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REVIEW

Decoding the 'embryonic' nature of embryonal rhabdomyosarcoma

R. Lav*, R. Heera and L. M. Cherian

Department of Oral Pathology and Microbiology, Government Dental College, Gandhinagar, Kottayam, India

Embryonal rhabdomyosarcoma is one of the major defined histologic variants of rhabdomyosarcoma that is mainly reported in children. The histologic appearance of this neoplastic entity recapitulates normal myogenesis. The tumor cells variably exhibit the different cellular phases of myogenesis ranging from undifferentiated mesenchymal cells to elongated myoblasts, multinucleated myotubes and differentiated muscle fibers. The carefully orchestrated embryonic signaling pathways that are involved in myogenesis, conceivably also result in the genesis of rhabdomyosarcoma; albeit as a corollary to an imbalance. We have attempted to review the pathogenesis of embryonal rhabdomyosarcoma in an endeavor to understand better, how closely it is linked to normal myogenesis in terms of its molecular dynamics and histologic presentation.

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Introduction

Rhabdomyosarcoma (RMS) is a relatively rare malignancy occurring mainly in children. It was first described by Weber (1854) and classified as embryonal, alveolar, botryoid and pleomorphic. The embryonal variant that accounts for about 60% of all reported RMSs,[1] was described by Bernard (1894), who termed it *'tumeur embryonnaire du muscle striae.'*[2]

Although more common in children, it exhibits a bimodal age distribution; the larger peak between 0 and 5 years and the smaller peak in adolescence – the latter exhibiting a male predilection. It is also reported in young adults with considerable rarity and is uncommon in patients older than 40 years of age.[1]

Histologic examination of this entity recapitulates normal myogenesis. The tumor cells variably exhibit the different cellular phases of myogenesis ranging from undifferentiated mesenchymal cells to elongated myoblasts, multinucleated myotubes and differentiated muscle fibers. It is this latter phase of terminal differentiation that is believed to prevail following chemotherapy and radiotherapy,[2] and is aimed at by several newer differentiation inducing treatment modalities targeting molecular signaling pathways.

This review is aimed at understanding the link between normal myogenesis and the pathogenesis of embryonal RMS at the molecular and histologic level. We have also attempted to discuss the genetic and epigenetic factors possibly responsible for the genesis of this neoplasm.

*Address for correspondence: R. Lav, Department of Oral Pathology and Microbiology, Government Dental College, Medical College P.O., Gandhinagar, Kottayam 686008, India
(Email rupali.lav@gmail.com)

Myogenesis

Mammalian skeletal myogenesis begins during embryonic life and continues through the developmental stages – from fetus to adulthood. The embryonic myogenesis phase is marked by the development of the primary myotome allowing the establishment of a basic muscle pattern. The subsequent fetal and neonatal myogenic phases involve generating the adult musculature thereby facilitating growth and development of muscle. In the final phase, adult myogenesis accounts for postnatal growth as well as regeneration and repair, utilizing satellite cells.[3] These satellite cells are believed to originate from multipotent cells of the somite and are housed within a specialized stem cell niche that supports their renewal while preventing differentiation. This niche dictates the differentiation of progenitor cells along specific cell lineages.[4] Satellite cells undergo stochastic division (one progenitor cell divides to give rise to two committed daughter stem cells thereby maintaining a constant progenitor pool) or asymmetric divisions (give rise to identical stem cell and a committed daughter cell).[5]

During muscle development, mononuclear progenitor cells undergo asymmetric division and differentiate along the myogenic lineage to form multinucleated myofibers. In the post-embryonic phase, a small population of muscle stem cells or satellite cells remains conserved, that aids in a certain degree of muscle regeneration following injury.

Myogenesis is guided by a myriad of signaling molecules that in turn induce intracellular pathways through the activation of cell surface receptors. Following transcription, these factors ultimately translate the extracellular signals to gene and microRNA expression, allocating a myogenic lineage to the undifferentiated progenitors. An understanding of the embryonic signaling

pathways involved in skeletal myogenesis is imperative to comprehend their role in the development of RMS.

Notch signaling pathway

Notch signaling pathway aids in the maintenance of muscle progenitors during both embryonic and postnatal development by suppressing myogenic differentiation.[3] Studies on mice have shown that activation of Notch signaling pathway has two notable operations: inhibition of muscle regulatory factors including MyoD in myoblasts(studied in mice)[6] and reduced transcription of promyogenic genes: myogenin and myocyte enhancer factor 2c (MEF2c).[7,8] It also promotes stem cell renewal during embryogenesis and satellite cells during postnatal myogenesis.

Wnt pathway

The role of the Wnt pathway in adult myogenesis is unclear with two seemingly opposite roles. Available literature states that activated β-catenin promotes myogenic differentiation while a contradictory role of satellite cell proliferation and inhibition of differentiation has also been established.

Wnt proteins are crucial regulators of satellite cell renewal and commitment during postnatal myogenesis. Myoblast differentiation is modulated by a transition from Notch signaling, which results in the expansion of the progenitor pool of adult skeletal muscle upon injury toward the canonical Wnt3a signaling. In addition, Wnt7a released in regenerating muscle fibers, signals through the non-canonical planar cell polarity pathway to result in symmetric division of the satellite cells.[4]

Hedgehog pathway

During embryonic and fetal myogenesis, hedgehog is required for survival, proliferation and maintenance of myogenic regulatory factors, that is, it plays a crucial role in survival and proliferation of the developing myotome. Studies have shown that hedgehog signaling inhibits apoptosis in muscle precursors. In addition, it has been shown that hedgehog signaling represses terminal differentiation of myoblasts.[3]

Bone morphogenic proteins (BMP)

BMPs play a contradictory role to Shh and Wnt by inhibiting expression of certain myogenic genes. Ono *et al.* demonstrated that BMP signaling allows expansion of the satellite cell pool by stimulating proliferation and preventing precocious muscle development. The expression of BMP receptor type 1A on activated satellite cells facilitates response to BMPs during their proliferation. Interference in the interactions between BMPs and their receptors by BMP antagonist Noggin, was shown to induce precocious differentiation.[9] This subclass of TGF-β superfamily exerts its action through serine-threonine kinase receptors, leading to the activation of SMAD protein and subsequent activation/repression of the target genes.[10]

Transcriptional regulation of myogenesis

Skeletal muscle differentiation is largely governed by muscle regulatory factors (MRF), also referred to as the MyoD family; and myocyte enhancer factor-2 (MEF2), which act in conjunction to activate muscle specific genes. The MRFs are basic helix-loop-helix proteins that bind specific DNA motifs (E boxes) and are key regulators of differentiation. The MyoD family comprises Myf5, myogenic differentiation 1(MYOD), myogenin (MYOG) and myogenic factor 6 (MYF6) that are exclusively expressed in skeletal muscle. MEF2 binds to MyoD and other basic helix-loop-helix factors to activate transcription of genes responsible for muscle differentiation during myogenesis. Among the factors regulating MEF2 activity, histone deacetylases (HDAC) 4,5,7 and 9 have been of keen scientific interest. These HDACs are present in the nucleus of myoblasts (precursors) and bind to MEF2, thereby blocking its transcriptional regulation of muscle differentiation. Calcium/calmodulin dependent kinase phosphorylates HDACs, releasing MEF2 and are subsequently transported into the cytoplasm.[11–13] Ongoing research is aimed at exploring the therapeutic potential of HDAC inhibitors that induce differentiation, growth arrest or apoptosis in a broad range of cancer cell lines.

Pathogenesis of RMS

RMS is thought to arise from skeletal muscle precursors that fail to undergo appropriate terminal differentiation.[14] Two possible scenarios to explain the genesis of RMS are:

(a) Somatic mutation hinders the normal differentiation of skeletal muscle precursor cells resulting in an expansion of the progenitor pool, which in turn wager accumulation of further mutations.
(b) Derangement of signaling pathways in different cells results in dedifferentiation and neoplastic transformation.[15] The fact that RMS occurs even at sites that are expected to be devoid of skeletal muscle and its progenitors, corroborates this scenario.

It has been hypothesized that RMS may potentially arise from uncommitted mesenchymal progenitor cells with more widespread tissue distribution (including the non-muscle tissues).[16] The much debated cellular origin of RMS is still largely obscure, owing mainly to the complexity of its presentation. Considerable variability is noted among the histologic subtypes in their clinical behavior and biological mechanisms. These individual subtypes may thus have distinct cellular origins.[17]

The main embryonic signaling pathways mainly Notch, Wnt and hedgehog are known to orchestrate the careful balance between proliferation/self-renewal and differentiation fates of muscle progenitor cells.[3] A derangement in this careful balance may thus potentially aid in tumorigenesis by increasing the progenitor pool susceptible to mutations; promoting proliferation of mutated progenitors; or facilitating dedifferentiation of skeletal muscle cells.

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Understanding the myriad factors involved in myogenesis has allowed us to fathom how a minor glitch can result in an entity as grave as RMS. The main attributable factors conceivably involved in the genesis of this malignancy are: tumor suppressors such Rb and p53; oncogenes such as Ras and Myc; tyrosine kinases such as IGF and cMet; specific chromosomal translocations seen in alveolar rhabdomyosarcoma (ARMS) – the more commonly encountered t(2;13)(q35;q14), and the infrequently occurring t(1;13)(p36;q14) – each giving rise to a unique fusion protein. Embryonal rhabdomyosarcoma (ERMS) which is more common than the ARMS subtype is disparate in terms of the underlying molecular mechanisms responsible for neoplastic transformation/genesis.

Genetic basis

The gradual decline in the postnatal proliferation rate in somatic myogenic lineage cells has been attributed to the declining expression of growth promoting genes including Dlk1, Mest, Igf2, Plag11 and Peg3. Rezvani *et al.* have demonstrated that this postnatal down regulation of growth promoting genes fails to occur in some embryonal cancers, notably in RMS, thereby resulting in persistent rapid sarcoma cell proliferation. In addition, the failure of control mechanisms overlooking deceleration of somatic growth may promote tumorigenesis.[18] Most ARMS have chromosomal translocation involving PAX3 or PAX7 and forkhead transcription factor.[19] ERMS, on the other hand, are mostly associated with allelic loss at 11p15.5(where the gene for insulin like growth factor is located) and so far, no consistent chromosomal translocations have been detected in this variant.[20,21] Studies have shown that the growth factor IGF2, functions as an autocrine growth factor in RMS. Consequently, inhibition of binding of the insulin like growth factor receptors suppresses tumor growth.[22]

Scientific evidence suggests that both ERMS and ARMS exhibit collaborating alterations affecting common targets including p53 and Rb pathways. Derangements in these pathways involve amplifications of genes such as MDM2 and CDK4; however, these amplifications have been detected more commonly in ARMS than in ERMS. This suggests that, despite the similarity in the downstream targets of genetic alterations, the cytogenetic differences between the two subtypes indicate distinct molecular etiologies.[20] Rubin *et al.* demonstrated that loss of p53 in maturing myoblasts mostly results in ERMS, while satellite cells tend to give rise to undifferentiated pleomorphic sarcomas (UPS). Their study also highlighted the role of Rb1 as a modifier of tumor phenotype to mimic UPS, regardless of the cell of origin. Their findings indicate a continuum of the same disease with UPS and ERMS on either end of the spectrum.[23]

Syndromic association

Although most cases of RMS occur as a result of sporadic genetic aberrations, this entity has also been associated with familial syndromes such as neurofibromatosis and Li-Fraumeni Syndrome.[24] RMS has also been observed in association with Beckwith–Wiedmann syndrome, a heterogeneous overgrowth syndrome associated with inherited chromosomal alterations at 11p15.5 region.[20]

Environmental influences

As such the influence of environmental factors in the genesis of RMS remains largely obscure. The demographic predisposition for childhood RMS led investigators to suspect factors potentially influencing early development. Such studies suggested an influence of factors including prenatal X-ray exposure, maternal drug (cocaine and marijuana) exposure, an increased maternal age as well as a maternal history of stillbirths.[25–27] In addition, the risk of childhood RMS has also been associated with increased intra-uterine growth suggesting a possible role of fetal growth factors in its pathogenesis.[28] The data obtained in most epidemiological investigations is essentially limited in sample size owing to the relatively low incidence of this soft tissue sarcoma.

Deregulation of oncogenic pathways

As anticipated in the neoplastic phenomenon, deregulation of oncogenic pathways results in failure of the cells to exit the cell cycle in addition to the interference with myogenic differentiation. Saab *et al.* identified four areas of deregulation that have been studied in experimental models and to some degree, in human samples. These include deregulated Cyclins/Cdk/Rb; uncontrolled mitogenic signaling (mainly mitogenic fibroblast growth factors, hepatocyte growth factor/scatter factor and insulin-like growth factors); failed activation of p38 MAPK; and defects in mitogenic regulatory factors (e.g., deregulated twist expression, week expression of MyoD/E-proteins).[15]

Deranged developmental signaling pathways in RMS

Notch signaling pathway

It has been found that transcription factor HES1 (that reverses cell quiescence) acts via Notch dependent signaling pathway and aids in initiation and progression of RMS by preventing irreversible cell cycle arrest and suppressing MyoD dependent differentiation. This exemplifies how tumor cells may adopt pathways normally used by quiescent cells, to suppress entry into irreversibly arrested states.[29]

Furthermore, in an *in vitro* study investigating the role of Notch-Hey1 pathway inhibition in ERMS cell lines, it was found that reduction of Notch target gene Hey1 resulted in an increase in early differentiation.[30]

Wnt signaling pathway

Many human cancers have been shown to harbor changes in the Wnt pathway resulting in upregulated β-catenin activity and target gene expression. Our current knowledge about the role of Wnt in RMS is limited. Singh *et al.* investigated the Wnt

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pathway in ERMS cells from p53/c-fos double mutant mice and reported a down regulation in the canonical Wnt-β catenin signaling pathway in the tumor cells as compared with normal myoblasts. Activating this pathway promoted myogenic differentiation in the tumor cells. This, they proposed could be viewed as a potential therapeutic modality, as induction of differentiation through upregulation of Wnt may potentially terminate the proliferation of immortal progenitor cells.[31] In RMS, a downregulated Wnt pathway aids in tumorigenesis.

Hedgehog signaling pathway

Deregulation of the mammalian hedgehog (Hh)-Gli signaling pathway has been implicated in ERMS that originates from the progenitor population that does not normally receive active Hh signaling. Scientific evidence indicates that Hh-induced post-natal ERMS arises from Hh/Gli-quiescent non-myogenic populations.[14] Activation of sonic hedgehog signaling in adipocytes through expression of a constitutively active smoothened (receptor protein of hedgehog signaling pathway) allele in mice has been shown to give rise to ERMS.[32] These findings attest the role of hedgehog pathway deregulation and the potential of non-myogenic lineage cells to be involved in the genesis of RMS. Heterozygous state of *Ptch 1* (which inhibits smoothened) is characterized by abnormal hedgehog signaling in ERMS and other tumors associated with Gorlins syndrome.[33]

MicroRNA mediated control and regulation in myogenesis and RMS

MicroRNAs are evolutionarily conserved small non-coding RNAs that associate with the 3' untranslated regions of target mRNA to induce their translational repression. miRNAs involved in myogenesis include both muscle specific miRNAs (selectively expressed in muscle tissue) and ubiquitously expressed miRNA that play a role in the myogenic process.[34] Those that aid in controlling cell fate determination of myogenic precursors and muscle tissue homeostasis have been termed 'MyomiRs.'[35] MiRNA – 206 is a member of the muscle specific miR-1 family of myomiRs. This miRNA is unique among the muscle specific miRNAs in that it is exclusively and highly expressed in skeletal muscle.[35–37] miRNA that is regulated by MyoD and targets Pax3 and Pax7 mRNA is known to regulate skeletal muscle differentiation. Khanna *et al.* identified miRNA-146b as a novel positive regulator of skeletal myoblast differentiation. They reported an upregulation in the expression of miRNA-146b during myoblast differentiation *in vitro* and muscle regeneration *in vivo*.[38] Another key myomiR is miRNA-489, that is essential for the maintenance of quiescence in satellite cells and is downregulated during their activation.[39]

miR-1 and miR-133a have been found to be remarkably decreased in embryonal and alveolar RMS cell lines compared with differentiated myoblasts and skeletal muscle tissues.[40]

miR-1 has been shown to be remarkably downregulated in primary RMS (alveolar and embryonal subtypes).[41] Wang *et al.* demonstrated that in RMS and other primary tumors that exhibit impaired differentiation, non-muscle specific miR-29 is epigenetically silenced (by an activated NF-κ B YY1 pathway). Conversely, its reconstitution in RMS mice models was found to inhibit tumor growth and differentiation of cells which suggests that miR-29 acts as a tumor suppressor through its promyogenic function.[42]

Cancer stem cells in RMS

The cancer stem cell hypothesis states that a small subset of tumor cells resembles stem cells and possesses the ability to self-renew, evade therapy and proliferate to drive tumorigenesis. Langenau *et al.* were able to identify and isolate a distinct fluorescent labeled cancer stem cell population in zebrafish ERMS. It was proposed that the genetic expression of this subset of cells shares similar self-renewal programs as those found in satellite cells.[21]

Later, Walter *et al.* reported the formation of 'RMS spheres' containing a cancer stem cell enriched population. They found stem cell genes such as oct4, nanog, c-myc, pax3 and sox2 to be significantly upregulated in rhabdospheres with potential to differentiate into multiple lineages. They also reported an upregulated CD133 genetic expression in rhabdospheres that correlated with poor overall survival, suggesting its role as a potential prognostic marker for ERMS.[43] Another study demonstrated the co-expression of CD133 and nestin in RMS tissue as well as cell lines, which in conjunction with functional assays suggested a possibility of the presence of cancer cells with a 'stem like' phenotype in these tumors.[44]

Histopathology

As mentioned above, the histologic appearance of ERMS exhibits similarity to different stages of embryonic myogenesis; with variability in terms of degree of differentiation. This variability renders the poorly differentiated tumors a diagnostic challenge without ancillary immunohistochemical investigations. The well differentiated tumors, however, offer favorable diagnostic clues such as the presence of fetal muscle fibers with cross striations. Rhabdomyoblasts at different stages of cytologic differentiation may be evident only in a few areas, thus mandating adequate sampling.

Poorly differentiated tumors constituting the lower end of the differentiation spectrum, may exhibit diffuse proliferation of round to ovoid and occasional fusiform cells (Fig. 1a). Such tumors reciprocate to developing muscle at the 5–8th week of the embryonic myogenesis phase. Most cases are devoid of identifiable rhabdomyoblasts and offer no clues indicating a skeletal muscle origin/lineage. Occasionally, tumors may be comprized of a relatively monomorphic round cell population with minimal cytoplasm and remarkable cellular and nuclear pleomorphism – constituting the lower end of the

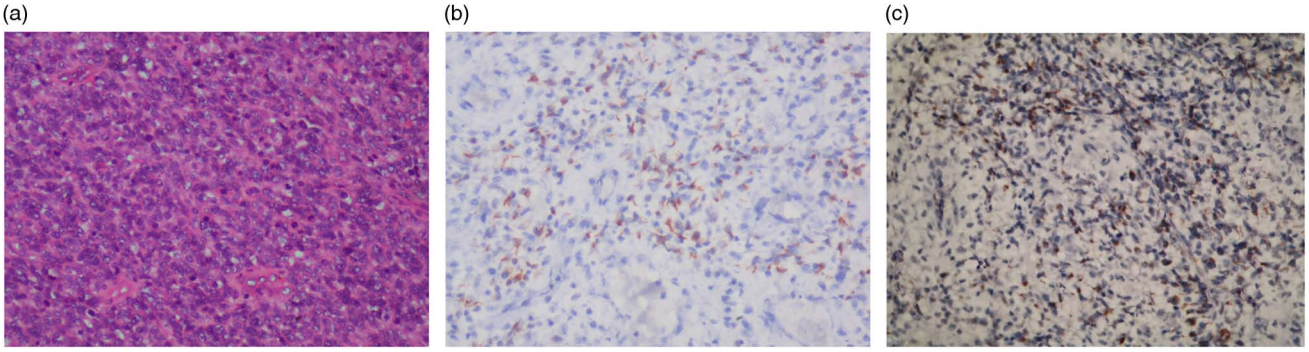

Fig. 1. (*a*) Hematoxylin and eosin stained section exhibiting a highly cellular, diffuse proliferation of round to ovoid and occasional fusiform cells with vesiculated nuclei and prominent nucleoli. The proliferating cells exhibit remarkable cellular and nuclear pleomorphism. (*b*) Tumor cells exhibiting scattered desmin immunopositivity. (*c*) Tumor cells exhibit scattered, strong immuno-positive myogenin staining.

differentiation spectrum. The stromal density may be non-uniform ranging from loose myxoid areas to moderately collagenous areas; the latter exhibiting scattered collections of the tumor cells. Glycogen is demonstrable in most cases of RMS regardless of the histologic subtype.[45]

A positive expression of markers indicating a skeletal muscle lineage, such as desmin (Fig. 1b), myogenin (Fig. 1c), MyoD1, muscle specific actin and sarcomeric α-actin, aid in lineage determination.[45–47] There is no clarity in terms of marker expression pattern corresponding to the degree of differentiation of the tumor cells.

Conclusion

Understanding myogenesis and its regulation has provided us a deeper insight into the molecular dynamics of embryonal RMS. The stages of muscle development are recapitulated by the variable histologic presentation of this neoplastic entity, in accordance to the degree of differentiation. Ancillary diagnostic aids are usually required when encountered with poorly differentiated cases.

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Conflicts of Interest

None.

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