Sarcomagenesis

Sarcomagénesis

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Resumen

Los sarcomas representan un número heterogéneo de neoplasias que surgen de la transformación de algunas células mesenquimales primitivas. La evidencia ha aumentado de forma considerable respecto de las células pluripotenciales que dan origen a estos tumores y que parecen ser responsables de la iniciación, el mantenimiento, la diferenciación y la proliferación del osteosarcoma, sarcoma sinovial, rabdomiosarcoma y del sarcoma de Ewing. Se han adoptado diferentes métodos para la identificación de células primitivas en los sarcomas, tales como el uso de marcadores de superficie, la citometría de flujo para el aislamiento de células con elevada actividad de la aldehído deshidrogenasa y la realización de análisis de población celular. Esta revisión resume y analiza datos sobre la tumorigénesis de los sarcomas, evaluando su posible papel en la sensibilidad y resistencia a diferentes intervenciones clásicas (quimio y radioterapia), así como nuevas terapias dirigidas molecularmente.

Palabras clave: Sarcoma, célula pluripotencial, célula mesenquimal, genotipo.

Abstract

Sarcomas represent a heterogeneous group of neoplasms arising from the malignant transformation of mesenchymal cells. Evidence has increased considerably regarding the origin of sarcomas having putative sarcoma stem cells which are responsible for the initiation, maintenance, differentiation and proliferation of osteosarcoma, synovial sarcoma, rhabdomyosarcoma and Ewing's sarcoma. Different methods have been adopted for identifying primitive cells in sarcomas such as identifying surface markers, using flow cytometry for isolating cells having aldehyde dehydrogenase activity and performing side population analysis. This review summarizes and discusses data regarding the tumorigenesis of sarcomas, assessing their potential role in sensitivity and resistance to different classical interventions (chemotherapy and radiotherapy) as well as new molecularly-directed therapies.

Key words: Sarcoma, stem cell, mesenchymal cell, genotype.

Introduction

Sarcomas represent a heterogeneous and uncommon group of malignancies, arising from connective tissues whose primary function is to support an organism and its systemic integration. Together, they account for over 20% of all pediatric solid malignant tumors but less than 1% of all adult malignancies. The vast majority of diagnosed sarcomas arise from soft tissues, while malignant bone tumors make up just over 10% of all sarcomas[1]. Sarcomas affect ~11.000 individuals annually in the USA and around 200.000 worldwide[2,3]. Risks for sarcomas developing can be divided into environmental exposure, genetic susceptibility, and an interaction between them. Radiotherapy has been strongly associated with secondary sarcoma development as the history of hernias has revealed a greater risk of Ewing's sarcoma (EWS) developing among children[4,5]. Bone development during pubertal growth spurts has been associated with the development of osteosarcoma and exposure to chemicals such as herbicides whilst chlorophenols have also been linked to how sarcomas originate[1].

Sarcomas have been historically grouped into two main types according to tumor location: soft tissue sarcoma (STS) and primary bone sarcomas; however, an alternative genetically-based classification has divided sarcomas into two broad categories since 2002[6],

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each including clinically-diverse tumor subtypes. The first includes sarcomas having near-diploid karyotypes and simple genetic alterations including translocations or specific activating mutations (alveolar rhabdomyosarcoma, myxoid liposarcoma, EWS and synovial sarcoma); the second covers tumors having complex and unbalanced karyotypes characterized by genome instability resulting in multiple genomic aberrations (leiomyosarcoma, malignant fibrous histiocytoma and osteosarcoma)[7,8] Such genomic subtypes seem to be related to a common subpopulation of self-renewing cells capable of initiating sarcomas and maintaining them in the long-term. Increasing evidence has suggested that multi-potent mesenchymal stem cells (MMSC) reproduce human sarcomas upon the overexpression of specific fusion oncoproteins or disruption of key signaling pathways[9]. *Ex vivo* MMSC have certain dominant characteristics including adhesion plasticity.

There is CD105, CD73 and CD90 expression and lack of reactivity to CD45, CD34, CD14, CD11b, CD79b, CD19 and HLA-DR when MMSC are kept in standard culture conditions. Likewise, MMSC should be capable of differentiating into osteoblasts, chondroblasts and fat cells *in vitro*[10]. The exact nature and localization of MMSC *in vivo* remain poorly understood, but recent data has indicated that sarcoma precursors could have a perivascular distribution[11,12], their niche including several cell subsets spanning different stages of mesodermal development having distinct potency, ranging from multi-lineage stem cells to unilineage precursors or even fully-differentiated cells[13]. The expression of embryo markers, such as Oct-4, in tumor and in aged MMSC is another finding supporting a common origin for sarcomas[14].

The present review has been aimed at presenting and discussing evidence related to the origin of sarcomas, following the hierarchical principle of a primordial cell model.

The genetic taxonomy of sarcomas

Most sarcomas involving simple genetic alterations have translocations and account for around a third of such neoplasms; they tend to be presented *de novo* and some of the cytogenetic damage is retained through clonal evolution. Most fusion genes encode chimeric transcription factors causing transcription alterations, whilst others encode proteins having tyrosine kinase or growth factor activity[15].

By contrast with sarcomas derived from well-recognized translocations, the second group involves complex karyotype modifications arising from less aggressive forms and runs through different stages of the disease, each having greater complexity. The liposarcoma, peripheral nerve-derived tumors and chondrosarcomas are clear examples of such subgroup. The main mechanisms triggering sarcomagenesis are associated with transcriptional deregulation producing aberrant fusion proteins arising from genomic rearrangements as well as the presentation of somatic mutations in driver genes from differing signaling routes and abnormalities regarding the number of DNA copies. Likewise, the importance of genome integrity associated with telomere maintenance has been recognized. Major telomerase activation in the absence of alternative lengthening of telomeres (ALT) characterizes sarcomas having specific chromosome translocations; nevertheless, ALT occurs more frequently in sarcomas having non-specific complex karyotypes[16,17]. Lafferty-Whyte *et al*., have described a genetic signature which led to classifying telomerase and changes in ALT for pluripotent cell mesenchymal transition[18].

Sarcomas having non-specific complex karyotypes are sometimes found which have no association with translocations regularly present in hereditary syndromes produced by genomic instability, such as the Werner (WRN), Nijmegen Breakage (NBS1) and Rothmund-Thomson (RECQL4) syndromes[19-21].

Studies of the genome's complete sequence have found that around 35% of osteosarcomas and 18% of chordomas have chromothripsis; this involves hundreds of chromosome rearrangements occurring during a particular cell crisis. Such catastrophe has been described in up to 3% of neoplasms but appears in a quarter of high-grade bone tumors and in medulloblastoma of children predisposed by germinal mutations in p53[22-24]. The most representative examples of transcriptional regulation amongst sarcomas are associated with the PAX3-FOXO1 fusion protein whose direct objective would include myogenic genes such as myogenic differentiation 1 (MYOD1) and myogenic factor 5 (MYF5), as well as other biologically-active elements such as fibroblast growth factor receptor 4 (FGFR4), anaplastic lymphoma kinase (ALK), mesenchymal epithelial transition growth factor (c-MET), insulin like growth factor 1

receptor (IGF1R) and myelocytomatosis viral related oncogene, neuroblastoma-derived (MYCN)[25,26].

The ASPSCR1 gene becomes fused to transcription factor TFE3 (IGHM enhancer 3) in alveolar sarcoma to form a chimeric protein retaining the TFE3 DNA binding domain (containing the CACGTG recognition site). Recognition studies have found that such alteration is related to activation of MET 38, uridine phosphorylase 1 (UPP1) and CYP17A1 genes (cytochrome P450 17A1)[27].

A somewhat more complicated picture has emerged concerning EWS which affects Ewing sarcoma breakpoint region 1 (EWSR1) and Friend leukemia virus integration 1 (FLI1) genes[28]. Several ChIP-seq datasets have been produced in EWS cell lines with endogenous EWS-FLI1, all using the same FLI1 antibody for immunoprecipitation of EWS-FLI1-bound DNA. The amount of bound genomic regions in such studies has varied widely[14-16]. ChIP-seq has demonstrated that most EWS-FLI1-bound genomic regions were intergenic and that EWS-FLI1 binds avidly to GGAA microsatellites through its FLI1-derived ETS family DNA-binding domain[28,29]. Microsatellites containing 6 or more GGAA repeats (the core ETS domain binding sequence) are associated with EWS-FLI1 target gene upregulation[28,30]. These repeats are often more than 200kb upstream of the target gene transcription start site, suggesting that chromatin looping brings distant regions together in a transcriptional hub to allow EWS-FLI1 to modulate gene expression. EWS-FLI1 also binds to more conventional, non-repetitive ETS motifs and such sites are associated with genes repressing or activating transcription[30]. A subset of EWS-FLI1 target regions has shown co-enrichment of sites for E2F, nuclear respiratory factor 1 (NRF1), and nuclear transcription factor Y (NFY), thereby raising the possibility of specific cooperative interactions[31].

On the other hand, some EWS cell lines may be able to reprogram themselves, as such events have been documented after the EWS-FLI1 gene has been silenced, producing a more similar expression profile to that of mesenchymal stem cells (MSC) which might then be induced to become differentiated by adipogenic or osteoblast linage[32,33]. For example, EWS-FLI1 has induced limited expression of a neuroeptodermal gene which can program and impose an osteogenic differentiation mold by inhibiting Runt-related transcription factor 2 (RUNX2) which is related to other genes promoting bone maturation. EWS-FLI1 expression in MSC has induced EWS in a reverse experiment; on the contrary, EWS-FLI1 expression provoked apoptosis in other differentiated cells presenting intact ARF-p53[34].

EWS-FLI1 directly upregulates the polycomb group repressor enhancer of zeste homolog 2 (EZH2) in human MSC[35], and has induced expression of embryonic stem cell genes POU5F1 (also known as OCT4), SRY-box 2 (SOX2) and NANOG, at least partly by repressing miR-145 expression[36]. Interestingly, EWSR1 also fuses with POU5F1 itself, albeit rarely, in undifferentiated bone sarcoma[37,38], myoepithelial tumors of the soft tissue[39], and in certain salivary gland tumors[8,40].

Synovial sarcomas contain fusions between the SS18 (SYT) SSX1 or the SSX2 genes. Analogously to that found in EWS-FLI1, synovial sarcoma cell lines also express POU5F1, SOX2 and NANOG. Silencing SYT-SSX fusion in such cell lines has increased their differentiation potential regarding adipogenic, osteoblast or chondrogenic linages[41]. Synovial sarcoma formation in mice accompanied by the conditional expression of SYT-SSX2 in myoblasts or in other cell linages has provided additional information about fusion protein nuclear reprograming in a compromised variety of mesenchymal linages. Some myxoid liposarcoma fusions, such as FUS-DDIT3 (SHOP) and ARMNS (PAX3-FOXO1), seem to have been able to transform mesenchymal progenitors in murine models. Figure 1 describes the genetic ancestry of different sarcomas.

Mutations and signaling routes in sarcomas

Excluding gene fusions in sarcomas having translocations, it can be stated that few driver genes have recurrent mutations. The most representative examples would be angiosarcomas, an aggressive vascular tumor which has been shown to overexpress tyrosine quinase receptors in some transcription profiles, including KDR (VEGFR2), TIE1, SNF related kinase (SRNK), TEK and FMS-related tyrosine kinase 1 (FLT1)[42]. Sequencing these 5 genes has revealed that 10% of angiosarcomas have mutations in KDR, and that when mutant VEGFR2 proteins have expressed COS-7 cells, there has been independent ligand activation. Large-scale genomic analysis of seven types of sarcoma has identified mutations in TP53, NF1 and PI3KCA[43]; 17% of pleomorphic liposarcoma have mutations in TP53, such finding being consistent with the fact that such alterations are frequent in tumors having complex karyotypes. On the contrary,

in translocation-associated sarcomas, alterations in TP53 and homozygous deletions in cyclin-dependent kinase inhibitor 2A (CDKN2A) have been less common, but when present have usually been related to a very aggressive clinical course (44); 18% of myxoid/round cell liposarcomas have mutations in PI3KCA, thereby suggesting their role as modifications cooperating with the fusion protein (FUS-SHOP) in developing sarcomagenesis[45]. Curiously, mutations found in PI3KCA have been located in the two hotspots observed in epithelial tumors: the helical domain (E542K and E545K) and the kinase domain (H1047L and K1047R). Patients having mutations in the helicoid domain have a lower chance of survival attributable to the disease; they have increased AKT phosphorylation in CREB-regulated transcription coactivator 2 (TOR2) and in pyruvate dehydrogenase kinase 1 (PDK1)[45].

Another recent finding has concerned precise NF1 mutations or deletions being present in 10% of mixofibrosarcomas and 8% of pleomorphic liposarcomas. This finding has been associated with individuals presenting neurofibromatosis type 1 (alterations in the germ-line and somatic mutations) but has not been described previously in subjects having sporadic tumors[43].

A special chapter deals with genomic alterations of gastrointestinal stromal tumors (GIST); mutations in KIT and, to a lesser extent, in PDGFRA are considered primary effectors of the disease, meaning that they are routinely identified in clinical practice before treatment is begun. Physiologically, these receptors are activated after ligand binding, thereby triggering receptor dimerization followed by auto-phosphorylation of the intracellular tyrosine kinase domain and final activation of multiple substrata included in the signaling pathway, such as PI3K/AKT, RAS, MAP and JAK/STAT. Mutations in KIT and PDGFRA are mutually exclusive in GIST and around 10% of these tumors have a wild genotype; some recent series have described the presence of the BRAF gene V600E mutation in up to 7% of these patients[45,46]. Until quite recently, no mutations had been detected in KRAS in GIST patients having alterations in KIT; however, Antonescu *et al*., have identified mutations in codon 12 (G12D: GGT->GaT), 13 (G13D: GGC->GaC), and a concomitant variation (G12A/G13D: GGT->GcT and GGC->GaC) in KRAS in three patients without prior exposure to imatinib (5%)[47]. Another group of GIST patients (children) has overexpressed IGF1R MRNA and its protein, even though the mechanism for such alteration remains unknown. In fact, most pediatric tumors have diploid genomes[48].

Alterations in the number of gene copies

DNA copy-number alterations provide the third route for sarcomagenesis. Sarcomas have a range of complexity among human malignancies regarding their copy-number alterations[49]. They vary from translocation-associated sarcomas with few copy-number alterations (broad or focal) to karyotypically-complex subtypes that are heterogeneous, unstable and profoundly altered regarding their genomic copy number. Moreover, a recent high-resolution array-based copy-number analysis has revealed an intermediate complexity group characterized by few, yet highly recurrent, amplifications exemplified by undifferentiated liposarcomas[43]. Information from another copy-number analysis has shown that the third category can be subdivided into sarcomas having few chromosome arm or whole chromosome gains or losses and sarcoma genomes having a high level of chromosomal complexity[50].

Intermediate complexity sarcomas, such as well-differentiated and undifferentiated liposarcomas, are driven by chromosome 12 alterations, often generating extra-chromosomal episomes, ring chromosomes and larger markers[51]. These 12q gains have high prevalence (80-90%) and co-amplified oncogenes cyclin-dependent kinase 4 *(CDK4)* and *MDM2* can serve as confirmatory diagnostic markers[52] and as targets[53]. Another gene affected by 12q amplification is *HMGA2,* which often loses its 3′ untranslated region (UTR), disrupting microRNA-mediated repression[54]. This genetic remodeling of chromosome 12 is likely the result of progressive rearrangement and amplification in an evolving amplicon rather than a single catastrophic event such as the recently proposed chromothripsis. Similar 12q amplifications occur at lower frequencies in other mesenchymal tumors such as osteosarcomas[55]. Other remarkable, and less frequent amplifications in the intermediate sarcoma group occur on 1p and 6q, these amplifications, which appear to be mutually exclusive, span genes in the p38 and JNK pathways of MAPK signaling including, on 1p, *JUN* and, on 6q, *TAB2* and *MAP3K5 (ASK1)*[56,57]. Another genomic amplification alteration is telomerase reverse transcriptase *(TERT)* located on 5p[43]. Some targets of genomic amplification appear to be

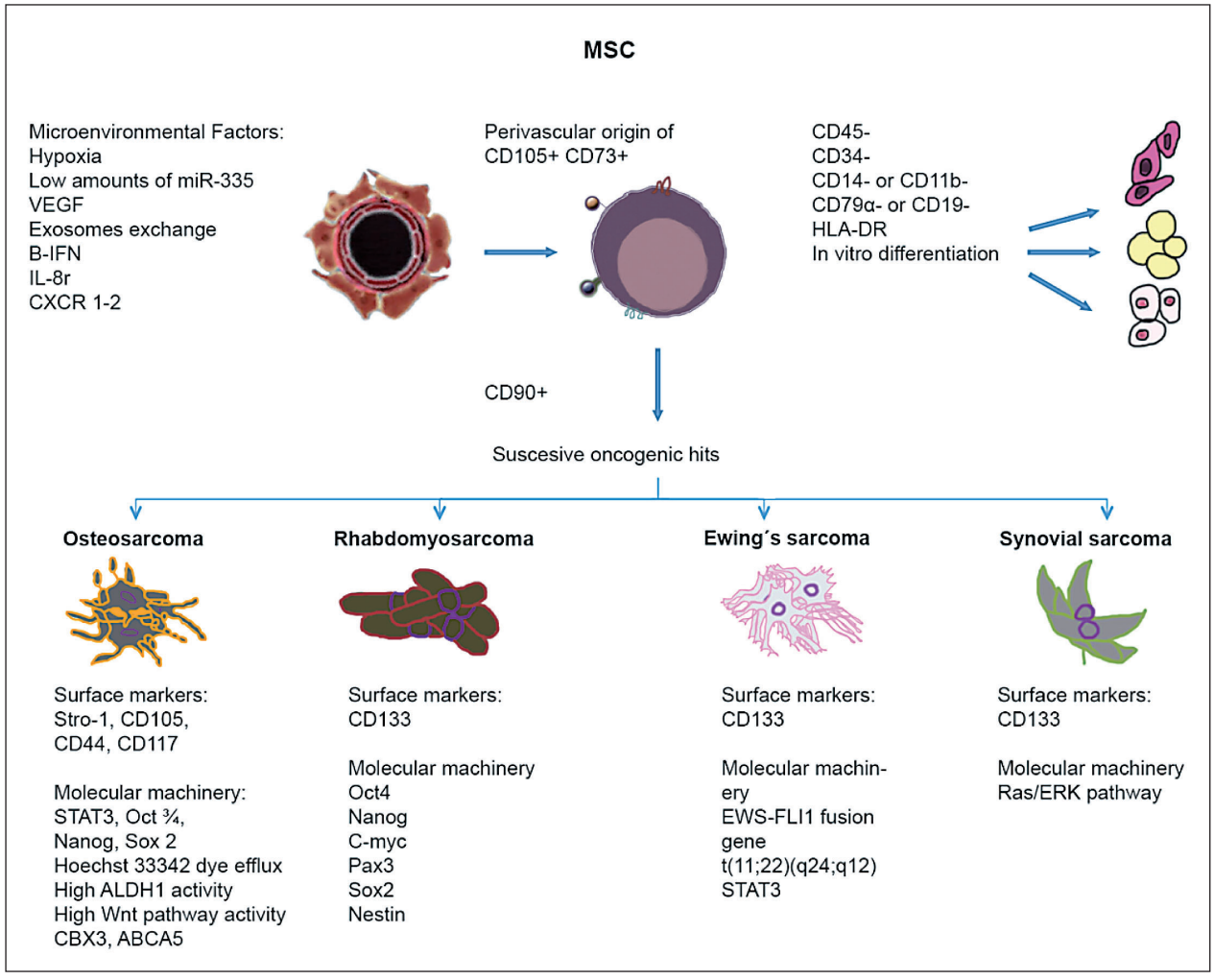

Figure 1. Genetic ancestry of different sarcoma's subtypes.

shared among a subset of both intermediate and highly complex sarcomas, including Yes-associated protein 1 *(YAP1)* and vestigial like 3 *(VGLL3)* on 11q22 and 3p12, respectively[58].

On the other hand, highly complex sarcomas harbor multiple numerical and structural chromosome aberrations that are similar to those previously described in epithelial tumors. Molecular classification of these subtypes reflects varying levels of similarity in their genomic aberrations; some subtypes may be considered a single entity[59], while others are distinct[60]. Broad amplifications of several chromosome arms (such as 5p)[61] often occur in combination with deletions affecting well-established tumor suppressors such as *CDKN2A, CDKN2B, PTEN,* retinoblastoma 1 *(RB1), NF1* and *TP53*. In fact, several of these genes play a direct role in maintaining chromosome integrity[62] and their loss of function may be an early event leading to genomic instability in highly complex sarcomas. In other subtypes, such as leiomyosarcoma, genomic deletions are more common than amplifications[62].

Genesis of primary sarcomas

It has been established recently that transformed MMSC may initiate sarcomagenesis *in vivo*. Efforts have been directed towards characterizing such transformation and also to prospectively generating specific models for different sarcomas. These studies include both spontaneous and induced transformation of MMSC mediated by specific alterations such as the accumulation of chromosome instability, p53 mutations or loss of CDKN2A/p16. Mouse MMSC is especially predisposed to gain these alterations after long-term *in vitro* culture favoring clonal selection[63-66]. p53-depleted mouse adipose-derived MSC (mASC) have been capable of originating

leiomyosarcoma-like tumors after injection into immunodeficient mice. This finding has been supported by a differentiation-based microRNA study which identified leiomyosarcoma as an MSC-related malignancy[67,68]. Another study determined that complete loss of p53 expression in p21–/–p53+/– mASC after culture induced cell growth, karyotype instability and loss of p16INK4A which prevents senescence, thereby resulting in the formation of fibrosarcoma-tumors *in vivo*[69]. Overexpression of c-MYC in p16INK4A–/–p19ARF–/– bone marrow mouse MMSC results in osteosarcoma developing, accompanied by a loss of adipogenesis. Similarly, the loss of other cell cycle regulators, such as Rb, has not transformed mMSC but its deficiency has potentiated tumor development of p53-deficient mouse MMSC, generating further undifferentiated sarcomas[70].

Although Rb-deficient mice develop normally, Rb deficiency synergizes with p53 deletion to accelerate sarcoma formation and increases the frequency of poorly-differentiated sarcomas.

In other mouse models where mutations have been restricted to muscle, the expression of oncogenic K-RAS or the mutation of endogenous K-RAS has been needed to efficiently induce sarcoma formation in p53-deficient tissue[71].

Sarcomas developed in these models have been characterized as pleomorphic rhabdomyosarcoma and high-grade sarcomas with myofibroblastic differentiation. Interestingly, deletion of the INK4A-ARF locus could substitute the p53 mutation in such K-RAS mutation-based model of sarcoma development[72].

Human MMSC do not undergo malignant transformation as easily as mouse primitive cells. For instance, as opposed to mouse MMSC, inactivation of p53 or p53 and Rb does not induce transformation in humans, although p53-/Rb-deficient human MMSC display a higher growth rate *in vitro* coupled to an extended lifespan[73,74].

Several oncogenic events must be combined to promote *in vivo* sarcomas from human MMSC, including introducing the human telomerase catalytic subunit (hTERT), HPV-16 E6 and E7 (abrogating p53 and Rb family member functions), SV40 small T- or large T-antigens (resulting in c-MYC stabilization and inactivating Rb and p53, respectively) and oncogenic H-RAS (providing a constitutive mitogenic signal)[75,76].

In one striking model, transforming human MMSC has been associated with a gradual increase in genomic hypomethylation, although this is not necessary for sarcomagenesis. Using a different basic approach, another research group has transformed human MMSC through ectopic expression of hTERT, H-RAS and BMI-1 thereby inhibiting the expression of genes controlled by polycomb response elements, including p16INK4A[77].

It has also been reported that some hTERT-transduced human MMSC lines lose contact inhibition, acquire anchorage-independent growth and form tumors in mice after long-term *in vitro* culture. This has been associated with the deletion of the Ink4a/ARF locus and with acquiring an activating mutation in K-RAS. Overall, *in vivo* tumors originating from most of these transformed human MMSC have been classified as undifferentiated spindle cell sarcomas[76].

Besides inactivation of cell cycle regulators, hMSC transformation has been related to alterations in several signaling pathways. It has been reported that the PI3K-AKT-mTOR signaling pathway plays a critical role in the development of leiomyosarcomas.

Thus, mice carrying a homozygous deletion of PTEN in the smooth muscle have developed leiomyosarcoma. PTEN and PI3KAKT involvement in leiomyosarcoma has been implicated by the fact that these signaling pathways are dysregulated in leiomyosarcoma-forming p53-decifient mouse MMSC[78].

The WNT/β-catenin pathway plays a major role in the balance between self-renewal, differentiation, regulation and invasion of human MMSC. The loss of WNT characteristics in MMSC leads to malignant transformation and reduces apoptosis; accordingly, a recent study has supported a role for aberrant β-catenin stabilization in promoting MMSC-derived tumorigenesis[79]. Similarly, inactivation of WNT signaling upon treatment of previously SV40-immortalized human MMSC with the WNT inhibitor DKK1 has led to full malignant transformation of these cells and the consequent *in vivo* formation of malignant fibrous histocytoma[80].

Conversely, restoring WNT signaling in sarcoma cells has allowed them to differentiate amongst different mesenchymal lineages. It has been reported that key components of the WNT pathway are down-regulated in osteosarcoma compared to normal human MMSC and MMSC differentiated into osteoblast[81].

Conclusions

Sarcomas are generally studied when the full transformation events have already occurred and therefore, the mechanisms of transformation and pathogenesis are not amenable to analysis with patient samples. Thus there exists the need to establish bona fide mouse and human based models to recapitulate sarcomagenesis *in vitro* and *in vivo.* Over recent years, mounting evidence indicates that MMSCs from different sources may represent the putative target cell of origin for a variety of human sarcomas, thus linking MMSCs and cancer. Future research should be aimed at defining precisely the specific phenotype of the MMSC populations at the origin of the different types of sarcomas as well as at dissecting the mechanisms governing MSC transformation. We envision that experimental research based on MMSCs coupled to whole-genome sequencing of different types of primary sarcomas will advance our attempts to develop accurate MSC-based models of sarcomagenesis and to decipher the underlying mechanisms, provide a better understanding about the onset and progression of mesenchymal cancer, and lead to the eventual development of more specific therapies directed against the sarcoma initiating cell.

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