Review Article

Notch, Wnt, and Hedgehog Pathways in Rhabdomyosarcoma: From Single Pathways to an Integrated Network

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Rhabdomyosarcoma (RMS) is the most common type of soft tissue sarcoma in children. Regarding histopathological criteria, RMS can be divided into 2 main subtypes: embryonal and alveolar (ERMS and ARMS, resp.). These subtypes differ considerably in their clinical phenotype and molecular features. The prognosis of ERMS is more favourable than that of ARMS. From a molecular point of view, the majority of ARMS (80% to 85%) contain one of the reciprocal chromosomal translocations: embryonal and alveolar. These subtypes differ considerably in their clinical phenotype and molecular features. Abnormal regulation or mutation of signalling pathways that regulate normal embryonic development such as Notch, Hedgehog, and Wnt is a recurrent feature in tumorigenesis. Herein, the general features of each of the three pathways, their implication in cancer and particularly in RMS are reviewed. Finally, the cross-talking among these three pathways and the possibility of better understanding of the horizontal communication among them, leading to the development of more potent therapeutic approaches, are discussed.

1. Introduction

Rhabdomyosarcoma (RMS) is the most common type of soft tissue sarcoma in children. RMS can be divided into 2 main histopathological subtypes: embryonal and alveolar (ERMS and ARMS, resp.). These subtypes differ considerably in their clinical phenotype and molecular features. The prognosis of ERMS is more favourable than that of ARMS. From a molecular point of view, the majority of ARMS (80% to 85%) contain one of the reciprocal chromosomal translocations: either t(2;13) (q35;q14) or t(1;13)(p36;q14). These translocations generate the anomalous fusion genes PAX3-FOXO1 and PAX7-FOXO1, respectively [1, 2]. The resulting chimerical proteins have potent transforming effects and are thought to inhibit myogenic differentiation. However, no characteristic translocations have been described in ERMS. The ERMS is typically characterised by loss of heterozygosity on the short arm of chromosome 11 (11p15.5) [3], and gains in chromosomes 2, 7, 8, 11, 12, 13, and 17 are also common in this subtype [4].

Notch, Wnt, and Hedgehog pathways are known to play critical roles in the development of pluricellular organisms. Knowledge of the oncogenic role (by mutation or deregulation) of these pathways has been widening in recent decades. In paediatric malignancies, evidence of the possible significance of these pathways in the promotion of oncogenic phenotype has been accumulating. Although the understanding of the roles played by these pathways in paediatric tumours is advancing, it is far from that of better known adult malignancies.

2. Notch Signalling

Notch signalling plays a critical role in tissue development in organisms ranging from nematodes to mammals. The notch genes encode 4 highly conserved cell surface receptors that are activated by its ligands (Delta and Jagged in vertebrates). The Notch intracellular domain (NICD) is then proteolysed and released by the γ-secretase complex and translocates to the nucleus where it binds to CSL transcription repressors, converting them into transcriptional activators. The paradigmatic targets of these transcription factors in vertebrates are the HES and HEY genes [5, 6].
2.1. Notch and Cancer. The oncogenic potential of the Notch pathway was first described in acute T-cell lymphoblastic leukaemia (T-ALL) in the late 1980’s. In normal conditions, Notch signalling is necessary for correct maturation of T-cell progenitors; however, constitutive activation of the pathway leads to abnormal T-cell proliferation causing T-ALL [7]. An abnormal upregulation of the Notch pathway has also been reported in ovarian [8], breast [9], and other cancers (cervix, head and neck, endometrium, kidney, lung, pleural mesothelioma, malignant melanoma, Hodgkin’s lymphoma, anaplastic large cell lymphomas, some acute myeloid leukaemias, and chronic B-cell lymphocytic leukaemia, among others) [10]. With respect to paediatric malignancies, Notch signalling appears to contribute essentially to osteosarcoma metastasis [11] and proliferation [12]; Notch signalling also promotes medulloblastoma cancer stem cell survival [13] and contributes to angiogenesis in neuroblastoma [14].

2.2. Notch and RMS. During normal muscle development, the Notch pathway is involved in satellite cell activation and in cell fate determination during postnatal myogenesis [15]. Activation of Notch pathway is known to inhibit myogenesis [16, 17]. However, the role of the pathway in RMS is barely known. Our group recently showed that the Notch pathway is widely and consistently activated in both ARMS and ERMS patients; a clear implication in the regulation of motility and invasiveness of ARMS and ERMS cells was also reported in the same work [18]. The existence of a wide range of pharmacological Notch inhibitors renders this pathway a promising therapeutic target in the fight against metastases; however, the cross-talk with other pathways such as Hedgehog and Wnt—as will be discussed below—may negatively influence the efficacy of therapeutic approaches if directed exclusively against one pathway.

3. Hedgehog Signalling

Hedgehog (Hh) signalling was first described in 1980 as a gene exerting a direct effect on embryonic development in Drosophila [19]. Hedgehog genes are considered to be key regulators of development in organisms ranging from the fruit fly to mammals, since they control multiple embryonic processes such as tissue patterning, proliferation and differentiation. Hedgehog signalling also plays important roles in adult organisms such as stem cell maintenance and tissue repair and regeneration. The 3 Hedgehog proteins present in mammals, Sonic (SHh), Indian (IHh), and Desert (DHh), need a maturation process to achieve their active forms. This maturation process implies autocatalytic cleavage of the protein to release its active N-terminal peptide, with subsequent N-palmitoylation and the formation of a C-terminal cholesterol adduct [20]. Mature Hh proteins are ligands of patched receptors (Ptch1 and Ptch2). Ligand-free Ptch inhibits the activation of Smoactivity [21]. Although the exact endogenous small molecule that modulates Smo activity has not been identified, sterol-like molecules have emerged as leading candidates [21]. In the absence of active Smo in the membrane, GLI family zinc finger proteins (Gli1, Gli2, and Gli3) in a complex with SuFu (suppressor of fused homolog) are proteosomally processed. Upon binding of a Hedgehog ligand, active Smo is detected in the membrane and prevents Gliproteosomal processing. Gli is then translocated to the nucleus where it binds to Glispecific promoters. Gli1 and Gli2 mainly function as transcriptional activators, whereas Gli3 exists in two forms, either as a full-length transcriptional activator (Gli3A) or an amino-terminal fragment that functions as a repressor (Gli3R). The three best known direct targets of the pathway are Gli1, Ptc1, and Hipp, genes of the pathway itself [21–23].

3.1. Hedgehog and Cancer. The Hedgehog pathway also has major implications in several cancers. Mutation or deregulation of the pathway may lead to tumorigenesis in a wide variety of tissues. The initial link between Hedgehog signalling and human cancers was established when mutations in human PTCH1 were found to be associated with a rare hereditary disease called Gorlin’s syndrome. Patients with Gorlin’s syndrome have a high incidence of basal cell carcinoma, medulloblastoma, and rhabdomyosarcoma. The molecular origin of this syndrome is a constitutive activation of the Hedgehog pathway caused by mutations in the PTCH1 gene [24, 25]. Hedgehog pathway alterations—mainly loss of function of Ptc1 and SuFu or activating mutations in Smo, Hh, or Gli—are thought to be oncogenic in a considerable number of other cancers (gliomas, breast, lung, prostate, ovarian, colon, and endometrial carcinomas, multiple myeloma, and chronic myeloid leukaemia, among others) [26].

3.2. Hedgehog and RMS. The role of Hedgehog signalling in the genesis of RMS was first described in the Patched knockout mouse by Hahn et al. in 1998 [27] who reported that mice heterozygous for Ptch1 not only develop features consistent with Gorlin’s syndrome, such as generalised overgrowth of the body and a variety of neural and skeletal abnormalities, but also have a high incidence of ERMS. Tumours in heterozygous Pch1 mice exhibited elevated transcript levels of Gli1 and Pch1 itself, indicating that abnormal Hedgehog signalling may be common to the various tumours associated with Gorlin’s syndrome [27]. The formation of RMS in Pch1-mutant mice has been associated with the ability of tumour cells to resist apoptosis [28]. The role of epigenetic regulation of Pch1 expression seemed to be crucial in heterozygous Pch1 mice, since a combined treatment with the DNA-methyltransferase1 inhibitor 5-aza-2’-deoxycytidine and the histone deacetylase inhibitor valproic acid efficiently prevented RMS and medulloblastoma formation in this model [29]. Currently, a consistent activation of the pathway is well established and generally accepted in RMS. A higher degree of Hh activation in ERMS and translocation-negative ARMS than in translocation-positive ARMS has also been reported [30]. In the same work, Zibat
et al. analysed the pathway in a large cohort of RMS patients and hypothesised that the activation of the pathway confers poor prognosis in ERMS and translocation-negative ARMS and suggested an inverse correlation between Hh pathway activation and muscular differentiation [30]. Conversely, Pressey et al. stated recently that neither GLI1 nor PTCH1 mRNA transcripts in ERMS tumours correlated with survival or other clinical characteristics analysed [31]. Another recent work showed positive staining by immunohistochemistry of 78% of samples for SHH, 100% for PtcH, and 78% for GLI1 in a panel of 18 RMS and reported, in disagreement with the work of Zibat et al., higher Gli1 expression in alveolar than in embryonal subtypes [32]. On the other hand, several publications that combine in vitro and in vivo works with xenografted rhabdomyosarcoma models agree about the possibility to effectively reduce tumour growth by Hh pathway inhibition mediated by betulinic acid, GANT-61, and forskolin [33–35]. Although these treatments did not achieve total remission of the tumour, the significant reduction in tumour growth suggests that Hh signalling plays a leading role in RMS oncogenicity and that the pathway can be considered a potential molecular target for new treatment strategies in this neoplasia. In fact, derivatives of cyclopamine and other small molecular antagonists of Smo have recently entered clinical phase I and II trials for basal cell carcinoma, with encouraging results particularly for GDC-0449 [36, 37].

4. Wnt Signalling

The first description of the gene Wingless in Drosophila and the subsequent discovery of its orthologous genes in vertebrates laid the keynote of an evolutionary conserved signalling pathway now commonly referred to as the Wnt pathway. This pathway is involved in the establishment of the body axis at the earliest stages of embryogenesis and is also later required for development of many organs in organisms ranging from C. elegans to mammals. Two variants of the pathway, canonical and noncanonical, have been described. The Wnt canonical pathway is generally thought to regulate cell fate determination and the non-canonical one to control cell movement and tissue polarity [38].

In the canonical pathway and in the absence of Wnt ligands, APC and Axin bind to β-catenin (the central actor in the canonical pathway) thereby permitting its phosphorylation by casein kinase 1 alpha (CK1α) and glycogen synthase kinase 3 beta (GSK3β) and, sequentially, its polyubiquitination and proteosome-mediated degradation. The interaction of Wnt ligands with Frizzled receptor and Lrp5/6 coreceptor inhibits the degradation of β-catenin owing to the formation of Frizzled-Dishevelled and Lrp5/6-Axin-FRAT complexes and the inactivation of GSK3β via Dishevelled (Dvl). Stable β-Catenin is translocated to the nucleus where it binds to T-cell factor/lymphoid enhancer factor (TCF/LEF) and also to Legless family docking proteins (BCL9 and BCL9L) associated with PYGO family coactivators, thereby promoting transcription of the target genes of this pathway: FGF20, DKK1, WISP1, MYC, and CCND1, among others [38]. The non-canonical pathway is also initiated by Wnt ligands (exemplified by Wnt5a and Wnt11) and Frizzled receptors; however, while the canonical pathway leads to β-catenin and TCF/LEF-mediated gene expression, non-canonical Wnt signalling is mainly mediated by activation of PKC and JNK [39, 40].

4.1. Wnt and Cancer. Anomalous activation of the Wnt pathway has been reported in several adult cancers such as non-small-cell lung cancer, colorectal carcinoma, prostate cancer, and breast cancer among others. Deregulation of the Wnt pathway in carcinogenesis is often attributed to a mutation in the β1-Catenin gene (CTNNB1). Oncogenic involvement of the Wnt pathway has also been shown in different embryonal tumours, such as hepatoblastoma, nephroblastoma (Wilms’ tumour), pancreatoblastoma, and medulloblastoma. Up to 75% of hepatoblastomas and 15% of Wilms’ tumours display CTNNB1 mutations, approximately half of which affect exon 3 [41–43]. Other cancer studies revealed a downregulation of the tumour suppressor Wnt5a, related to the non-canonical pathway, the deletion or reduced expression of which can occur in several cancers including sarcomas [44–46].

4.2. Wnt and RMS. Very few works on the role of Wnt pathway in RMS have been published. Soglio et al. concluded that there was no evidence of β1-Catenin mutation in the genesis of RMS and that this protein did not constitute a useful marker for distinguishing between ERMS and ARMS [47]. Analysis of the CTNNB1 gene sequence in 8 ERMS and 3 ARMS revealed no β1-Catenin mutations in this patient cohort. In the same work, an immunohistochemical study of β1-Catenin location showed cytoplasmatic staining with cytoplasmatic membrane reinforcement and without nuclear staining [47]. Another work recently published by Singh et al. provided supporting observations that β1-Catenin-activating mutations did not contribute to ERMS tumorigenesis [48]. They found the Wnt/β1-Catenin signalling pathway to be inhibited in an ERMS cell line derived from p53/c-fos double-mutant mouse tumours. Although these cells showed higher expression of Wnt2, Wnt10a, and Wnt8b compared to normal myoblasts, the most highly expressed genes were Wnt pathway inhibitors such as sFRP2, sFRP4, Dkk1, and Nkd1. Wnt receptors (Frizzled1, 3, and 5), the signalling mediator Dvl and factors involved in recruiting and forming the activation complex with β1-Catenin in the nucleus (LEF-1 and Pygo) were downregulated. Moreover, the majority of downstream target genes of this pathway showed no expression differences compared to normal myoblasts, thereby suggesting absence of oncogenic activation of the pathway. On the other hand, some Wnt genes involved in myogenesis showed an expression pattern that may lead to impaired muscular differentiation. Thus, those authors reported a down-regulation of Wnt7b, which promotes myogenic differentiation, and up-regulation of 2 proteins, sFRP2 and Wnt2, which inhibit myogenic differentiation. Furthermore, the activation of the Wnt pathway raised MyoD and MyHC expression levels and promoted myoblast fusion, a fact confirming that Wnt signalling directly promotes myogenic differentiation [48]. Taken together, the results of these experiments suggest that the activation of Wnt pathway in RMS may mainly promote antioncogenic effects.
5. Notch, Wnt, and Hedgehog Compensative Cross-Talking

A general drawback when pharmacological pathway inhibition is attempted is that only moderate decreases in the expression of downstream targets are achieved. This observation is often attributed to incomplete pathway inhibition by the drugs or compounds used. However, deeper understanding of the complex nature of cells and their adeptness at rewiring molecular circuitry to evade target-specific agents may permit the identification of new molecular targets and lead to the development of novel and more powerful therapeutic approaches.

In support of this idea, Ingram et al. recently reported in mesodermal and neural cells that Hes1 transcription can also be activated by Hedgehog ligands by a mechanism that is absolutely independent of Notch pathway signalling [49]. Therefore, this mechanism of Hes1 activation bypasses γ-secretase inhibition—or any other kind of Notch inhibition—and may maintain significant Hes1 expression even in the complete absence of Notch activation. Moreover, the Notch target Hes1 can directly bind the Gli1 promoter by acting as a transcriptional repressor and may therefore influence Hh signalling in glioblastoma [50]. Interestingly, that work suggested that γ-secretase-mediated Notch inhibition may lead to a rise in Gli1 levels which may produce alterations...
in Hh signalling that in turn may promote tumour survival by Hh overactivation.

The Notch ligand Jagged1 has also been described as a link between the Notch and Wnt pathways. The first evidence reported stemmed from an in silico phylogenetic analysis by which the Jagged1 promoter was identified as a conserved target of Wnt/β-Catenin signalling based on the conservation of specific consensus binding sites [51]. Three years later, Rodilla et al. showed Jagged1 to be the pathological link between the Wnt and Notch pathways in colorectal cancer [52]. Hence, that work established that Notch can be a downstream target of Wnt via β-Catenin-mediated transcriptional activation of the Notch ligand Jagged1. Other Wnt-pathway-belongs proteins such as Dvl and GSK3β have also proved to be involved in a cross-talking with Notch. GSK3β directly binds to the Notch2 ankyrin repeats in the HEK-293 (human embryonic kidney 293) cell line [53] while Dvl binds Notch2 within its C-terminal region in Drosophila [54]. In both cases, Notch activity was reduced. The capability of β-Catenin to modulate the level and transcriptional activity of Notch1/NICD through its direct interaction has also been described in HEK-293 cell line [55].

Finally, the ability of the Hedgehog pathway to modulate Wnt signalling has also been reported. The negative regulation of Wnt signalling by Gli3R activity has been described in mouse and chick embryos [56]. Furthermore, Yanai et al. showed that Gli1 overexpression suppressed Wnt transcriptional activation and found an inverse correlation between Wnt and Hh activation in human gastric tumours [57]. Additionally, the ectopic expression of Gli1 increased the levels of secreted Frizzled-related protein-1 (sFRP1) by direct binding to its promoter in gastric cancer cells [58]. In colorectal cancer, Gli1 was shown to inhibit the proliferation of cancer cells by suppressing activation of the Wnt signalling pathway [59]. The main interactions described in this section for the three pathways are summarised in Figure 1.

In Notch γ-secretase-mediated inhibition, we observed that the reduction in Hes1 protein levels in RMS cells in vitro after GSI treatment was only moderate [18]. Likewise, the reduction in Hes1 in a RMS xenograft mouse model treated with γ-secretase inhibitors was also low. The low inhibition achieved could be explained by low efficiency of the drug or concentration used. Alternatively, the remaining Hes1 expression may be explained, at least in part, in terms of compensatory mechanisms based on pathway cross-talking. Similarly, several works with xenografted rhabdomyosarcoma models agree on the possibility of reducing, but not abolishing, tumour growth by Hh pathway inhibition [33–35]. The compensative activation of other pathways could explain this partial resistance to Hh-specific therapies. The current lack of knowledge on pathway cross-talking in RMS renders it impossible to substantiate these hypotheses; however, the information recently gathered on cross-talking in other tissues may provide us with the guidelines for future research in this field.

Knowledge accumulating in recent years has rendered the extensive cross-talk among signalling pathways clearly manifest. The simplistic view of the pathways as linear entities should give way to a vision of a complex network formed by longitudinal and transverse interactions as a prior step towards improvements in future pathway-targeted therapies. At least in some cancers, deeper understanding of the adeptness of cells at rewiring molecular circuitry to evade target-specific therapies should aid the development of more successful molecular therapies.

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