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Review

Chordoma: the entity

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ABSTRACT

Chordomas are malignant tumors of the axial skeleton, characterized by their locally invasive and slow but aggressive growth. These neoplasms are presumed to be derived from notochordal remnants with a molecular alteration preceding their malignant transformation. As these tumors are most frequently observed on the skull base and sacrum, patients suffering from a chordoma present with debilitating neurological disease, and have an overall 5-year survival rate of 65%. Surgical resection with adjuvant radiotherapy is the first-choice treatment modality in these patients, since chordomas are resistant to conventional chemotherapy. Even so, management of chordomas can be challenging, as chordoma patients often present with recurrent disease. Recent advances in the understanding of the molecular events that contribute to the development of chordomas are promising; the most novel finding being the identification of brachyury in the disease process. Here we present an overview of the current paradigms and summarize relevant research findings.

Contents

1. Introduction

Chordomas are relatively rare neoplasms with an overall incidence of approximately one per million population [1]. These tumors have a

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slowly aggressive and locally invasive character and predominantly arise at the cranial (clivus) and caudal (sacrum) ends of the axial skeleton. Due to the close relationship with vital structures, especially at the skull base, managing these tumors surgically has proven to be a challenge [2]. Therefore, an increasing number of novel (radio)surgical and pharmacological strategies are currently being investigated [3,4]. However, despite combining gross total resection with particle radiation therapy, frequent local recurrence remains a reality, and the 5- and 10 year overall survival rates are circa 65% and 35%, respectively [5–7]. Additional chemotherapy is not implemented in the standard treatment, as chordomas are known to be relatively resistant to conventional chemotherapies [8]. With the aim of improving the management of these tumors, histopathological and molecular research on chordoma tissue and cell lines has been carried out to improve our understanding on the origin of chordoma. So far, most of the research has focused on well-known molecular markers found in other oncologic diseases. While these studies have contributed to our basic understanding of the pathophysiology of chordoma, recent experiments have reflected a paradigm shift away from focusing on important cell-cycle characters towards the investigation of more differentiating molecular markers that are primarily discovered by genome-wide experiments. Proteins involved in the cell-cycle regulation, including the p53 pathway and downstream messengers of the receptor tyrosine kinase pathway, have been mentioned in previous publications. However, the interpretation of more recent genome-wide projects together with associated smaller case–control studies is lacking. It is our understanding that a holistic approach will benefit research on chordomas and will result in an improved strategy for clinical management. In this article, we present an overview of the current paradigms and summarize relevant research findings.

2. Embryology

2.1. Formation of the notochord

The current line of thinking is that chordoma cells originate from remnants of the embryonic notochord [9–11]. For the purpose of clarifying the pathophysiology, we will discuss the function of the notochord and the formation of the vertebral axis. During embryogenesis, in the third week of human development, gastrulation takes place, in which the body axis and the three primary germ layers (ecto-, meso- and endoderm) are formed. One of the key events following this phenomenon is the formation of the notochord; an elongated rod of cells derived from the intraembryonic mesoderm initiated at the midpoint of the vertical axis (primitive node) with a caudo-cranial extension [12].

In the developing embryo, the notochord plays a critical role by producing and secreting important signaling factors (e.g. sonic hedgehog [Shh] [13], bone morphogenetic protein [bmp] [14]) to the surrounding tissue in order to direct organogenesis by providing fate and positional guidance [15]. The notochord also exhibits cartilage-like and epithelial properties [16], with considerable significance as it not only functions as the embryonic backbone, but also orchestrates the formation of the axial skeleton [15]. In this regard, several heterotopic grafting studies have illustrated a crucial role for chordal cells in the formation of somites [17–20]. These paired rectangular shaped segments which bilaterally propagate alongside the notochord are derivatives of the para-axial mesoderm, and in later development (imposed by chordal cells) [21] further evolve by an epithelial-to-mesenchymal transition to form the ventral sclerotomes [22]. Soon after this transformation, the loosely arranged mesenchymal cells will coalesce around the notochord and neural tube (primordial spinal cord) to instigate the formation of the initially unsegmented *perinotochordal* or *perichordal sheath* [19,23–25]. The subsequent events and ultimate fate of the chordal and mesenchymal cells are still not fully understood.

2.2. Formation of the vertebral axis

It has been proposed that mesenchymal cells of the perichordal sheath become cartilaginous (chondroblasts/chondrocytic cells) via a condensation process with a central role for the secreted factors Shh [26] and epimorphin [27] and the production of collagens (types 1, 3 and 9) and type 2A procollagen [28] (all derived from the notochord). Subsequently, in the span of the perinotochordal sheath, condensation of mesenchymal cells is shown to occur in a metameric fashion, where less condensed areas containing the chondrocytic cells form a cartilage template (primordial vertebral body). Formation of this cartilage anlagen and its subsequent ossification ensues in a process called "endochondral ossification" [29]. During this process, the chondrocytic cells become hypertrophic and alter the matrix, enabling it to become mineralized (ossified) by calcium phosphate [30]. Concurrently with the vertebral body morphogenesis, the confined notochordal cells probably undergo apoptosis as mechanical forces increase, or will be pressed out of the primordial bodies into the adjacent more condensed zones [24,31]. In these more condensed areas, where mesenchymal cells still enclose an intact notochordal tube, both cell types will later be replaced by a mature intervertebral disk (IVD). The outermost portion of the IVD (the annulus fibrosus) is generally accepted to originate from the mesenchymal cells, whereas the source of the "*chondrocyte-like*" cells which are found primarily in the inner portion (nucleus pulposus) are shown to differ between chordates [28]. In humans, the notochordal cells in the nucleus pulposus (NP) are hypothesized to either progressively degenerate after coordinating the formation of the NP, or differentiate into the chondrocyte-like cells [32]. Either way, the ultimate fate of the notochordal tissue in the vertebral axis resides in the NP and is usually undetectable after the end of the first decade [33,34].

Occasionally, notochordal cells remain in the IVD — noted as *notochordal rests* or *vestiges*, or can be witnessed in *notochord-like tissue* in the intravertebral region — in which case they are described as "*benign notochordal cell tumors*" (BNCT) [35,36]. These latter lesions also show morphologic characteristics that are different to typical notochordal rests or chordomas (discussed later). Furthermore, it is presumed that from these BNCT, which are found in approximately 20% of autopsies, the malignant variant *chordoma* arises [36]. Evidence in favor of this concept is currently accumulating as Yamaguchi and colleagues described a classical chordoma to closely adjoin a benign notochordal cell tumor in a single section [37]. Confirmation of this association was further provided by Nishigushi et al. [35], who described a 59-year old woman with similar findings in the lumbar vertebra.

Differentiating BNCTs from chordomas is crucial, as BNCTs tend to have a much better prognosis. These benign counterparts can be managed by a wait and scan policy, whereas patients with chordoma benefit from immediate gross surgical resection [38]. In addition, (immuno)histological [39] and anatomical differences have recently become more apparent (for review see Amer and Hameed [38]). Even so, differentiation between the two entities can still be challenging.

2.3. Formation of the skull base

In a similar fashion to the genesis of the vertebrae, the formation of the skull base is predominately preceded by a cartilage intermediate that becomes ossified by the same mechanism of endochondral ossification. This intermediate framework, termed the chondrocranium, forms by the fusion of several precursor cartilages. A set of three cartilage structures — the parachordal, hypophyseal and prechordal cartilages (trabeculae cranii) — together with the cartilage formed from the occipital sclerotomes — fuse to form a continuous platform that makes up the midline of the future skull base. The *occipital sclerotomes* are, as in case of the vertebrae, derived from somites, and the (subsequently formed) cartilage is combined with the *parachordal cartilage* to craft

the cartilaginous continuum called the basioccipital cartilage (part of the future occipital bone). As the name already suggests, the parachordal cartilages lie alongside the notochord, which elongates and stretches cephalically to terminate at Rathke's pouch. Rathke's pouch, the primordial adenohypophysis positioned at the mid-sphenoid synchondrosis, demarks an important border, as the cartilage formed caudal to this structure is derived from mesodermal cells and is influenced mainly by molecular signaling of the notochord. Chondrocranial precursors rostral to this juncture (prechordal cartilages) are derived from neural crest tissue and require induction by signaling of the preoral gut endoderm. The *hypophyseal cartilage*, as an intermediary part between the perichordal and parachordal cartilages, develops around the pituitary, and is formed by differentiation of mesenchymal cells. By the end of the eighth week, the precursor cartilages have fused and the chondrocranium becomes apparent [40].

At the same point in intrauterine life, numerous ossification centers arise and transform most of the chondrocranium into the four major bones that form the mature cranial base (the ethmoid, sphenoid, occipital and temporal bones), leaving only small segments of cartilage to function as growth plates in the spheno-occipital and spheno-petrous sutures. This cartilage at the spheno-occipital synchondrosis, which is responsible for much of the cranial lengthening, becomes ossified at pubertal onset [41]. Even though only investigated in a mouse model, this growth and ossification appears to be regulated by the hedgehog signaling pathway which is induced by signaling originating from notochordal remnants [42].

These notochordal remnants, which can persist throughout adult life, are also recognized in the region of the human skull base in 0.4–2% [43,44] of autopsies, and were first described by Lushka and Virchow in 1857 [45,46]. While Lushka and Virchow both describe the histological appearance of vacuolated cells ("physiliphoren") in the lesions, and their resemblance to the chorda dorsalis, Virchow appointed them as "*Ecchondrosis Prolifera*", suggesting a cartilaginous origin. Müller [10] in 1858, discarded this description and proposed that the lesions have a notochordal source, renaming them as "*Ecchordosis* physaliphora Spheno-occipitalis" (EP). In 1894 [47] and 1895 [48] Ribbert first suggested the term "*chordoma*" to describe a resembling lesion that could be reproduced experimentally by puncturing the nucleus pulposus. It was not before the following century that Hortwitz associated chordal ectopia to the existence of chordoma [49]. Before doing so, Stewart and Burrow [43] had emphasized the clinical desire for differentiation between the two lesions. These authors proposed a nomenclature in which small extravertebral ectopic notochordal rests (jelly-like nodules) with very limited proliferative ability must be considered EPs, and the larger variant which causes symptoms and eventually death should be regarded as chordomas. In the last century, additional features have been proposed to help differentiate between these two lesions. EP patients are described to be usually younger, to have a better prognosis and their lesions are proposed to have a lower MIB-1 labeling index (MIB-1 LI) compared to chordomas, and there is a reduced tendency for contrast enhancement and a frequent location intradurally at the prepontine cistern, typically connected to the clivus with a cartilage/osseous stalk [50,51]. Even so, differentiation is still a challenge, as chordomas can sporadically occur in the intradural space, and these chordomas show a similar prognosis to EP tumors [52]. Histological findings are almost identical as well, as both tumors show similar morphological and immunohistochemical patterns [53,54]. Thus, since differentiation is very difficult and both show similar biological behavior, the question remains whether such a differentiation should be made, or if we should consider these different entities on the same spectrum of pathology [52]. On the contrary, a clear distinction in terms of survival can be made when both are compared to conventional extradural chordomas.

3. Formation of chordoma

Hitherto, a model has been described where chordomas arise via transformation of benign notochordal cells (Fig. 1). However, the question still remains as to what drives the notochordal cells to differentiate into the benign or malignant variant. The recent and popular cancer stem cell (CSC) theory sheds more light on these transformations. This hypothesis states that a small subpopulation of tumor cells which exhibit stem cell characteristics (such as self-renewal, resistance to apoptosis, and the potential for multidirectional differentiation), are the driving force behind tumor growth and the presence of different neoplastic cells [55]. Although debated [56], such CSCs, can be observed as "spheres" by starvation of tumor cells, as this method selects for the most primitive cells by eliminating the differentiated cells that are unable to survive. Recently, in relation to chordoma, Hsu et al. [57] illustrated the formation of sarcospheres in the human chordoma cell line CHJ-7, and reported that these cells have a higher expression of the stem cell marker ALDH1 compared to typical chordoma cells. After injecting these sarcospheres into an athymic mouse, the authors revealed a transformation of these cells in the typical chordoma cells, suggesting a hierarchical model for the differentiation of typical chordoma cells. As this offers an interesting perspective, further investigations concerning the initiating factors for this differentiation step are crucial for our understanding of the development of chordoma.

4. Molecular pathways in chordoma

4.1. Embryological pathways in chordoma

Although a plethora of components involved in the control of embryological development have been investigated, only some of these developmental factors are thought to be crucial for notochordal development and maintenance. Since the notochord is hypothesized to be the predecessor of chordoma, proteins implicated in preservation of this vital structure have also been investigated in chordoma. These experiments have generated interesting results, with one of the most important and promising findings reported by Vujovic et al. [58]. The authors compared thirty-five chordoma tissue samples with 33 normal and 323 other tumors, using a cDNA microarray technique, and reported the expression of the transcription factor *Brachyury* (Bra or T) to be very specific for the notochord and for notochordal derived tumors. This assumption has since been confirmed by the finding of a high protein expression level (90–100%) in chordoma and none in genitourinary neoplasia, metastatic germ cell tumors and clear cell renal cell carcinoma [59,60]. However, using RT-PCR amplification of cDNA samples derived from normal and tumoral tissues, Palena et al. [61] indeed confirmed that there was no abnormal expression of brachyury in the normal tissue, but illustrated an overexpression in tumors of the small intestine, stomach, kidney, bladder, uterus, ovary, and testis. Even so, brachyury has been shown to help in the differential diagnosis of not only chordoma compared to other malignancies, but also to its assumed benign predecessor BNCT, making it a crucial marker which might be implicated in events preceding chordoma formation [62–65].

Following the article of Vujovic and coworkers, the same group [66] reported their genetic analysis of brachyury in 181 tumor samples using *fluorescent in situ hybridization* (FISH) and real-time PCR (qPCR), and found a copy number gain of the brachyury gene in 54%. Interestingly, regardless of the presence of amplification or any other type of chromosomal abnormality involving chromosome 6q27 (brachyury locus), all 23 investigated tumors showed high expression of brachyury mRNA on qPCR analysis. Furthermore, it was shown that RNA-interference mediated silencing of brachyury expression in the U-CH1 cell line results in morphological changes of the tumor cells and in initiation of cell cycle arrest. With the use of a similar experimental design using a novel CHJ7-cell line, Hsu et al. [67] recently confirmed this inhibiting

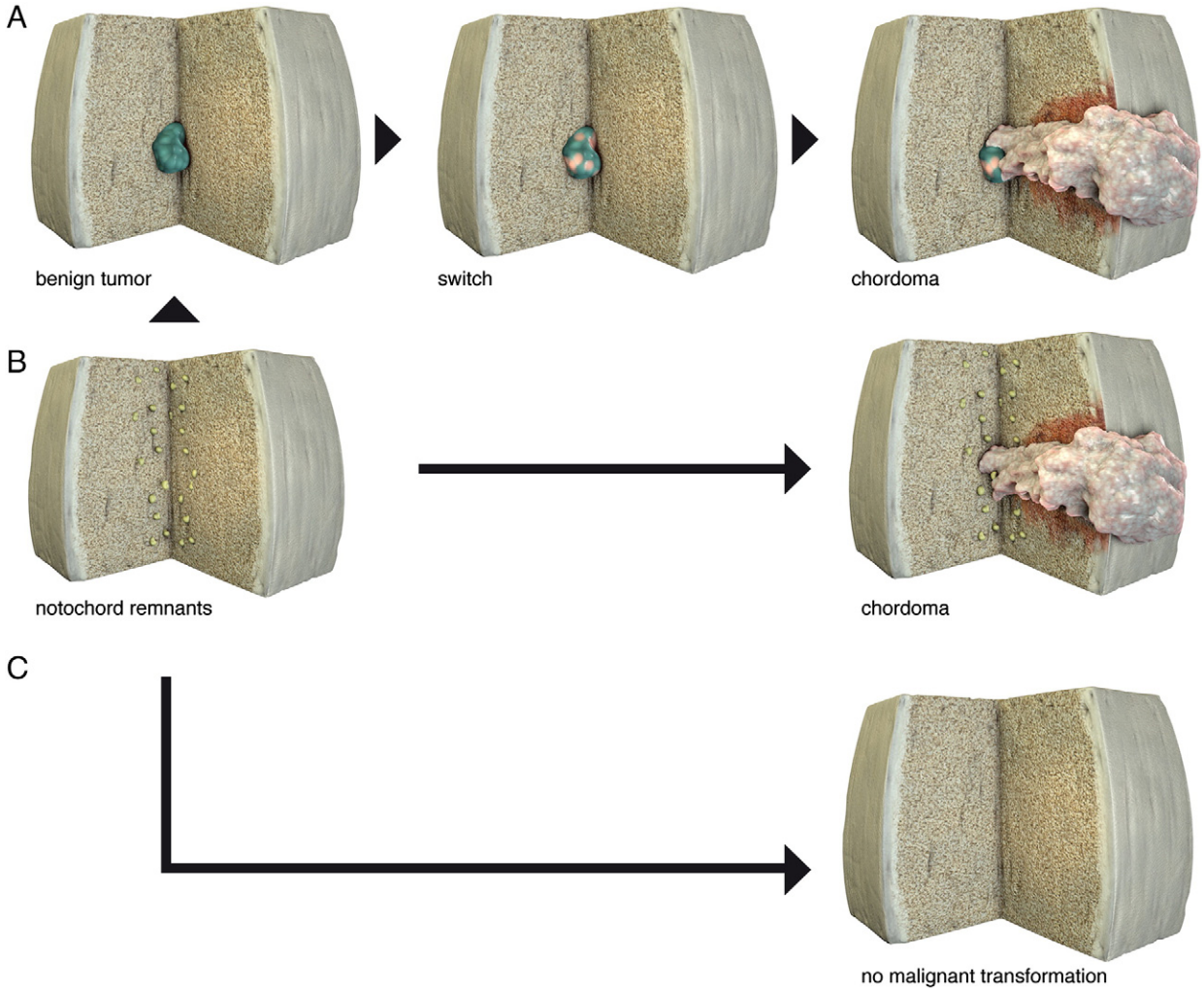

Fig. 1. A hypothesized model of chordomagenesis. Notochordal remnants are observed in 2% of autopsies. Although considered a separate entity, benign notochordal tumors show close resemblance to these lesions due to a common notochordal cell nature. A chordoma might be derived from these benign notochordal tumors, since chordomas have been located next to these lesions only separated by a fibrous septae. A certain malignant transformation in phenotype (switch) would in that case be responsible for the chordomagenesis. (A). Chordomas might also be a result of direct malignant transformation of the notochordal remnant, without a benign notochordal tumor intermediary stage. (B). However, as notochordal remnants are observed more frequently than chordomas, these cells in most cases do not result in a malignant phenotype or benign tumor and are expected to go in apoptosis or to advance in further differentiation. Lastly, chordomas might not be derived from notochordal cells, but might only mimic notochordal morphology due to altered genetic expression (not depicted).

effect. Consequently, as brachyury overexpression in chordoma seems to be a crucial marker for cell cycle progression, and this does not rely on alterations in the genetic material, understanding protein–protein interaction may improve our current insights. In line with this, Shalaby et al. [68] attempted to evaluate the involvement of the potential upstream regulator fibroblastic growth factor receptors (FGFRs), their adaptor signaling protein fibroblast growth factor receptor substrate 2 alpha (FRS2α), and downstream mediators, such as ERK1/2. Although 94% of tumors were positive with immunohistochemical stainings for at least one of the four members of FGFRs, with phosphorylated FRS2α present in a subset of these tumors, no directly mediated effect on ERK1/2 and other downstream moderators, including brachyury, was demonstrated. Moreover, screenings for mutations and amplifications showed no genetic alteration. It thus seems that alternative mechanisms are responsible for activation of brachyury expression, which remain to be unraveled.

In addition to brachyury, the expression of the cellular membrane protein galectin-3 has been implicated in the pathophysiology of chordomas. Galectin-3 expression was first described to be present in the human notochord in 1997 by Götz et al. [69], and the expression was noted to increase as gestation proceeded beyond the 8th week. In the same report, the authors illustrated positive galectin-3 immunostainings in chordoma specimens, contributing to the notochordal origin of chordomas. Recently, this insight was confirmed as it was demonstrated that benign notochordal cell tumors showed almost no positive staining when compared to an aligning chordoma [39]. However, while its expression has been characterized in chordoma, no reports have been made with regard to the functional aspect of this protein. Future studies might hold much promise, as galectin-3 has been associated with more malignant behavior in other tumors [70,71].

Besides brachyury and galectin-3, the canonical Wingless (Wnt) pathway is thought to maintain the notochord fate during development [72] and has been associated with multiple neoplasms [73]. Furthermore, Wnt proteins and the downstream effector molecule β-catenin regulate cranial base development, and β-catenin deficient mice are shown to lack typical growth plate zones at the synchondroses in addition to delayed endochondral ossification [74]. Moreover, in *Xenopus laevis*, Wnt signaling is shown to be essential for the expression of brachyury [75,76]. Notwithstanding the fact that Wnt regulate intracellular mechanisms in an intricate manner, several interactions of the canonical Wnt pathway have been identified. In these series of events, Wnt signaling is exerted by binding one of the large family of Wnt glycol–protein molecules to the cell-surface receptor Frizzled and co-receptor LRP5–LRP6. In the absence of this binding, the intracellular

β-catenin is targeted for degradation by a cytoplasmic 'destruction complex' via phosphorylation by glycogen synthase kinase 3β (GSK3β) and casein kinase 1α (CK1α) [77,78]. Therefore, non-activated Wnt signaling results in low levels of cytoplasmic β-catenin. However, when activated, Frizzled receptor transfers the signal by inactivation of the destruction complex through recruitment of disheveled (Dvl1) to the receptor complex, which consequently results in cytoplasmic accumulation of β-catenin [77]. β-Catenin is subsequently found to be localized in the nucleus, indicating a nuclear transposition, and finally serves as a transcription factor for multiple genes [78].

Accumulation of β-catenin has been encountered in 56–78% of chordoma cases. However, there is a remarkable report by Cho et al. [79], who, in 14 chordoma patients, found no positive staining, except for one weak staining. This controversial result might be due to the use of different primary antibodies compared to those used in the other studies. Aside from the amount of positive stainings, Horiguchi et al. [80] and Triana et al. [81] stated that their staining of β-catenin represented membranous immunoreactivity, reflecting a mechanism other than that suggested in the canonical pathway. One way to explain these findings is by the interaction of β-catenin with the cell adhesion molecules E-cadherin and N-cadherin. In line with this, Hendriksen et al. [82] showed that upon Wnt3a stimulation, dephosphorylated β-catenin is recruited to the plasma membrane. This migration occurred only in E-cadherin deficient cells, and not in E-cadherin expressing cells, suggesting a new step in the conveying of the Wnt signal. Furthermore, Wahl et al. [83] showed that N-cadherin–catenin complexes are formed prior to the transportation to the plasma membrane where linkage to the actin cytoskeleton can be established. Both of these findings become particularly interesting as analysis of multiple cell adhesion molecules in chordoma illustrate an overexpression of N-cadherin and a downregulation of E-cadherin. Thus, protein–protein regulation is again likely to be responsible, as there have been no reports of frequent aberration on the coding sequences containing these adhesion molecules.

Chromosomal aberrations are observed for chromosome 3 and 1p36 in many chordoma patients, with the latter also providing a connective link to familiar chordoma. These chromosomal changes might be accountable for the overexpression of β-catenin as chromosome 3 entails the GSK3β gene and locus 1p36 comprises the adaptor molecule Dvl1. Although no sequence analysis has been performed on GSK-3 β, the gene transcript of Dvl1 has been noted by Riva et al. [84] to encompass an abnormal 350 bp spliced transcript fragment in 50% of the tumors, and a complete absence of the Dvl1 transcript in the remaining half. The effect of this loss of transcript on β-catenin's cellular action has yet to be analyzed in chordoma cell lines.

Thus, the Wnt pathway and the closely related cadherins appear to play a considerable role in the cellular process of chordoma. Further investigation should reveal the dimension of this impact, perhaps by suppressing their expression at the transcriptional level.

Finally, and conceivably the most well-known pathway central to the genesis of the human embryo, is the *hedgehog* (*HH*) *signaling pathway*. Although this pathway is observed to be altered in many tumors, there is a relative paucity of information in the literature regarding its involvement in chordoma. *Shh*, one of three family members of the hedgehog ligands, is the best studied molecule in the hedgehog family, and known to be involved in vertebrate development [85]. Upon binding to the transmembrane receptor *Patched1* (PTCH1), Shh exerts its biological signal by inducing a conformational change of the PTCH1 receptor. Consequently, PTCH1 loses its ability to repress the constitutive signaling activity of *Smoothened* (SMO), an additional transmembrane receptor. Subsequent activation of downstream effector molecules Gli1, Gli2 and Gli3 mediated by SMO ensues via complex mechanisms which are not entirely understood. However, these transcription factors have been associated with multiple intracellular actions, including transcriptional activation of many genes involved in development and cross-talk with the Wnt and p53 pathway [86,87]. Concerning HH-signaling in chordoma, Cates and co-workers [88] investigated the expression of *Shh*. In their analysis of twenty-three chordomas, they found *Shh* to be overexpressed in twenty-two cases (96%), and 89% of these cases also showed a positive staining for PTC1 receptor. However, this is not a chordoma specific marker, as anti-*Shh* antibodies also reacted in 50% of the notochordal cases, and in 81% of chondrosarcoma cases. Nevertheless, greater understanding of the involvement of this pathway in chordoma might unravel new therapeutic strategies.

4.2. Other pathways in chordoma

Numerous studies have been carried out in an attempt to describe the molecular–pathological pathways involved in the formation of chordoma. Even if no effective medical treatment is currently available for chordomas, novel molecular biomarkers are increasingly being identified for the development of targeted therapies. In the following sections, we will summarize the current knowledge on the pathophysiology of chordoma.

4.2.1. Genome wide investigations

The genetic profile of chordomas has been intensively investigated. Although varying data are published on this matter, some genetic aberrations have been consistently demonstrated. Most experiments concerning genetic integrity have focused on chromosomal segregation in cases of sporadic chordoma. Familial chordomas, however, rarely occur and have been linked to susceptibility of loci 1p36 [89] and 7q33 [90] (for an overview of families see Larizza et al. [91]). More recently, with the isolation of brachyury as a characteristic feature of chordomas, Yang et al. [92] linked the brachyury coding sequence to affected individuals in four families with a frequent occurrence of chordoma.

Reports on sporadic cases of chordoma, however, reveal a considerable discrepancy regarding karyotype integrity. The incidence of aneuploidy in these cohorts ranges from 26% to 100% (mean 53%) [93–100], with an average of 3.2 losses and 4.2 gains per tumor [97]. Aneuploidy and chromosome instability are frequently attributed to gains or losses of either whole chromosomes or specific regions, which typically include losses of 1p [84,96,97,99,101–103] and 3p [93,96,97,99,101–103] and gains of chromosome 7 in most studies [97,99,101,102]. The frequency of aneuploidy seems to be comparable for the classic and chondroid subgroup, but occurs more often in dedifferentiated chordomas [95,100].

In the same vein, Almefty et al. [93], in an analysis of sixty-four patients, demonstrated a substantial increase in recurrence rate for patients with abnormal as compared to normal karyotypes. Detailed analysis revealed odds ratios (OR) for recurrence for patients with abnormality on chromosomes 3, 4, 12, 13 and 14, compared to cases with intact copies of 10, 7.5, 22, 24 and 18, respectively. The overall OR for recurrence calculated for an abnormal karyotype was 12.45. Besides recurrence rate, recurrence-free survival and overall survival was shown to be significantly impaired in patients with abnormal karyotypes, suggesting that these findings may have a possible role in the prediction of prognosis, as previously suggested by Schoedel et al. [104] in 1995.

Possible explanations for the origin of chromosomal instability include faulty cell-cycle regulation and telomerase activity [105,106]. Increased telomerase activity and telomere shortening are observed in various tumors, and have been related to the stability of the genome and to a gradual increase in chromosomal aberrations over time [107–112]. Thus far, five studies have been published on the subject of telomere length or telomerase activity in chordomas. Three of these originated from the group of Butler et al. [113–115], who showed increased telomere length in a total of 10 chordomas (considering no double report of patients) and three patients of which one had increased telomerase activity. Expression of human telomerase reverse transcriptase (hTERT) messenger RNA was investigated by Pallini et al.

[116] in 26 chordoma cases in a study which yielded remarkable findings. Firstly, it was shown that despite the fact that only 14 out of 29 (48.3%) classic chordoma specimens expressed hTERT, none of the four chondroid and both of the two more malignant dedifferentiated variants did. Moreover, this seems to affect tumor behavior, as these authors reported that patients with expression of hTERT have shorter tumor doubling time, increased proliferation, an association with mutation of the p53 tumor suppressor gene, and a decrease in recurrence-free probability. In addition, in a different report from the same group, similar proportions (42%) of hTERT expression were shown in a group of seven patients, and found exclusively in recurrent chordomas [117]. However, although it seems that telomerase activity is present in a proportion of chordoma cases and has prognostic value, the basic mechanisms underlying hTERT and further downstream effects are still not well understood.

Besides the enhancement of chromosome aberration by hTERT expression, another phenomenon in tumor biology and chordomagenesis, called chromothripsis, was recently postulated by Stephens et al. [118]. This potential mechanism for genomic rearrangement refers to a single catastrophic event resulting in multiple genomic rearrangements, and appears to be more frequent in bone cancers. What defines this single event and its cause has yet to be clarified.

4.2.2. Cell cycle regulation

Denominated as the guardian of the genome, p53 exhibits multiple functions in the regulation of cellular processes in response to various forms of stress. Following DNA damage, p53 exerts its tumor suppressive function by inducing a reversible cell cycle arrest or apoptosis [119]. Therefore, loss of p53 function is an important process in malignant progression, and may result from alteration in the gene encoding the protein, or from diminished activation due to protein–protein interactions [120]. Mutations in the p53 gene, which is located on 17p13.1, occur in more than 50% of human cancers [121]. In relation to chordoma, relatively few studies have focused on genetic mutations of the p53 gene, while many have focused on its protein expression. Naka et al. [122] screened thirty-seven chordoma samples for mutations on specific exons 5, 7, and 8, and found no changes. However, cytogenetic analysis using fluorescence in situ hybridization exposed the 17p13.1 locus to be deleted in 17.6% of cases with a higher occurrence in recurrent tumors [101]. Loss of heterozygosity (LOH) on 17p13 was also reported for 52% of 23 cases, but this was not correlated to either overall survival or expression of the (impaired) p53 protein product [123]. This might indicate that alterations on the gene level are located on different exons or LOH accounts for other closely located genes. DNA sequencing of the full p53 gene would clarify such ambiguity, but to date has not been reported.

However, data concerning p53 protein overexpression are available but varying expression has been reported between 0% and 53% [117,122–127]. Even though all of the studies are based on immohistochemical techniques, the discrepancy in outcome might be explained by the use of semi-qualitative versus quantitative methods of analysis, and by differences in interpretations (cut-off points) of the data. In addition, positive staining for p53 should be interpreted carefully, as this does not necessarily indicate overexpression of a mutant protein, even though wild type p53 is usually not detected at high concentration because of its relatively short half-life of approximately 20 min [128]. Other factors such as protein–protein interactions and a disruption of the degradation pathway should therefore also be considered.

Concerning both mechanisms, the mouse double murine 2 (MDM2) protein negatively regulates the function of p53 by binding at the N-terminal end and, by means of ubiqitination, modifies it for subsequent degradation. Expression of MDM2 itself is also controlled by p53 transcription, making it a negative feedback regulatory system [129]. In chordoma, MDM-2 gene amplification is noted in 15% of cases, however no sequence analysis has been reported to account for possible mutations [122]. Moreover, a significant correlation has been reported for p53 protein expression and MDM2 overexpression and, when present, both separately show a higher MIB-1 LI [122]. Considering their cellular behavior, one might not expect to find an overexpression of both in a dividing cell. A potential explanation for this phenomenon might be that the mutant p53–MDM2 complexes are responsible for a "gain of function" phenotype [130]. Accumulation of p53 might also occur if the wild type protein is altered by an additional protein (e.g. HSP90) [131], which opposes or counteracts the binding capability of MDM2. Furthermore, MDM2 might necessitate additional proteins for the ubiqitination of p53. In keeping with this notion, a crucial regulator has been shown for the retinoblastoma (Rb) protein [132].

Direct inactivation of the tumor suppressor Rb1 via mutation/deletion of the gene transcript (e.g. osteosarcoma) and functional inactivation due to (lack of) interaction with upstream regulators, including CDK4 (e.g. glioblastoma), cyclin D1 (lung carcinoma), and p16/INK4A (e.g. T cell lymphoma), are often described in many human neoplasms [133–136].

Loss of heterozygosity for the Rb gene transcript in chordoma was described by Eisenberg et al. [137] in 1996, who reported loss of heterozygosity in two introns (17, 20) in 2 out of 7 cases. Furthermore, alterations of chromosome 13, where the Rb gene is located (13q14.2), are associated with frequent recurrence and a very short survival [93]. Moreover, chromosomal loss on chromosome 13 occurs in 56% of the patients, with involvement of the specific 13q14.2 locus in an additional 18% [93]. Similar proportions have been reported for Rb protein expression, which are absent in 52% of the chordoma tissues [122]. Similar protein expression level were reported for cyclin D1, which was absent in 57% of the cases. Only one paper has been published regarding the genomic integrity of this enzyme's transcript, and reported a loss of heterozygosity in 2 out of the seven chordomas investigated [137].

Conversely, cytogenetic aberrations have been frequently reported for another associated upstream regulator, $p16^{INK4A}$. Tumor suppressive function of this protein is mediated via an inhibitory effect on the phosphorylation process of the Rb protein, by preventing CDK4/cyclin D1 complex formation, confining the cell to the G1 cell cycle arrest [138]. The CDKN2A gene, which encodes the $p16^{INK4A}$ protein, is located on 9p21 and was shown by Hallor et al. [139] to be homo- or heterozygously lost in 70% of chordoma tumors. Immunocytochemistry has also revealed a 74% to 100% absence of p16 protein expression in several cohorts [122,140,141]. Analysis of the 9p21 region has also been shown to be of prognostic value, as deletion of this specific area will potentially show a more aggressive clinical course and shorter overall survival [123]. It thus appears to be the case that activation of the Rb protein might have a significant impact on the clinical condition of patients with chordoma, and that this is most probably mediated by a frequent deletion of the CDKN2A gene. Further reports on the expression of CDK4 activation in chordoma might confirm this notion, and also offer a potential mechanism for chemotherapy, as CDK4 inhibitors are shown to have anti-tumoral activity [142].

In addition to the production of $p16^{ink4a}$ and its effect on the Rb-pathway, another protein product of the CDNK2A gene is formed, called $p14^{arf}$. This protein has been reported to interfere with the function of MDM-2, and by blocking the MDM-2 mediated ubiquitination, facilitates the action of p53.

Deregulation of the function or expression of cell cycle regulatory proteins is one of the hallmarks of cancer. P53 overexpression and loss of the intact CDKN2A gene are the main findings reported by studies on chordoma patients. As much as we know about the presence or absence of these factors, information about their functional effects is entirely absent. Moreover, aside from these two findings, information regarding other cell-cycle regulators is very limited.

4.2.3. Receptor tyrosine kinase

Receptor tyrosine kinases (RTKs) are important mediators of extracellular induced intracellular signaling, and if constitutively activated are

Table 1
Overview of molecular analysis performed on receptor tyrosine kinases in chordoma. Abbreviations: SM = silent mutations, AM = activating mutations, M541L = exon 10 mutation, AS 9&15 = alternative splicing of exons 9 and 15, n.s. = not specified. IHC = immunohistochemistry, WB = western blotting, IP = immunoprecipitation, N. = non-phosphorylated, P. = phosphorylated.

Receptor tyrosine kinases (RTKs)					
Marker	IHC N.	WB/IP N.	WB/IP P.	mRNA overexp. (exon)	Sequence analysis (exon)
PDGFα				100% (n.s.) [148]	
PDGFRα	100% [148,146]	100% [148]	100% [148]	17% (10, 12, 14, 16, 18) [148]	20% SM, 0% AM (10–22) [148]; 0% (12 & 18) [228]
PDGFβ				98% (10–20) [148,149]	
PDGFRβ	100% [148,146,149–229]	98% [148,149]	100% [148,149]	25% (10–20) [148]	45% SM, 0% AM (10–20) [148]; 0% (12 & 18) [228]
EGF				100% (18–20) [149]	
EGFR	62% [149,145,154,151]	77% [149]	88% [149]		0% (18–21) [149,154]
p-EGFR	50% [145,154]				
HER2	18% [145,151]	43% [149]		0% (n.s.) [149]	
c-Met	86% [145,150–231]				
c-Kit	49% [148,146,145]	86% [148]	100% [148]	18% (n.s.) [148]; 0% (11) [228]	20% SM, 100% AS 9 & 15, 0% AM (8–21) [148]
pIGF1R/pIR	41% [140]				
NGF	96% [232]				
TrkA	93% [232]				

important for malignant transformation and tumor proliferation. As a large group of transmembrane proteins which include families such as the epidermal growth factor receptor (EGFR) family, the platelet-derived growth factor receptor (PDGFR) family, and the mesenchymal–epithelial transition factor receptor (c-MET), RTKs are generally composed of two domains, a ligand-binding extracellular domain, and a catalytic intracellular kinase domain. For many RTKs, binding of a ligand results in *induced proximity* and *transautophosphorylation*, processes, in which two receptor chains dimerize and, as both kinase domains interact, subsequent cross-phosphorylation on multiple tyrosines takes place [143]. Besides phosphorylation of the kinase domains, adjacent tyrosines are also phosphorylated and serve as docking sites for specific intracellular proteins, with the best-characterized being the SH2-domain-containing proteins. These proteins often serve as transmitters of downstream signaling by activating second messenger modules that lack the SH2 domain. Ras, superfamily of monomeric GTPases, exemplifies one of the most important second messenger modules. Activation of Ras by promotion of its GTP-bound state occurs via RTK adaptor molecules grouped as guanine exchange factors (GEF, e.g. SOS and Grb2). Once activated, GTP-Ras instigates an intricate network of intracellular phosphorylation cascades, of which activation of the RAS/RAF/MEK/ERK pathway and the PI3K/RAS/Akt/mTOR pathway are most frequently described. Even though several intracytoplasmic crosslink's with other pathways occur, both of these classical pathways have been extensively described as being responsible for transmission of proliferative signals from the RTK, and dysregulation of this pathway results in the uncontrolled proliferation and malignant transformation of naïve cells [144].

Many molecular studies have focused on the expression of receptor tyrosine kinases and their related secondary messengers in chordoma (Tables 1, 2a and 2b). Despite some varying results, these experiments have improved our understanding in regard to their involvement in the pathophysiology. While it has been shown that some RTKs are infrequently expressed, others are demonstrated to have repeated high expression levels and therefore seem to play a considerable role in chordoma's makeup. The platelet derived growth factor receptor (PDGFR), for instance, is observed consistently in the phosphorylated active form in almost all chordoma tissues [145–149]. Real time RT-PCR analysis on the PDGFR alpha and beta comparing a group of 23 chordoma cases to a pool of normal mesenchymal tissues revealed a higher median expression of PDGFR beta ($2^{-\Delta\Delta Ct} = 2.34$), but did not show an overall increased transcribed status for PDGFR alpha ($2^{-\Delta\Delta Ct} = 0.7$) [148]. Considering that no activating mutations have been found, and the fact that FISH analysis on the associated chromosomes demonstrated diploidy in 79% of the cases, it seems likely that activation of the receptor is ligand mediated. Para- or autocrine activation might account for the activated state as mRNA encoding both PDGFα and β ligands are present in nearly all of the tumors [148,149]. However, quantification of the protein levels of these ligands compared to healthy tissue is required, since mRNA-expression microarray analysis carried out by Vujovic et al. [58] showed a conversely low expression mRNA expression of PDGFα in chordoma cells as compared to other tumors.

Table 2a
Overview of downstream second messengers of RTK's signaling.

Second messengers of RTK's signaling				
Marker	IHC normal	WB/IP normal	WB/IP phospho	Mutations (exons)
ERK		100% [149]	100% [149]	
p-ERK	88% [145,140]	100% [149]		
p110 (PI3K)				0% (4, 5, 7, 9 & 20) [149,141]
p85 (PI3K)		95% [149]		
PDK1		100% [158]		
p-PDK1		100% [158]		
PTEN	80% [141,163,154]	95% [149]		0% (5–9) [149]
Akt		93% [149,141]	100% [149]	
p-Akt	85% [145,141]			
TSC1	35% [141]			
TSC2	100% [141]			
p-TSC2	96% [141]			
mTOR	75% [141]	93% [149,141]	100% [149]	
p-mTOR	27% [141]	17% [141]		
Src		100% [166]		
pSrc		100% [166]		

Table 2b
Overview of downstream effector molecules of RTK's signaling.

Effector molecules of RTK's signaling			
Marker	IHC norm.	WB/IP norm.	WB/IP phosph.
S6K	100% [141]	100% [141]	
pS6K	62% [141]	100% [141]	
S6		100% [141]	
p-S6	100% [163]	17% [141]	
4EBP1		91% [149]	65% [149]
p-4EBP1	97% [141,163]	83% [141]	
eiF-4E	98% [141]		
Stat3		100% [166]	
p-STAT3	92% [145,165]	100% [166]	
p-BAD (S75)	32% [140]		
p-BAD (S99)	77% [140]		
p-PRAS40	64% [163]		

Paracrine regulation by the surrounding tissues might also offer an alternative explanation for the phosphorylated form of the PDGFR.

Besides the PDGFR, the epidermal growth factor receptor (EGFR) has also received much attention in the literature. For this receptor, however, immunohistochemistry studies have shown varying degrees of positive signal in relation to chordoma, ranging from 32%, described by Walter et al. [150], to 92%, reported by Weinberger et al. [151]. These different findings make the interpretation of the extent of involvement rather difficult. A promising link between the gene loci of the receptor, which are located on the frequently amplified chromosome 7, was negated as Walter et al. showed this to not be correlated to the protein expression seen on immunohistochemical stainings [150]. However, the authors did illustrate a correlation between high expression of the mesenchymal–epithelial transition factor/hepatocyte growth factor receptor (c-MET) and chromosome 7 amplification. On average, immunohistochemical reactivity of this receptor is found to be present in 86% of the patients, and high expression is significantly correlated to the expression of matrix metalloproteinase-1 and -2, suggesting an effect on local invasion and metastasis. In fact, in an experimental design using a human chordoma cell line (CCL-3), Ostroumov and Hunter [152] illustrated the increased migratory capacity of chordoma cells following stimulation with its ligand HGF. Unfortunately, as these cells have been shown not to recapitulate chordoma cells, this concept has yet to be confirmed in an established chordoma cell line.

Concerning the downstream signaling following RTK's phosphorylation, a considerable number of second messengers are reported to be overexpressed, and these are cross-linked in a very complex manner [153].

As previously mentioned, downstream signaling subsequent to the transautophosphorylation process occurs via activation of adapter molecules to the phosphorylated non-kinase regions of the receptor. For the canonical MAPK pathway (RAS/RAF/MEK/ERK), subsequent activation of the RAS protein results in a cascade of phosphorylation signaling. This eventually leads to modification of proteins involved in cell cycle progression, transformation and cellular motility. In chordoma, some studies have focused on the genetic integrity of the family of RAS protein-sequences (KRAS, HRAS, NRAS and Rheb), but, using PCR and direct DNA sequencing, have found none to show alterations [68,141,149,154]. However, to the authors' knowledge, no study has investigated the qualification or quantification of the (active) Ras protein, making its active condition generally presumed rather than scientifically proven. The same argument applies to the downstream activated BRAF protein. This serine/threonine kinase is commonly shown to be activated by somatic point mutation in human cancer [155]. Mutated BRAF can exhibit constant activation and consequently results in high cellular activity of the extracellular signal-regulated kinase (ERK) mediated via the activation of MAPK/ERK kinase (MEK) [156]. However, although western blot analysis detected active phosphorylated ERK in nearly all chordoma tissue, denaturing high-performance liquid chromatography (DHPLC) for the BRAF gene did not show any genetic alterations on two frequently altered exons (11 & 15) [68]. This suggests that abnormal stimulation by auto- or paracrine growth factor is responsible for the constitutive RAS/RAF/MEK/ERK signaling, or that ERK activation might be subsequent to interaction with other proteins external to this classical pathway.

Besides signaling from the RAS/RAF/MEK/ERK pathway, the canonical signaling cascade of phosphoinositide-3-kinase (PI3K) becomes activated either through direct interaction of its p85 subunit with the intracytoplasmic phosphotyrosine tail of the RTKs, or indirectly as intermediate activators, such as RAS, enhance its catalytic p110 domain [157]. Once activated, the PI3K exerts its effect by converting the phosphatidylinositol (4,5) bisphosphate (PIP2) to the active phosphatidylinositol (3–5) triphosphate (PIP3), which recruits and activates phosphatidylinositol-dependent kinase 1 (PDK1). Schwab et al. [158] detected the presence of this active phosphorylated protein in all of their investigated chordoma material on western blot. Furthermore, like others, they have also reported on the presence of its associated downstream messenger protein kinase B (PKB/AKT), and noted it to be frequently phosphorylated, suggesting that it has an active function in chordoma. This function becomes more apparent as known subsequent targets of AKT, including GSK3β, mammalian target of rapamycin (mTOR), and the pro-apoptotic BCL2-associated agonist of cell death (BAD) [159,160] are shown to be implicated in chordoma in several studies. Activated mTOR is also recognized to act as an upstream regulator of many important proteins. One of the proteins acknowledged to be regulated by mTOR is the S6 kinase (S6K) [161]. After phosphorylation by mTOR, activated S6K is responsible for the activation of the ribosomal S6 protein and, via mechanisms which are still not completely understood, this pathway seems critical for several diverse and important cellular functions, including protein synthesis, metabolism and cell growth [161]. Since chordoma patients frequently exhibit active forms of both proteins, this might explain the large cells observed in microscopic analysis [149,162,163]. However, besides S6K, mTOR has been illustrated to elicit active forms of several other important proteins in cancer, of which the cytoplasmic transcription factor signal transducer and activator of transcription 3 (STAT3), and the eukaryotic translation initiation factor 4E (eIF4E), are particularly interesting [164]. Stat3, which can also bind directly to the EGFR, has been demonstrated to be highly expressed on immunohistochemical examination in half of the chordoma cases. It is also associated with aggressive tumor behavior and decreased overall survival in chordoma patients [165]. Blockage of this signaling by inhibition of the phosphorylated Stat3 and the oncogenic upstream regulator Scr by 2-cyano-3,12-dioxooleana-1,9 (11)-dien-28-oic acid-methyl ester (CDDO-Me), resulted in chordoma cell growth inhibition [166]. Even though no prognostic value has been proposed for eIF4E, an important molecule involved in the regulation of RNA translation, this protein has been proposed as an interesting therapeutic target against cancer [167]. In a normal functioning cell, eIF4E is prevented from binding to mature mRNA, through binding of hypophosphorylated eIF4E binding proteins (4EBPs), and so attenuates its mitogenic capacity. However, when 4EBP becomes phosphorylated, as it is the case in most chordoma patients, 4EBP loses its capacity to bind eIF4E, and in this way facilitates mitogenesis [141,149,163].

Since all these downstream molecules have been observed to be activated, an explanation has yet to be offered for the failed regulation by inhibitory phosphatases such as the tensin homology (PTEN), which is responsible for the regulation of both mTOR and STAT3 [164]. Some authors have suggested that more than half of chordoma cases lack the expression of PTEN [163]. Other larger series have demonstrated absence in only 10–15% [141,154]. In their analysis of 22 chordoma patients, Tamborini et al. [149] reported the expression of PTEN on immunoblot analysis in 95% of cases, and found no genetic mutation in any cases. Impaired function of this tumor suppressor might be induced by inactivation due to interactions with other proteins.

One such protein might be a member of the Src family of protein–tyrosine kinases, which play an important role in signal integration and have been shown to alter the function of PTEN [168]. As this protein has been shown to be overexpressed in a phosphorylated state in all chordoma cases, this hypothesis might clarify the increase in p53 observed in chordoma, as PTEN is also shown to regulate the transcriptional activity of p53 by antagonizing the Akt mediated activation of Mdm2 [169,170].

Two other tumor suppressor proteins — tuberous sclerosis protein (TSC) 1 and 2 — are also involved in regulation of the PI3K/AKT/mTOR pathway. In their normal cellular function, TSC1 and TSC2 form a complex that inhibits downstream signaling of mTOR and thereby prevents cellular growth and proliferation. Mutations on the encoding genes are associated with the tuberous sclerosis complex, a rare inherited autosomal disorder that is characterized by the presence of multiple hamartomas in possibly all organs [171]. In the literature, some reports have been made about chordomagenesis in a subgroup of these TSC-patients, with an occurrence almost exclusively in the pediatric population [172–178]. As this on its own is a remarkable finding, it must be noted that such a tumor might easily be mistaken for chordoma, while it represents a giant notochordal hamartoma of intraosseous origin (also known as BNCT) [179]. Additional evidence in support of this notion comes from McMaster et al. [180], who illustrate the substantial difference in survival in pediatric chordoma and TSC associated chordoma (favoring the last). However, in order to gain further insight and to improve discriminative capacity, further detailed investigation (e.g. immunohistochemical analysis) is required [39].

Taken together, studies performed on RTKs and their downstream messengers illustrate active signaling via the canonical RAS/RAF/ERK and PI3K/PDK/AKT/mTOR pathways.

Most of these second messengers are also shown to be present (in a large proportion of chordoma patients) in their active phosphorylated state, suggesting an important involvement in the disease process. Constraining signaling in this frequently activated pathway via pharmacological reagents has been proven to be effective in a number of case reports and small clinical trials [181]. Recently, Stacchiotti et al. [182] reported the results of their phase II clinical trial using imatinib mesylate, a kinase inhibitor primarily targeting PDGFR. The authors reported very promising findings, but also discussed the presence of PDGFR in patients with no response to the drug. This highlights the possible implication of other members of the RTK family of proteins in the (in)effect of this drug, as suggested by previous data [183,184]. It might therefore be relevant to investigate the expression pattern of multiple RTKs in these patient cohorts.

4.2.4. Cell adhesion molecules and matrix metalloproteinases

Cell adhesion molecules (CAMs) encompass particular properties which are involved in cell–cell and cell–matrix interactions, and as a result participate in the induction and maintenance of cellular differentiation, proliferation, and migrations [185]. The large number of CAMs is divided into four major families, of which the cadherins and the immunoglobulin superfamily are shown to be present in chordoma tissues [80,185].

Cadherins are highly conserved Ca^{2+}-dependent cell-surface receptors that are known for their involvement in crucial developmental steps in early embryonic morphogenesis and carcinogenesis [186]. These proteins form trans-interactions on (mostly hemophilic) opposing cells by binding their extracellular domain, whereas the intracellular domain is responsible for modification of intracellular signaling by constituting a protein complex with members of the catenin family and the p120 protein [187,188]. The classic E-, P- and N-cadherin were the first cadherins to be identified and have been extensively studied in the pathophysiology of multiple cancers [189,190]. Several mechanisms have been proposed for their implication in neoplastic cell progression and metastasis, including cadherin switch and loss of contact inhibition.

Cadherin switch refers to a process by which cells alter their expression of cadherin isoforms. Although this represents a common phenomenon during development, in epithelial cancers a certain isoform-switch from E- to N-cadherin is associated with an invasive and metastatic phenotype [191]. In general, the "invasion-suppressor" E-cadherin initiates strong cell–cell bonds by clustering in complexes, whereas upregulation of N-cadherin is shown to induce a scattered morphology, increased motility and invasive potential [192]. Interestingly, this pro-invasive character seems to be independent of E-cadherin's existence in the cell, indicating a dominant role for N-cadherin [192]. This same principle of cadherin-switch has been demonstrated in chordoma. Triana et al. [81] described a patient with recurrent chordoma who exhibited a high E-cadherin expression on primary resection and a twentyfold decrease in the recurrent tumor. The opposite was illustrated for N-cadherin, in that a threefold upregulation was noticed in the recurrent tumors. As previously described, a relationship with survival has been proposed, although this appears to differ according to the investigative technique used. More investigation is needed in order to understand this switching.

In addition to cadherin switch, loss of contact inhibition probably indicates one of the most important mechanisms for an infiltrative and metastatic phenotype. This principle reflects an inhibition of cell growth by the binding of two or more cells to each other. E-cadherin seems to play a crucial role in the underlying mechanism, as loss of cell–cell adhesion of this transmembrane protein has been illustrated to induce early invasion and metastasis [193]. Loss of functional E-cadherin might be due to several factors, including aberrations in the reading frame, transcriptional repression, or protein alteration by protein–protein interaction. Research concerning chordoma has mainly focused on the presence of the cadherin protein product, its relation to survival, and the distinctive character of chordoma in contrast to other diagnosis [79,80,194–196]. In this regard, E-cadherin is variably detected in chordoma, but if present can differentiate between chondrosarcoma, where its expression is reported to be absent [194,195]. Besides the variable expression, ranging from a positive intense expression in most cells reported by Naka et al. and Mori et al., to very low/no staining described by Horiguchi et al. and Laskin et al., E-cadherin expression seems to be a dynamic protein localized either on the bordering membranes of neighboring tumor cells or inside the cell's nucleus [80,194–196]. In addition, Laskin and colleagues described this expression to be stronger in the physallyphorous cells and absent in the spindle cells, indicating a possible marker for chordoma cell differentiation.

However, besides protein expression, evidence for E-cadherin's function in chordoma is still lacking. In particular, analysis of the coding sequence integrity and its relation to intracellular signaling might shed more light on the pathological mechanisms of chordoma. In cancer, the regulation of cadherin in signaling has also not been fully elucidated, though it is believed to be mediated via clustering of its intracellular affixed catenin proteins, or by altering receptor responsiveness of RTKs [197]. In regard to the latter, it has been shown that an intact ectodomain of the E-cadherin protein is required for regulation of RTK-mediated signaling and, as this domain is cleaved by matrix metalloproteinase 7 (MMP-7), this might be an interesting target to investigate, and could explain the high activity of c-Met and EGFR in chordoma [198,199].

In addition to cadherins, most of the studies carried out on CAMs in chordoma also focus on the immunoglobulin superfamily of cell adhesion molecules (IgCAMs). This family consists of complex transmembrane proteins capable of multiple molecular interactions mediated by expression of repeated immunoglobulin-like domain at their extracellular N-termini [200]. Neural CAM1 (NCAM1), as a member of the IgCAMs that primarily mediate hemophilic interactions, is frequently expressed in chordoma where it can be witnessed on immunohistochemical analysis in 75–93% of cases [79,80,195]. Horiguchi et al. [80] demonstrated, in addition to NCAM, the immunoreactivity of other members of IgCAMs: vascular CAM (VCAM) and intracellular CAM (ICAM), and showed a far less frequent involvement in 56% and 25% of the immunostained cases

respectively. Moreover, the authors illustrate NCAM and not ICAM or VCAM to be present in the early notochord, suggesting a possible reactivation. However the underlying consequences in terms of morphology, cell growth or clinical parameters has not yet been investigated. Even so, considerable evidence in the literature implicates NCAM in the activation of FGFR, which is shown to be frequently expressed in chordoma [201–205]. Furthermore, new insight has been gained into the potential crosstalk between NCAM and EGFR in mammals, which might also account for the overexpression of the latter in chordogenesis [206]. Thus, experimental studies addressing these interactions in chordoma might offer interesting new perspectives.

The adhesion of cells to each other and to the extracellular matrix can be explained by the altered expression of other CAM subfamilies, including integrins, selectins and cartilage-linked proteins. However, as there are no in depth studies yet concerning their function in chordoma, evidence for the involvement of some members (CD44 [80], integrin β4 [207] and CD24 [58,207]) has been reported. In addition, and also in line with the altered expression of cell adhesion molecules, changed expression matrix metalloproteinases (MMPs) and other proteases in chordoma have sparked the interest of several investigators, as chordomas are known for their locally aggressive and invasive character, and even if infrequent, have a known metastatic potential [208]. Generally, from the tumor cells' point of view, the ability to degrade and destroy extracellular matrix is very important because it provides a mechanism to divide while being embedded in a matrix. Additionally, it enables tumor cells to pass through this barrier. MMPs belonging to a family of highly conserved zinc atom-dependent endopeptidases are known to fulfill such roles. Currently, more than twenty family members have been identified, but the gelatinases MMP-2 and MMP-9 are particularly interesting for chordoma research. These members have been implicated in the tumor biology of several epithelial tumors, and have been shown to be of prognostic significance, signifying the potential presence in chordoma [209–216]. Indeed, in chordoma, high levels of MMP-2 expression have been shown by Naka et al. to relate to a significant decrease in overall survival [217,218]. In addition, this expression was shown to be more enhanced in primary lesions at sites where bone infiltration of the tumor was noticed. On the other hand, expression of the other gelatinase, MMP-9, has been described to be limited to only a few cells for a small number of cases. Although these cells were also observed by the authors' near bone invasion fronts, the authors did not report on their possible prognostic significance [218]. However, in a cohort of eleven patients, Rahmah et al. [219] reported that ten (91%) exhibited positive staining for MMP-9, and also showed high expression to be associated with recurrence within the first two years after the final treatment. No such relation was found for MMP-2, for which expression was noticed in six (55%) of the cases. Other authors confirmed the prognostic significance of MMP-9, as patients harboring an immune-positive sacral chordoma revealed a significantly shorter continuous disease-free survival time [220,221]. Besides these gelatinases, other proteinases of the MMP family (MMP-1) — cysteine proteinases (cathepsin B and K) and serine proteinases (urokinase plasminogen activator (uPA)) — appear to be implicated in chordoma, and all illustrate an increased expression at the bone invasive fronts, suggesting the involvement of multiple enzymes in the invasive character of chordomas [218,222]. This is corroborated by the finding that high expression of both MMP-1 and uPA are related to a significantly worse prognosis for survival.

However, to date, an explanation for the overexpression of these proteinases is still lacking, as attempts to prove a significant down-regulation of the tissue inhibitors of metalloproteinases (TIMP-1 and TIMP-2) and plasminogen activator inhibitor type 1 (PAI1) at the same areas of bone infiltration only demonstrate a non-significant trend. Regulatory intra- or extracellular factors responsible for the altered expression of proteinases and their inhibitors thus seem attractive investigative targets. Further investigation of the RTKs and their downstream pathway might offer new insights, since high expression of c-Met in chordoma significantly correlates with MMP-1 and -2 [223].

Altogether, it seems that increasing evidence points to an intricate but close relationship between cell adhesion molecules, proteinases, and RTKs. Inhibition of one might therefore also have an effect on the mode by which another exerts its cellular effect.

4.3. Epigenetics and the formation of chordoma

Epigenetics, a process by which gene expression is modified irrespective of changes in the primary genomic sequence, has received increasing attention in many areas of research. This dynamic process entails a broad range of components, including DNA-methylation and non-coding RNA expression, which are recognized as important contributors in tumor formation [224]. DNA-methylation, a biochemical alteration of distinct regions on the gene, so called CpG islands, is held responsible for phenotypic changes in the cell, such as morphologic conversions and increased proliferation rates. In relation to chordoma, Longoni et al. [225], reported the methylation status of the TNFRSF8 gene in thirteen specimens, and found seven to have methylated DNA that correlated with transcriptional silencing of the gene. This association was not observed for the methylation status in the promoter region of the tumor suppressor proteins CDNK2A and PTEN. In the chordoma patients investigated by Le et al. [226], the authors observed a methylated DNA sequence in two and four of the fifteen cases for CDNK2A and PTEN, respectively. However, the authors illustrate that loss of protein expression could be attributed to loss of chromosomal regions containing the gene transcript rather than the methylation status.

Next to DNA methylation, the well-established inhibitory process, known as RNA interference (RNAi), is based on the presence of endogenous non-coding micro-RNA (miRNA) fragments that bind and consequently impede target messenger RNA (mRNA) expression. Using miRNA microarray technology, Duan et al. [227] analyzed differentially expressed miRNAs in chordoma specimens and cell lines and compared them to the expression in skeletal muscle. They found a different set of miRNA to be either up- or downregulated, with one of the most promising downregulated miRNA being miRNA-1. miRNA-1 knockdown was then validated and subsequently demonstrated to have a significant effect on the cell growth of the UCH1 cells as transfection of miRNA-1 into these cells inhibited the growth rate substantially. In addition, the authors showed that downstream targets of miRNA-1, MET and HDAC4 were overexpressed and reacted in a dose-dependent manner. However, further studies are warranted to evaluate the potential of miRNA-1 as a therapeutic target.

5. Discussion

Research unraveling the mechanisms underlying the initiation and further progression of chordoma cells has gained substantial interest in the last decade. As time progresses, accumulating evidence supports the notion of a notochordal origin of chordoma. Even so, only limited progress has been achieved in discovering the presence and function of embryologically active proteins in chordoma. Research focusing on *brachyury*, one of the most promising and recently found markers in chordoma, has revealed this transcription factor to be a crucial aspect of chordoma, and highlights the impact of such embryological factors in the pathophysiology of chordoma. As we reviewed the importance of some other notochordal factors (Shh, Wnt, galectin-3, NCAM) with regard to their effect in notochord formation and in chordoma, we would like to emphasize that more functional studies are required to gain a better understanding of their existence and interactive roles. Other, previously discovered molecular pathways involved in this tumor include the overexpression of cell cycle regulatory pathways and an activated receptor tyrosine kinase-pathway (Fig. 2). With regards to the former, expression of p53 and the loss of the CDKN2A

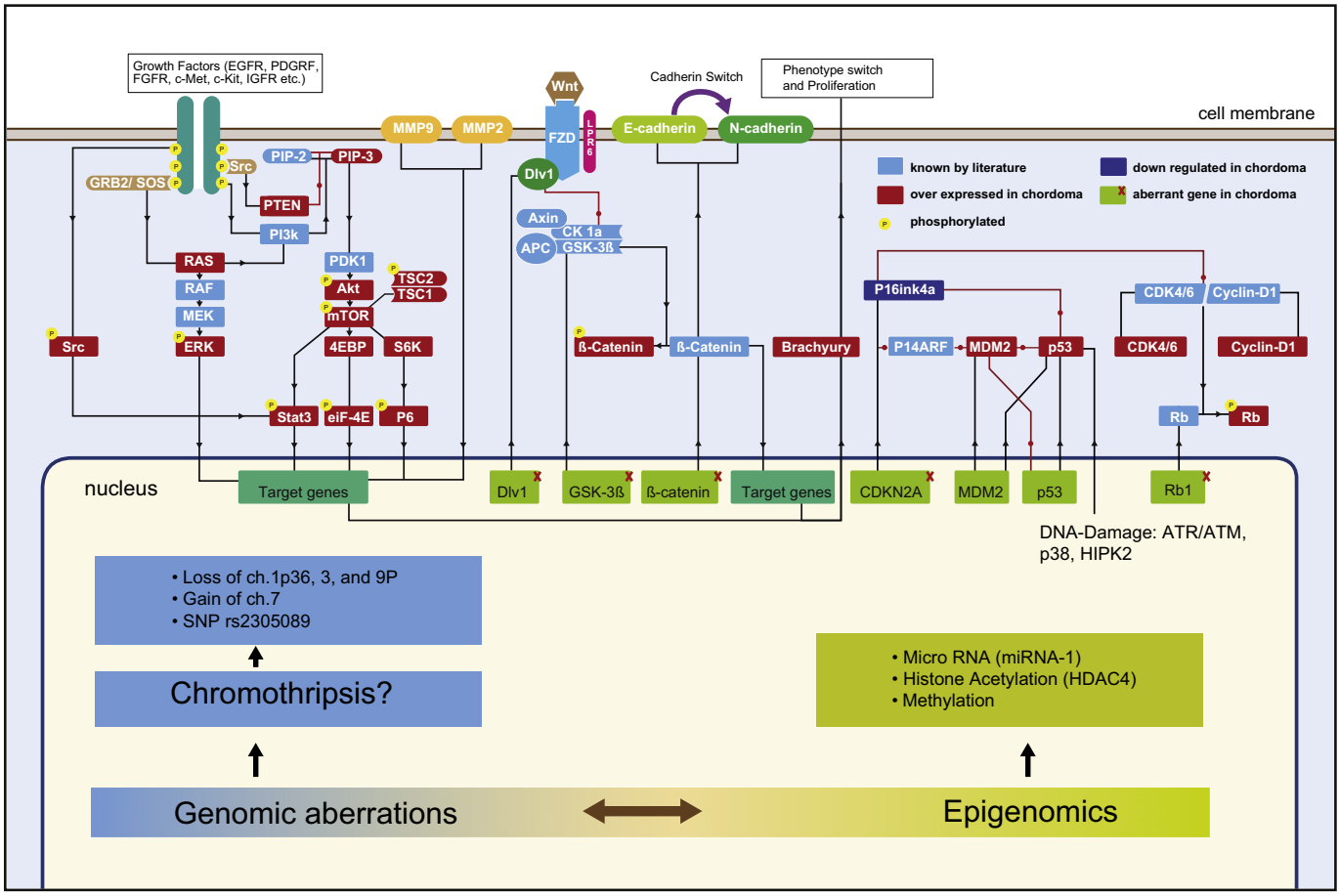

Fig. 2. A simplified overview of the molecular cell biology of a chordoma cell.

gene have been related to a worse prognostic clinical course. However, no functional implication has been proposed for these aberrations in the tumorigenesis of chordoma and they may be a frequent result of tumor progression. More recent data have been collected on receptor tyrosine kinases and their downstream signaling pathway, which are shown to play a role in the pathophysiology of chordoma. Inhibition of the activity of these receptors via pharmacological interference (imatinib mesylate, erlotinib, cetuximab, gefitinib) has shown promising results in a small cohort of studies. However, as these receptor tyrosine kinase inhibitors are impeding the activity of multiple RTKs, it might be interesting to examine the phenotype of (non)responding patients with respect to the expression of a particular member of this protein family. The use of patient selection with such an approach would improve clinical management significantly. In line with this patient-tailored management are the multiple genome-wide and karyotype integrity studies that are becoming more widespread. Here, the chordoma karyotype seems to be frequently altered in very heterogeneous manner, with amplifications of chromosome 7 and deletions of chromosomes 1p and 3p as the most stable observations. These karyotype changes might be due to an overexpression of the hTERT protein or a recently suggested phenomenon called chromothripsis. However, as several studies report an overexpression on the RNA or protein level with an unaltered genetic sequence, evaluation of the epigenetic changes in chordoma are becoming increasingly attractive. Expression of miRNA-1 has shown to be a potent inhibitor of the proliferative capacity of chordoma cells. Additional experiments which are dedicated to other epigenetic regulations of transcription (acetylation, methylation etc.) are required to fully comprehend the biology of this disease.

6. Conclusion

Chordomas are shown to be a management challenge, requiring multimodal therapy with a poor overall survival. The pathophysiology underlying this lethal disease is demonstrated to be complex. Molecular research has so far yielded significant findings, with the most prominent being the discovery of brachyury's involvement in this disease. Future fundamental research would benefit from the discovery of other associated embryological markers, as more evidence is gathered supporting a notochordal origin for chordoma. In addition to brachyury, other promising targets have been identified to be crucial in chordomagenesis. However, as the genetic sequence is inconsistently altered, epigenetic changes are an exciting and relatively unexplored field of research in chordoma.

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