Defining the Extracellular Matrix of Rhabdomyosarcoma

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Abstract

Background: Rhabdomyosarcoma (RMS) is the most common soft-tissue sarcoma of childhood with a propensity to metastasize. Current treatment for patients with RMS includes conventional systemic chemotherapy, radiation therapy and surgical resection; nevertheless, little to no improvement in long term survival has been achieved in decades – underlining the need for target discovery and new therapeutic approaches to targeting tumor cells or the tumor microenvironment.

Methods: To evaluate cross-species sarcoma extracellular matrix production, we have used murine models which feature knowledge of the myogenic cell-of-origin. With focus on the RMS/undifferentiated pleomorphic sarcoma (UPS) continuum, we have constructed tissue microarrays of 48 murine and 4 human sarcomas to analyze expression of 7 different collagens, fibrillins and collagen-modifying proteins, with cross-correlation to RNA deep sequencing.

Results: We have uncovered that RMS produces increased expression of type XVIII collagen alpha 1 (COL18A1), which is clinically associated with decreased long-term survival. We have also identified significantly increased RNA expression of COL4A1, FBN2, PLOD1 and PLOD2 in human RMS relative to normal skeletal muscle.

Conclusion: These results complement recent studies investigating whether soft tissue sarcomas utilize collagens, fibrillins and collagen-modifying enzymes to alter the structural integrity of surrounding host extracellular matrix/collagen quaternary structure resulting in improved ability to improve the ability to invade regionally and metastasize, for which therapeutic targeting is possible.

Background

Rhabdomyosarcoma (RMS) are highly malignant tumors known to phenocopy some of the early events in skeletal muscle embryogenesis but are also known to arise from tissues not known to contain striated muscle or muscle stem cells(1). Embryonal rhabdomyosarcoma (eRMS) and alveolar rhabdomyosarcoma (aRMS) are the major histological subtypes. eRMS are the most common subtype accounting for half of all RMS cases and occurs most often in the head, neck, and genitourinary tract(2). aRMS usually occurs in adolescents and young adults and is commonly found in the trunk and extremities.

To address the pressing and unmet clinical need for new treatments of soft tissue sarcomas, we have generated multiple genetically-engineered mouse (GEM) models of aRMS, eRMS and the undifferentiated pleomorphic sarcoma (UPS) subtype of non-rhabdomyosarcoma soft-tissue sarcoma (NRSTS)(3-7) (Figure 1). Lymphatic and hematogenous (pulmonary) metastasis is a predominant feature(4) and a primary cause of mortality in these models(8). We characterized these soft tissue sarcoma models and demonstrate them to be representative of the human diseases by histopathology, gene expression and other features(3, 4, 9, 10). Furthermore, these conditional models have the special features of knowing the cell-of-origin as well as the mutational profile making them a valuable tool to study RMS (Figure 1).

Very little is known about the composition of the RMS tumor microenvironment (TME). In this study, we have uncovered that type XVIII collagen alpha 1 (encoded by *COL18A1*) is expressed highly in RMS. Interesting, the variant of rhabdomyosarcoma cell cultures that produce COL18A1 is the 'alveolar' subtype, thus called because rich collagen stroma encasing the tumor cells is reminiscent of lung histology.

In addition to analyzing the expression of collagens in RMS, we also examined the expression of the post-translational collagen-modifying enzymes such as prolyl 3-hydroxylase (P3H2, *LEPREL1*), lysyl hydroxylase 1 (LH1, *PLOD1*) and lysyl hydroxylase 2 (LH2, *PLOD2*) which facilitate collagen maturation (reviewed in(11)). Whereas prolyl 4-hydroxylation of the Yaa position of Gly-Xaa-Yaa by P4H enzyme is ubiquitous and provides stability to the collagen triple helix, prolyl 3-hydroxylation by P3H2 in the Xaa position of a Gly-Xaa-4Hyp sequence can be far less frequent – yet biologically essential for processes such as the maternal-fetal interface with respect to collagen IV(12). Like type IV collagen, type XVIII collagen (COL18) contains a relatively large number of Gly-Pro-Hyp sequences(13). Lysyl hydroxylases target the Yaa position lysine residues in Gly-Xaa-Lys tripeptides(11). This post-translational lysyl hydroxylation allows subsequent glycosylation of hydroxylysine. Hydroxylysines and O-linked glycosylation of hydroxylysines within procollagen are essential to the formation of intra- and inter-molecular crosslinks(11). The critical underlying biology of the extracellular matrix not only has implications for histological appearance, but may also create therapeutic opportunities(14), which motivated this study.

Methods

Murine RMS Cell Cultures

Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin (P/S) and 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES). At confluency, cells were washed with Phosphate Buffered Saline (PBS) and further incubated in the medium without FBS. Freshly-made ascorbate was added daily at 50 µg/mL concentration to the medium.

Immunoblotting

For the analysis of secreted proteins, the medium was collected and proteins were precipitated with 0.2 µg/mL ammonium sulfate, centrifuged, dissolved in Tris Buffered Saline (TBS) and run on the SDS-PAGE. Western blotting with antibodies against COL18A1 (anti-NC1, 1087+; anti-NC11, 1112+ from Dr. Takako Sasaki) was performed after transfer to a nitrocellulose membrane. Cells were also fixed in methanol and stained with rabbit polyclonal antibody against murine type IV collagen from PF-HR9 cells (15), rabbit polyclonal against murine NC2 domain of collagen XVI (trimeric form, native conformation) purified using antigen-coupled column from serum (Hans Peter Bächinger's Laboratory, unpublished), antibodies against murine COL18A1 (anti-NC1, 1087+; anti-NC11, 1112+ from Dr. Takako Sasaki) and antibody against murine fibrillin-1 (9543 from Dr. Lynn Sakai, Oregon Health & Science University).

Tissue Microarrays

Four samples of formalin-fixed paraffin embedded (FFPE) human RMS were available to include in a custom mouse model tissue microarray (TMA) (1 x aRMS, 3 x eRMS). Forty-eight murine model sarcomas were also included, representing developmental stages and genotypes including early myoblast (origin), postnatal stem cell (origin), maturing myofiber (origin), *Pax3:Foxo1*-expressing, *Trp53* wild type or mutated and *Rb1* wildtype or mutated (5, 7, 10).

The TMA was stained with a standard H&E stain for histologic verification. Co-author AM classified each tumor as non-rhabdomyosarcoma and rhabdomyosarcoma. The latter was further divided into aRMS, eRMS, pleomorphic RMS and RMS not otherwise specified (RMS NOS).

Immunohistochemical (IHC) staining

All 7 IHC stains were performed on the TMA by an immunoperoxidase technique using the following commercial antibodies: anti-COL18A1 (rabbit polyclonal, 1:50), anti-PLOD1 (rabbit polyclonal, 1:100), anti-PLOD2 (rabbit polyclonal, 1:100), anti-FBN1 (mouse monoclonal, 1:400), anti-FBN2 (rabbit polyclonal, 1:400), anti-COL4A1 (rabbit polyclonal, 1:50) and anti-COL4A2 (rabbit polyclonal, 1:50). All antibodies were purchased from LifeSpan Biosciences (Seattle, WA).

Each IHC stain was scored by co-author SHG for intensity and percentage of positive cells in each individual tumor sample (some tumors were represented more than once in the TMA). The intensity of each stain was scored accordingly: 0-negative; 1-indeterminate; 2-weak positive; 3-strong positive. The intensity and percentages were averaged for all cases containing more than one TMA fragment. Scoring of intensity was further simplified in a binary scheme where average intensity of greater than 1.5 was considered positive, and 1.5 or less was considered negative.

Statistical Analysis

Binary immunohistochemistry expression values were compared between histologic groups using the Fisher exact test. RNA expression comparisons between histologic groups were analyzed using one-way analysis of variance (ANOVA) with Tukey contrasts for multiple pairwise comparison of means and these analyses were carried out in log₂ units. Statistical significance was set at **P* < 0.05, ***P* < 0.01, and ****P* < 0.001. Error bars indicate mean ± SD or SEM. Comparisons with a *p*-value less than 0.05 were considered statistically significant. All statistical analyses were performed using R statistical software (version 3.3.1; R Foundation, Vienna, Austria) with the R commander graphical user interface.

Results

COL18A1 expression is elevated in RMS

COL18A1 showed significantly increased expression at the mRNA level in human and murine aRMS and eRMS tumors relative to normal muscle (Figures 2 and 3). *COL4A1*, *FBN1* and *FBN2* also showed

significant overexpression versus normal muscle in both human aRMS and eRMS, and *COL4A1* was significantly expressed in murine aRMS (Figures 2 and 3). Fibrillin-1 (FBN1) and fibrillin-2 (FBN2) are two subtypes of the fibrillin glycoprotein incorporated into elastic tissue in the extracellular matrix, and FBN2 is a known immunohistochemical biomarker of eRMS (16), which was reflected at the RNA level for eRMS more so than aRMS. Increased expression of *COL4A2*, *PLOD1* and *PLOD2* were seen for human RMS tumors (Figures 2 and 3). For NRSTS, Col18a1 and Plod2 (but not *Fbn1* and *Fbn2*) were elevated in mouse tumors (Figure 4).

Given that RNA studies were conducted on whole tumor, which includes tumor cells and stromal cells, we sought to understand and further differentiate the source of these collagen and collagen-associated gene product expressions by immunohistochemistry on custom tissue microarrays. The expression of COL18A1, PLOD1, PLOD2, FBN2, COL4A1 and COL4A2 were present in both murine aRMS and murine eRMS (Table 1 and Figure 5). However, FBN1 was not expressed in murine aRMS and was weakly expressed in only one model of murine eRMS (Table 1 and Figure 5). Undifferentiated sarcomas, which includes a variety of morphologic phenotypes from spindled to epithelioid and pleomorphic expressed all collagen-modifying enzyme to varying degrees (Table 1). No statistically significant trends were found in human RMS due to small sample size.

Comparison of RMS versus undifferentiated sarcomas in mice showed a significantly higher expression of PLOD2 in RMS ($p=0.044$). When comparing aRMS to eRMS, a significantly higher expression of PLOD1 was seen in the aRMS subtype ($p=0.05$) (Table 1).

Table 1.

Immunohistochemical expression of collagens, fibrillins and collagen modifying enzymes in human and murine models.

	COL18A1	COL4A1	COL4A2	FBN1	FBN2	PLOD1	PLOD2
Human Model							
aRMS	100% (1/1)	0% (0/1)	100% (1/1)	0% (0/1)	0% (0/1)	0% (0/1)	100% (1/1)
eRMS	67% (2/3)	33% (1/3)	67% (2/3)	50% (1/2)	100% (3/3)	33% (1/3)	100% (3/3)
Mouse Model							
Rhabdomyosarcomas*	69% (20/29)	79% (23/27)	14% (4/29)	5% (1/22)	66% (19/29)	48% (14/29)	72% (21/29)
aRMS	86% (12/14)	93% (13/14)	7% (1/14)	0% (0/10)	64% (9/14)	71% (12/17)	79% (11/14)
eRMS	67% (4/6)	83% (5/6)	33% (2/6)	17% (1/6)	67% (4/6)	17% (1/6)	67% (4/6)
Lineage Origin:							
Early Myoblast (Myf5)	70% (7/10)	89% (8/9)	10% (1/10)	0% (0/8)	60% (6/10)	50% (5/10)	80% (8/10)
Postnatal Stem Cell (Pax7)	41% (7/17)	63% (10/16)	13% (2/16)	8% (1/12)	63% (10/16)	18% (3/17)	35% (6/17)
Maturing Myoblast (Myf6)	71% (12/17)	88% (14/16)	18% (3/17)	8% (1/12)	67% (12/18)	56% (10/18)	61% (11/18)
Undifferentiated Sarcomas**	47% (7/15)	64% (9/14)	14% (2/14)	10% (1/10)	60% (9/15)	25% (4/16)	31% (5/16)
*All subtypes of murine RMS							
**All murine sarcomas that could not be definitively characterized as RMS							

In general, murine sarcomas with *Rb1* nullizygosity were associated with the undifferentiated morphology, except for one case identified as aRMS which also showed lower expression of COL18A1, COL4A1 and PLOD2 compared to *Rb1* wildtype sarcomas ($p=0.0035$, 0.04 and 0.08 , respectively).

When comparing samples based on cell-of-origin (early myoblast, postnatal stem cell and maturing myoblast), a significant increase in PLOD1 was seen in cases of early myoblast and maturing myoblast

origin RMS compared to the postnatal stem cell origin($p=0.04$). No other significant differences in cell or lineage-of-origin were seen across the other IHC markers.

To visualize the intracellular versus extracellular localization of the ECM related protein in RMS cell lines, we performed immunocytochemistry (ICC) across several cultured murine RMS cells which showed expression of FBN1, the NC2 domain of COL16, COL4A1 as well as the NC1 and NC11 domains of COL18A1 (Figure 6). Intracellular and secreted(extracellular) COL18A1 was readily detectable by ICC (Figure 6). Secreted COL18A1 in the conditioned media was also detected by immunoblotting (Figure 7).

Clinical significance of the expression of COL18A1

Biopsy samples were studied for gene expression analysis as previously described(17). In the biopsy samples provided by the Intergroup Rhabdomyosarcoma Study-IV (IRS-IV)(17), higher expression of COL18A1, COL4A1 and COL4A2 were correlated with a worse outcomes in human RMS patients (Figure 8), with the alveolar subtype showing worsened survival compared to other RMS subtypes(Figure 8). Additionally, increased expression of FBN1 and FBN2 was correlated with worsened survival in aRMS(Figure 8); however, statistical significance is not seen for FBN1 and FBN2 when examining all RMS(Figure 8).

Discussion

The structural integrity of collagen fibers is reliant on a balance of post-translational modification and extracellular matrix enzyme composition, which can be significantly altered in tumors. To date, studies of the RMS TME are limited. However, mutations in *COL2A1* have recently been associated with chondrosarcoma risk(18).

As individual prognostic markers, increased COL18A1, COL4A1 and COL4A2 expression are all associated with decreased long-term survival in all pediatric RMS subtypes, with the alveolar subtype showing worsened survival versus other subtypes. aRMS is most often fatal when metastatic(19), yet recent studies of related soft tissue sarcomas suggests that collagen subtype and modification can determine the ease with which these sarcomas metastasize (14); furthermore, altering collagen modifications is therapeutically amenable with FDA-approved agents that suppress metastasis in mouse soft tissue sarcoma models(14). From the perspective of therapeutic opportunities, another study has introduced a 3D murine model recapitulating the *in vivo* structure of aRMS and the ECM, which has promising possibilities for tumor behavior and therapeutic exploration(20).

In this study, we have shown that RMS expresses specific collagen proteins (COL18A1, COL4A1) at significantly increased levels in pediatric RMS relative to normal muscle; this expression in turn represents a worse prognosis(*COL18A1*, *COL4A1*, *COL4A2*). Strong IHC expression of COL18A1 was shown to have a worse outcome in pulmonary carcinomas(21) similar to *COL18A1* RNA expression in RMS. Moderate to strong COL18A1 IHC staining is also seen in greater than two-thirds of murine and human RMS samples in our study. The dysregulated expression of COL18A1 and the worse prognosis associated with increased

expression raises the possibility that COL18A1 mediates RMS metastasis. Furthermore, modification or cleavage of COL18A1 (*e.g.* endostatin) perhaps alters tumor biology *e.g.*, dysregulating tumoral angiogenesis or increasing metastatic potential(22). In our exploration of tumoral production of collagen, we also sought to explore the possibility of RMS utilizing enzyme production to alter the collagen matrix of surrounding tissues. This phenomena in theory is an efficient way for a tumor originating in a stromal environment to quickly spread locally and gain access to blood supply/nutrition. Both *PLOD1* and *PLOD2* were shown to have increased overall RNA expression in human RMS relative to normal muscle. *PLOD1* is an enzyme responsible for catalyzing the hydroxylation of lysyl residues in collagen-like peptides and is known to be deficient in patients with kyphoscoliotic form of Ehlers-Danlos syndrome(23). Although *PLOD1* protein expression was present in less than 50% of RMS overall, a significantly larger number of murine aRMS samples showed *PLOD1* expression compared to eRMS; however, this finding did not correlate with RNA expression patterns as *PLOD1* expression in both murine aRMS and eRMS are statistically indistinguishable. A significant increase in *PLOD1* RNA expression was seen in both human aRMS and eRMS relative to normal muscle; unexpectedly, *PLOD1* protein expression is significantly higher in eRMS than aRMS.

Previous studies have shown correlation of *PLOD2* expression with metastatic potential in soft tissue sarcoma(24) akin to our discoveries in RMS. The fact that a larger number of aRMS show increased *PLOD1* expression compared to eRMS may be related to the possibility that the more aggressive alveolar subtype utilizes hypoxia-dependent mechanisms to break down collagen. However, *PLOD1/2* RNA expression did not correlate with worse outcome when looking at all RMS samples as a cohort and adjusting for stage.

In an attempt to isolate a specific tumoral mechanism based on the myogenic cell-of-origin, we analyzed whole transcriptome sequencing data segmented across murine aRMS and eRMS, and protein expression by IHC in RMS segmented by disease indication and by cell-of-origin (early myoblast, postnatal stem cell and maturing myoblast). Protein expression analysis by IHC yielded one important finding: significantly fewer cases of RMS with postnatal stem cell as cell-of-origin showed *PLOD1* positivity compared to other cells-of-origin. Given that cell-of-origin influences pharmacological response(5), absence of *PLOD1* positivity could potentially have diagnostic or even therapeutic value. Note that in the context of murine RMS models which have undergone whole transcriptome sequencing, the cell-of-origin status cleanly separated by histological diagnosis of aRMS or eRMS. Thus, we did not perform cell-of-origin based statistical comparison of extracellular matrix protein gene expression.

A more aggressive, undifferentiated morphology was associated with the *Rb1* nullizygous mouse sarcomas and was not seen in eRMS subtypes. The *Rb1* nullizygous sarcomas had a significantly lower number of tumors expressing COL18A1, COL4A1 and *PLOD2*. As described above, expression of COL18A1 and *PLOD2* are associated with increased tumor infiltration, metastasis and worse overall survival and thus offer potential therapeutic targets. This preliminary data suggests these targets are present in a higher number of aRMS/eRMS cases than in undifferentiated sarcomas and may represent a treatment opportunity.

Conclusion

Overall, our findings imply that RMS produce an imbalance in expression of a variety of collagens and collagen-modifying enzymes implicated in tumor growth and metastasis. COL18A1 expression is significantly increased in all RMS compared to normal muscle and is associated with worse overall survival. Our identification of overexpression of additional collagen markers at the RNA and protein level, specifically PLOD1 and PLOD2, may be considered for evaluation with potential FDA-approved or investigational therapies targeting these enzymes in future studies.

Abbreviations

aRMS: Alveolar rhabdomyosarcoma; COL16: collagen XVI; COL18A1: type XVIII collagen alpha 1; COL4A1: type IV collagen alpha 1; eRMS: Embryonal rhabdomyosarcoma; FBN1/2: Fibrillin-1/2; GEM: genetically-engineered mouse; NRSTS: non-rhabdomyosarcoma soft-tissue sarcoma; PLOD1/2: lysyl hydroxylase 1/2; RMS: Rhabdomyosarcoma; UPS: undifferentiated pleomorphic sarcoma

Declarations

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

AUTHOR CONTRIBUTIONS

JSB, AM, CK and NEB participated in the design or interpretation of the experimental results. NEB, JFS, MMC, XL, NB, ML, AB, TS, SPB, JSB, CH, EP and SHG participated in the acquisition or analysis of data. CK and JFS contributed resources to these studies. XL, NB, NEB, JSB and CK participated in writing the manuscript. CK, HPB, GS and AM directed studies. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All animal studies were conducted with the approval of IACUC. Muscle biopsies were originally collected for diagnostic purposes, at which time informed consent was obtained from all subjects or their

legal guardians. The biopsies were collected and tested according to the guidelines set out by the Human Subjects Institutional Review Board.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Svalina MN, Keller C. YAPping about differentiation therapy in muscle cancer. *Cancer Cell*. 2014;26(2):154-5.
2. Arndt CA, Rose PS, Folpe AL, Laack NN. Common musculoskeletal tumors of childhood and adolescence. *Mayo Clin Proc*. 2012;87(5):475-87.
3. Abraham J, Prajapati SI, Nishijo K, Schaffer BS, Taniguchi E, Kilcoyne A, et al. Evasion mechanisms to Igf1r inhibition in rhabdomyosarcoma. *Mol Cancer Ther*. 2011;10(4):697-707.
4. Nishijo K, Chen QR, Zhang L, McCleish AT, Rodriguez A, Cho MJ, et al. Credentialing a preclinical mouse model of alveolar rhabdomyosarcoma. *Cancer Res*. 2009;69(7):2902-11.
5. Abraham J, Nuñez-Álvarez Y, Hettmer S, Carrió E, Chen HI, Nishijo K, et al. Lineage of origin in rhabdomyosarcoma informs pharmacological response. *Genes Dev*. 2014;28(14):1578-91.
6. Hettmer S, Liu J, Miller CM, Lindsay MC, Sparks CA, Guertin DA, et al. Sarcomas induced in discrete subsets of prospectively isolated skeletal muscle cells. *Proc Natl Acad Sci U S A*. 2011;108(50):20002-7.
7. Rubin BP, Nishijo K, Chen HI, Yi X, Schuetze DP, Pal R, et al. Evidence for an unanticipated relationship between undifferentiated pleomorphic sarcoma and embryonal rhabdomyosarcoma. *Cancer Cell*. 2011;19(2):177-91.
8. Hosoyama T, Aslam MI, Abraham J, Prajapati SI, Nishijo K, Michalek JE, et al. IL-4R drives dedifferentiation, mitogenesis, and metastasis in rhabdomyosarcoma. *Clin Cancer Res*. 2011;17(9):2757-66.
9. Keller C, Arenkiel BR, Coffin CM, El-Bardeesy N, DePinho RA, Capecchi MR. Alveolar rhabdomyosarcomas in conditional Pax3:Fkhr mice: cooperativity of Ink4a/ARF and Trp53 loss of function. *Genes Dev*. 2004;18(21):2614-26.
10. Kikuchi K, Taniguchi E, Chen HH, Svalina MN, Abraham J, Huang ET, et al. Rb1 loss modifies but does not initiate alveolar rhabdomyosarcoma. *Skelet Muscle*. 2013;3(1):27.
11. Ishikawa Y, Bächinger HP. A molecular ensemble in the rER for procollagen maturation. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*. 2013;1833(11):2479-91.

12. Pokidysheva E, Boudko S, Vranka J, Zientek K, Maddox K, Moser M, et al. Biological role of prolyl 3-hydroxylation in type IV collagen. *Proceedings of the National Academy of Sciences*. 2014;111(1):161-6.
13. Okuyama K, Miyama K, Mizuno K, Bächinger HP. Crystal structure of (Gly-Pro-Hyp)₉: Implications for the collagen molecular model. *Biopolymers*. 2012;97(8):607-16.
14. Eisinger-Mathason TS, Zhang M, Qiu Q, Skuli N, Nakazawa MS, Karakasheva T, et al. Hypoxia-dependent modification of collagen networks promotes sarcoma metastasis. *Cancer Discov*. 2013;3(10):1190-205.
15. Bächinger HP, Fessler LI, Fessler JH. Mouse procollagen IV. Characterization and supramolecular association. *Journal of Biological Chemistry*. 1982;257(16):9796-803.
16. Grass B, Wachtel M, Behnke S, Leuschner I, Niggli FK, Schäfer BW. Immunohistochemical detection of EGFR, fibrillin-2, P-cadherin and AP2 β as biomarkers for rhabdomyosarcoma diagnostics. *Histopathology*. 2009;54(7):873-9.
17. Blandford MC, Barr FG, Lynch JC, Randall RL, Qualman SJ, Keller C. Rhabdomyosarcomas utilize developmental, myogenic growth factors for disease advantage: A report from the children's oncology group. *Pediatric Blood & Cancer*. 2006;46(3):329-38.
18. Tarpey PS, Behjati S, Cooke SL, Van Loo P, Wedge DC, Pillay N, et al. Frequent mutation of the major cartilage collagen gene COL2A1 in chondrosarcoma. *Nat Genet*. 2013;45(8):923-6.
19. Sokolowski E, Turina CB, Kikuchi K, Langenau DM, Keller C. Proof-of-concept rare cancers in drug development: the case for rhabdomyosarcoma. *Oncogene*. 2014;33(15):1877-89.
20. Pozzobon M, Saggioro M, D'Agostino S, Bisogno G, Muraca M, Gamba P. Alveolar Rhabdomyosarcoma Decellularization. In: Turksen K, editor. *Decellularized Scaffolds and Organogenesis: Methods and Protocols*. New York, NY: Springer New York; 2018. p. 317-25.
21. Chang H, Iizasa T, Shibuya K, Iyoda A, Suzuki M, Moriya Y, et al. Increased expression of collagen XVIII and its prognostic value in nonsmall cell lung carcinoma. *Cancer*. 2004;100(8):1665-72.
22. Walia A, Yang JF, Huang Y-H, Rosenblatt MI, Chang J-H, Azar DT. Endostatin's emerging roles in angiogenesis, lymphangiogenesis, disease, and clinical applications. *Biochim Biophys Acta*. 2015;1850(12):2422-38.
23. Yeowell HN, Walker LC. Mutations in the Lysyl Hydroxylase 1 Gene That Result in Enzyme Deficiency and the Clinical Phenotype of Ehlers–Danlos Syndrome Type VI. *Molecular Genetics and Metabolism*. 2000;71(1):212-24.
24. Eisinger-Mathason TSK, Zhang M, Qiu Q, Skuli N, Nakazawa MS, Karakasheva T, et al. Hypoxia-dependent modification of collagen networks promotes sarcoma metastasis. *Cancer discovery*. 2013;3(10):1190-205.

Figures

Figure 1

Figure 1

Conditional (Cre/LoxP) Mouse Models of Rhabdomyosarcoma (RMS) and Non-Rhabdomyosarcoma Soft Tissue Sarcomas, including like undifferentiated pleomorphic sarcoma (UPS). Phenotype of the sarcoma depends not only on mutational profile, but also on cell (lineage) of origin and timing of the initiation before or after birth (5-7, 10). Surprisingly, eRMS and UPS exist in a continuum (7). Also, to our surprise postnatal muscle progenitors almost never gave rise to aRMS.

Figure 2

Figure 2

Extracellular Matrix Gene Expression (ECM) gene expression in mouse and human aRMS. Boxplots are given for biopsies versus cell lines versus normal muscle (n=35 human aRMS biopsies, n=4 human aRMS cell lines, n=564 human normal skeletal muscle, n=13 mouse necropsy aRMS tumors, n=12 mouse normal skeletal muscle). HS_TU, human tumors. HS_CL, human RMS cell lines. HS_NM, human normal skeletal muscle. MM_TU, mouse tumors. MM_NM, mouse normal skeletal muscle.

Figure 3

Figure 3

ECM gene expression in mouse and human eRMS. Boxplots are given for biopsies versus cell lines versus normal muscle (n=57 human eRMS biopsies, n=5 human eRMS cell lines, n=564 human normal skeletal muscle, n=4 mouse necropsy eRMS tumors, n=12 mouse normal skeletal muscle). HS_TU, human tumors. HS_CL, human RMS cell lines. HS_NM, human normal skeletal muscle. MM_TU, mouse tumors. MM_NM, mouse normal skeletal muscle.

Figure 4

Figure 4

ECM gene expression in mouse NRSTS. Boxplots are given for biopsies versus normal muscle (n=6 mouse necropsy NRSTS tumors, n=12 mouse normal skeletal muscle). MM_TU, mouse tumors. MM_NM, mouse normal skeletal muscle.

Figure 5

aRMS

eRMS

Figure 5

Protein expression of collagens, fibrillins and collagen modifying enzymes in murine aRMS and eRMS. Representative images showing tissue microarray negative control immunohistochemical staining and positive tumoral immunohistochemical staining of COL18A1, COL4A1, COL4A2, FBN1, FBN2, PLOD1 and PLOD2. Note that the representative aRMS IHC image for FBN1 shown is nearly negative (scoring is 1). Antibody specificity is with tumor specimen ID in parentheses listed on the figure.

Figure 6

Immunocytochemical detection of ECM-related proteins in murine sarcoma models. Expression of ECM proteins in primary cell cultures derived from murine RMS. Immunocytochemistry using five independent antibodies against fibrillin-1, collagen XVI, collagen IV and two antibodies recognizing NC1 and NC11 domains of collagen XVIII. For each antibody, staining was intracellular and/or extracellular.

Figure 7

Figure 7

Biochemical analysis of secreted collagen XVIII in murine sarcoma models. Secretion of soluble type XVIII collagen molecules in cell cultures derived from murine RMS. Western-blot analysis of conditioned media from four cultures with two antibodies against type XVIII collagen. Antibody anti-NC1 recognizes the C-terminal non-collagenous domain (left panel), whereas antibody anti-NC11 recognizes the N-terminal non-collagenous domain (right panel). The bands depicted by arrow α are intact collagen XVIII. The bands depicted by arrow β are presumed collagen XVIII without heparan-sulphate. The bands depicted by arrow γ are presumed fragments of collagen XVIII without NC11 domain. The bands depicted by arrow δ are presumed endostatin/endostatin-containing fragments.

Figure 8

Overexpression of COL18A1, COL4A1, COL4A2, FBN1 and FBN2 at the RNA level is associated with worsened outcome. Decreased overall survival at 9 years for human RMS patients with elevated expression of COL18A1, COL4A1 and COL4A2, as well as human aRMS patients with elevated expression of FBN1 and FBN2, even when adjusted for other known clinical covariates (as per analysis in (17)). aRMS, fusion-positive RMS. eRMS, fusion-negative RMS.