostic tools. Among cancers, breast cancer (BC) is one of the most expensive health care costs with a high rate of diagnosis and deaths per year. The Wnt/β-catenin cascade and in particular the β-catenin content has been correlated with a dismal prognosis, high tumour grade, and metastasis formation. In addition in triple-negative breast cancer (TNBC) both the canonical and the non-canonical Wnt/β-catenin pathways have been reported as drivers of cancer dissemination, aggressiveness, early age of onset, and poor outcome. To add further complexity, the Wnt5a ligand was found to display both anti-tumour and tumour promoting properties depending on the tumour microenvironment (TME), the activation of specific signalling pathways, and the receptor availability in BC.

Likewise, an abnormal Wnt/β-catenin cascade has been shown to strongly contribute to ovarian cancer (OC) growth, stemness, and drug resistance.

In the last decades, particular attention has been dedicated to investigate the role of extracellular vesicles (EVs) released in the TME in cancer growth and progression. EVs are heterogeneous small membrane-bound carriers with a complex cargo contributing to cell-to-cell communication, tumour growth, invasion, and chemoresistance. Since EVs can be detected in the majority of biological fluids and in the TME, EVs have been proposed as diagnostic and/or prognostic tools, as well as useful therapeutic options. Indeed, EVs engineered with specific anti-tumour molecules or loaded with conventional anti-tumour drugs have been proposed as novel anti-cancer options.

Based on these notions, in the last decades, Wnt/β catenin targeting approaches have been explored to hinder tumour expansion. However so far, the most relevant limitation relies on the crucial role played by the Wnt/β catenin cascade in tissue homeostasis. Therefore, to develop targeting approaches the identification of the specific EV cargo driving tumour progression and the mechanisms accounting for the unbalanced Wnt/β catenin pathway in cancer should be considered as the most challenging issues.

Keywords: Wnt/β-catenin dependent pathway ; Wnt/β-catenin independent pathway ; colorectal cancer ; breast cancer ; ovarian cancer ; extracellular vesicles

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Cancer is one of the greatest public health challenges. According to the World Health Organization (WHO) 9.6 million cancer deaths have been reported in 2018. The most common cancers include lung, breast, colorectal, prostate, skin cancer (non-melanoma) and stomach. The unbalance of physiological signalling pathways due to the acquisition of mutations in tumour cells is considered the most common cancer driver. The Wingless-related integration site (Wnt)/β-catenin pathway is crucial for tissue development and homeostasis in all animal species and its dysregulation is one of the most relevant events linked to cancer development and dissemination. The canonical and the non-canonical Wnt/β-catenin pathways are known to control both physiological and pathological processes including cancer. Herein the impact of the Wnt/β-catenin cascade in driving cancers from different origin has been examined. Finally, based on the impact of Extracellular Vesicles (EVs) on tumour growth, invasion and chemoresistance, and their role as tumour diagnostic and prognostic tools, an overview of the current knowledge linking EVs to the Wnt/β-catenin pathway is also discussed.

1. Introduction

The human wingless-related integration site (Wnt) genes encode 19 evolutionarily conserved glycoproteins with 22-24 Cys residues. In the endoplasmic reticulum (ER), the Wnt ligands are post-translationally acetylated by porcupine, a membrane associated O-acyl transferase. Acetylation leads to palmitoylation, which is required for the release and binding of Wnt to the frizzled (*FZD*) receptors. This, in turn, drives the biological response [1].

The Wnt signalling pathway regulates crucial cellular processes including cell fate determination, organogenesis during embryonic development, normal adult homeostasis, motility, polarity and stem cell renewal [2]. Moreover, its contribution in cancer has been extensively investigated [3].

The Wnt pathway has been widely studied and reviewed, and a general understanding of the transduction cascade has been clarified. The Wnt cascade has been subdivided into different branches due to its complexity [4][5]. They include the canonical Wnt/β-catenin (Wnt/β-catenin dependent pathway) and the non-canonical Wnt/β-catenin pathway (β-catenin-independent pathway). The latter was further allocated into two additional branches, the planar cell polarity (PCP) and the Wnt/calcium pathways [2]. Both of them contribute to cancer development and dissemination.

2. Wnt Canonical Pathway: β-Catenin Dependent

The canonical pathway turns around the β-catenin intracellular level (Figure 1). In the absence of Wnt proteins, the β-catenin "destruction complex" keeps low β-catenin in the cell. The "destruction complex" mainly consists of two kinases: casein kinase 1α (*CK1α*), glycogen synthase kinase 3 β (*GSK-3β*) and two scaffolds: axis inhibition (*Axin*), and adenomatous polyposis coli (*APC*). Firstly, β-catenin undergoes phosphorylation by *CK1α* at serine 45 (Ser45), Ser33, Ser37 and threonine 41 (Thr41) by *GSK-3β*. Then, the E3 ubiquitin ligase, denoted as β-transducin repeat-containing protein (*βTrCP*), marks β-catenin ubiquitination and degradation [1]. This prevents β-catenin nuclear translocation while allowing histone deacetylation and chromatin compaction by the Groucho repressor, translating into the inhibition of gene transcription [6] (Figure 1a).

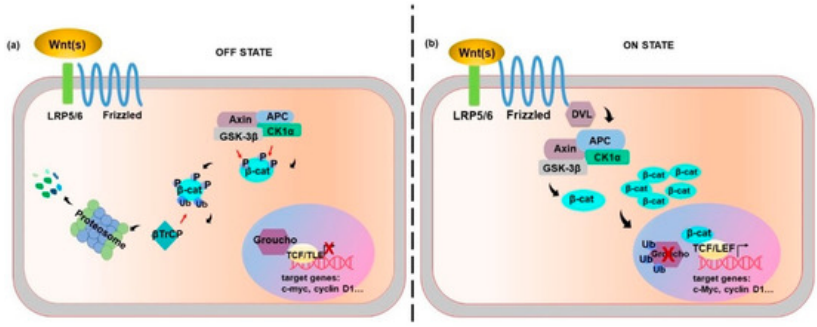

Figure 1. The Canonical Wnt signalling pathway. (**a**) OFF STATE. In the absence of Wnt ligands, β-catenin moves to the "destruction complex" consisting of casein kinase 1α (*CK1α*), glycogen synthase kinase 3 β (*GSK-3β*) and two scaffolds: axis Inhibition (*Axin*), and adenomatous polyposis coli (*APC*). β-catenin undergoes phosphorylation at Ser45 residue by *CK1α* and at Ser33, Ser37 and Thr41 residues by *GSK-3β*. Then, the E3 ubiquitin ligase β-transducin repeat-containing protein (*βTrCP*) marks β-catenin ubiquitination and proteasomal degradation. This prevents β-catenin nuclear accumulation while allowing chromatin compaction and Groucho-mediated promoter repression. (**b**) ON STATE. The Wnt ligands bind to frizzled (*FZD*) receptor and the low-density-lipoprotein-related protein 5/6 (*LRP5/LRP6*); this results in dishevelled (*DVL*) phosphorylation and β-catenin release from the "destruction complex", allowing β-catenin accumulation and nuclear translocation. In the nucleus, the Groucho repressor undergoes displacement, allowing β-catenin to interact with T-cell factor/lymphoid enhancer factor (*TCF/LEF*), chromatin remodeling and transcription of genes such as *c-myc* and *cyclin D1*.

The activation of the canonical Wnt signal requires both the *FZD* family receptors and the low-density-lipoprotein-related protein 5/6 (*LRP5/LRP6*) co-receptors, phosphorylation of which is essential for receptor activation. Wnt binding to its receptor results in dishevelled (*DVL*) phosphorylation, leading to *Axin* de-phosphorylation and decline of its cytoplasmic content [7]. Thereby, β-catenin can be released from the "destruction complex", and its degradation prevented while stabilization is allowed. Accumulation of β-catenin turns into its nuclear translocation [7].

Although several nuclear β-catenin binding partners have been involved in the control of gene transcription, the most relevant β-catenin partners are the members of the T-cell factor/lymphoid enhancer factor (*TCF/LEF*) family of transcription factors [7]. This complex binds to the promoter region of target genes and regulates their transcription.

Once in the nucleus, the engagement of β-catenin transiently converts the *TCF/LEF* into transcriptional activators, which displace Groucho and induce chromatin remodelling and transcriptional activity (Figure 1b).

A number of genes are targeted by Wnt-β-catenin. Among them, genes involved in positive- and negative-feedback regulation, cell-cycle progression, and stem cell homeostasis are the most commonly included genes.

3. Extracellular Vesicles and the Wnt Pathway

EVs are heterogeneous small membrane-bound carriers with complex cargoes released under both physiological and pathological conditions. Almost any cell can release EVs, which act as inter-cellular mediators modifying target cell fate at closed or distant sites [8].

Based on the biogenesis, size, content, mechanisms of release and function, three discrete EV subtypes are recognized: microvesicles (MVs), exosomes, and apoptotic bodies [8].

EVs-mediated transfers of specific molecules are known to dictate the phenotype of the recipient cell. They can act on proliferation, motility, EMT, migration, invasion, immune evasion, chemo-resistance, and TME reprogramming (Figure 2).

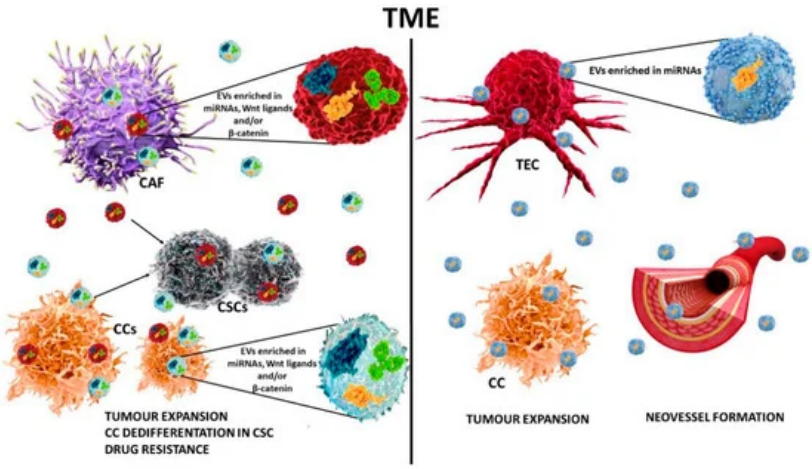

Figure 2. Schematic representation of cell-to-cell communication in the TME by EVs. EVs are released by almost all cell types in the TME. EVs serve as inter-cellular mediators transferring specific molecules (proteins including Wnt ligands and β-catenin, and miRNAs) to recipient cells, thus promoting tumour expansion, cancer cell dedifferentiation in CSCs, chemo-resistance, and neovessel formation. CCs: cancer cells; CSCs: cancer stem cells; TEC: tumour-derived endothelial cell; CAF: cancer associated fibroblasts.

Moreover, EVs derived from serum or other biological fluids have been proposed as tumour biomarkers. More importantly, EVs have gained attention as anti-cancer tools. Indeed, EVs can be used as drug delivery systems or potential cancer vaccines. Moreover, the transfer of Wnt ligands or β-catenin via EVs has been proposed as a Wnt signalling activation mechanism.

Kalra et al. [9] have shown that EVs released by CRC cells and containing the mutant β-catenin and high Wnt/β-catenin activity boost the expression of target genes as *c-myc* and *cyclin D1* when transferred to recipient cells (Table 1).

Table 1. EVs involved in several tumours, their alteration, targets, and impact on tumours.

EV Cargo	EV Source	Target Cells	Related Cancers	Expression Level	Pathway Interaction	Impact on Tumour Cells	Ref.
Mutant β-catenin in EVs	LIM1215	RKO	CRC	Upregulated	β-catenin	migration, metastasis tumour growth	[9]
14-3-3ζ in EVs	HEK293T	COS-7, SW480	CRC	Upregulated	β-catenin GSK-3β DVL2	survival migration	[10]
Wnt ligands in EVs	CAFs	CRC	CRC	Upregulated	β-catenin	dedifferentiation drug resistance colony formation	[11] [12]
β-catenin in EVs	milk	HCC	HCC	Silenced	β-catenin	proliferation tumour growth	[13] [14]

EV Cargo	EV Source	Target Cells	Related Cancers	Expression Level	Pathway Interaction	Impact on Tumour Cells	Ref.
DKK-1 in EVs	MM	MM	MM	Upregulated	β-catenin	osteoclast activity osteoblast differentiation	[15]
EVs	OSCC	OSCC	OSCC	Upregulated	β-catenin	metastasis stemness chemoresistance	[16]
Wnt5b in EVs	Caco-2 and PANC-1	A549	Lung cancer	Upregulated	β-catenin dependent and independent pathways	proliferation migration	[17]
EVs	CAFs	BC	BC	Upregulated	Wnt-PCP	cell growth and motility	[18]

The *14-3-3* are conserved molecules displaying regulatory functions and promoting cancer progression [10]. The *14-3-3ζ* isoform, which binds both β-catenin and *GSK-3β*, leads to the nuclear translocation and accumulation of β-catenin and enhances cell motility. Moreover, EVs enriched in *14-3-3ζ* and β-catenin, after internalization, promote cell survival and migration by activating the Wnt/β-catenin cascade [10] (Table 1).

Hu et al. [11] have investigated the mechanism of drug resistance in CRC and have proven that EVs released by fibroblasts drive dedifferentiation of CRC cells towards CSCs (Figure 2a). Additionally, they found that EVs derived from fibroblasts contain the Wnt ligands that activate the Wnt/β-catenin pathway in target cells, induce transdifferentiation of CRC cells into CSCs and increase drug resistance. Furthermore, it has been reported that collagen accumulation and the *APC* mutation in CRC cells stimulate the release of EVs and, under hypoxia conditions, fibroblast derived EVs boost CRC colony formation [12] (Table 1).

Accumulating evidence shows that EVs enriched in miRNAs are key determinants of human cancer cell growth, invasion and metastasis [19]. CAF-derived EVs enclose miR-92a-3p, which contributes to cancer progression, stemness, EMT, and drug resistance. Moreover, miR-92a-3p enriched EVs correlated with the activation of the Wnt/β-catenin pathway [19] (Figure 2a).

Long non-coding RNA-*APC1* (lncRNA-APC1) is a negative regulator of CRC. Low levels of lncRNA-APC1 correlate with metastasis, advanced clinical stage and poor prognosis in CRC patients. *APC*, via lncRNA-APC1, promotes cell-cycle arrest and suppresses angiogenesis by lowering the release of CRC cell-derived EVs. Finally, it has been shown that EV-derived from CRC are enriched in Wnt1 and enhance CRC cell proliferation and migration via non-canonical Wnt/PCP signalling [20].

Hepatocellular carcinoma (HCC) is one of the most common causes of cancer-related deaths worldwide. Constitutive activation of the Wnt/β-catenin pathway turns into the expression of the epithelial cell adhesion molecule (*EpCAM*) [13]. Ishiguro et al. [14] provided evidence that loss in β-catenin and reduced proliferation and invasion can be obtained by *EpCAM* positive liver cancer stem cells (LCSC) targeted by EVs engineered with a β-catenin specific siRNA (Table 1).

Multiple myeloma (MM) is a hematopoietic malignancy associated with an altered homeostasis of bone formation/resorption. MM-derived EVs enriched in *DKK-1* were found to boost the Wnt/β-catenin signalling and contribute to the abnormal osteogenesis. The inhibition of EV shedding, combined to chemotherapy, was found to impair tumour load, angiogenesis and osteolysis [15] (Table 1).

Furthermore, a recent study noticed that the release of EVs from HCC cells is increased in hypoxic conditions and linked to cancer cell proliferation, migration, invasiveness and EMT. Mechanistically, they have shown that miR-1273f enriched in EVs activates the Wnt/β-catenin signalling cascade by targeting the Wnt/β-catenin inhibitor LHX6 [21].

Overall, these data indicate a crucial contribution of EVs released by different cell sources in driving tumor development and dissemination. Several data suggest that these effects mainly rely on the transfer of their specific cargo into target cells. Therefore, approaches able to modify their cargo, particularly miRs and proteins involved in their tumor promoting action, have been proposed as useful therapeutic options. EV engineering by using siRNA for mutated protein has been tested and its effectiveness demonstrated in pancreatic cancer [22]. This suggests that using siRNA for mutant β catenin should be considered as an alternative option for CRC. Likewise, siRNA for different Wnt proteins or rearrangement of dysregulated EV miRs can be used to target the Wnt/β catenin cascade. Alternatively, EVs loaded with Wnt/β catenin inhibitors can be used as natural delivery tools.

4. Conclusions

Cell-to cell communication is part of the evolutional processes. Wnt ligands are essential for homeostasis and, in the last 30 years, genetic, biochemical, and molecular investigations have uncovered several Wnt signalling components [2][3]. Driving interest on this topic mainly relies on dysregulation of the Wnt/β-catenin signalling and cancer development/progression [3]. Moreover, Wnt/β-catenin cascade seems to contribute to the TME shape, which plays a crucial role in the control of tumour progression and immune regulation. Many different Wnt proteins have been described, and, among them, Wnt5a plays a critical role, taking part in both the canonical and the non-canonical Wnt/β-catenin pathway [23][24].

The identification of specific tools able to interfere with the Wnt/β-catenin cascade has been a hotspot for many years. This is particularly true for CRC, in which almost 70% of CRC patients display *APC* mutations [25]. Apart from CRC, the Wnt/β-catenin pathway is gaining attention in several malignancies, such as breast, ovarian, melanoma, prostate and paediatric osteosarcoma [26][27][28]. In this regard, BC and in particular TNBC are featured by the abnormal activation of both the canonical and non-canonical Wnt/β-catenin pathway [29][30]. Likewise, a hyper-active Wnt/β-catenin cascade has been shown to play a crucial role in the progression, stemness, and drug resistance in OC [31][32]. Several miRNAs have been identified to modulate this cascade and thereby widely studied as screening markers or targets in different tumour settings [33].

In the TME, intercellular communication has been recently reported as mediated by the transfer of EV molecular cargo and revised in [34]. Their cargo also includes a number of Wnt components. Of note, wild-type and mutant β-catenin, able to promote survival and proliferation of recipient cells and, in several instances, dedifferentiation towards a CSC phenotype, have been detected in EVs (Figure 2a). Moreover, their role in mediating drug resistance has been reported. Furthermore, since EVs are released within the TME, their contribution in cancer growth and progression has been extensively investigated [35]. EV shedding, blockade, or engineering have been proposed as innovative anti-tumour instruments for fine-tuning the Wnt/β catenin pathway [33][36].

In recent decades, several efforts have been directed to the development of Wnt/β catenin targeting approaches in order to interfere with tumour progression. However, these efforts have been limited by the crucial role of the Wnt/β catenin pathway in preserving tissue homeostasis. Therefore, future energies should be directed to clearly dissect the mechanisms driving the unbalanced Wnt/β catenin pathway in cancer, and the EV mechanism of action should be considered amid them. Should they be identified, targeting approaches would become a suitable anti-cancer option

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