European Journal of Therapeutics
pISSN: 2564-7784
eISSN: 2564-7040

Eur J Ther. 2024;30(5):675-681.
https://doi.org/10.58600/eurjther2260

Original Research

ECM Components are Essential for Proper i*n Vitro* Myogenesis

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Received: 2024-06-29 **Accepted:** 2024-08-05 **Published Online:** 2024-08-07

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ABSTRACT

Objective: *In vitro* models of skeletal muscle often utilize primary myoblast cells or myoblast cell lines. Myoblasts require adhesion to the extracellular matrix (ECM) to grow, proliferate, migrate, and differentiate in their natural environments *in vivo*. To meet the adhesion needs of adhesive cells under *in vitro* conditions, culture surfaces are coated with various biological or synthetic compounds. Within the scope of the study, the differentiation potential of H2K myoblasts, a cell line resembling primary myoblasts, were comparatively evaluated through morphological analysis on culture surfaces coated with various ECM and synthetic materials.

Methods: The culture surfaces were coated with fibronectin and laminin, the major adhesion proteins of ECM; gelatin, a molecular derivative of collagen; matrigel, an ECM extract; and PLL, a synthetic poly-amino acid. Cells were allowed to differentiate in each culture medium for 4 days and their capacity to adhere to the surface and differentiation rates from myoblast to myotube were evaluated by morphological analysis.

Results: In the uncoated culture environment, cells could only attach to 30-50% of the culture surface and myotube development was limited and not aligned with each other. On surfaces coated with PLL, no myotube development was observed and cells could only attach to 30-40% of the culture surface. Myotube development and alignment were similar on all surfaces coated with ECM components. On surfaces coated with ECM components laminin, fibronectin and matrigel, cells covered the entire culture surface and exhibited similar myotube development. However, on surfaces coated with gelatin, both cell adhesion to the surface and myotube development were limited compared to other ECM components. The mean myotube diameters of fibronectin, laminin, matrigel, PLL+laminin and gelatin were 49.71µm (±16.3µm), 52.31µm (±15.7µm), 51.9µm (±15.3µm), 53.06µm (±14.2µm) and 35.25µm (±11.4µm), respectively.

Conclusion: Within the scope of the study, it was revealed that coating the culture surface with only a cationic material such as PLL does not support myogenesis and ECM components are needed for cell viability and differentiation.

Keywords: Myogenesis, fibronectin, laminin, matrigel, gelatin, poly-l-lysine

INTRODUCTION

The human body is composed of approximately 650 skeletal muscles, which account for almost half of the entire body mass. Each skeletal muscle is composed of thousands of elongated, multinucleated cells, called muscle fibers, which are organized together through the extracellular matrix (ECM) to contract synchronously. The dynamic structure of this massive organization is kept under adaptive control by neural and physical stimulation, hormonal balance and nutrient availability [1-4]. Skeletal muscle performs many important functions, both mechanical and metabolic. It is connected to the bones by tendons and directs all movements of the body. It also maintains the posture of the body and keeps it in balance. It provides the power required for movement by converting chemical energy into mechanical energy during contractions. The heat generated during this process is used to maintain body temperature. Approximately 80% of circulating glucose is consumed by the muscles. Important compounds such as carbohydrates and amino acids are stored in the muscles. In case of starvation, they are able to utilize their stores for the energy the body needs for movement; they are also able to supply the amino acids needed to support protein synthesis in other tissues [5-7].

Deficiency of a protein in the skeletal muscle itself, as in Duchenne muscular dystrophy, or neurological diseases that prevent muscle innervation, as in amyotrophic lateral sclerosis, lead to chronic degeneration of skeletal muscles [8, 9]. Furthermore, metabolic diseases such as diabetes, sarcopenia and cachexia affect the normal availability of hormones, nutrients, metabolites and other important soluble modulators required for skeletal muscles, leading to skeletal muscle atrophy with a significant impact on quality of life [10-12]. For this reason, the molecules that both constitute the structure of skeletal muscle itself and provide its adaptive dynamism are the subject of intense research.

Main Points

- Myogenic cells need to interact with ECM in order to perform biological activities such as proliferation, migration and differentiation in their natural environment.
- Under in vitro conditions, a suitable adhesion environment must be provided to maintain myogenicity and to model the biological activities of myogenic cells.
- In order to mimic the adhesive environment of adhesion-dependent cells in vivo, the culture surface is usually coated with extracellular matrix components or synthetic polymers.
- This study well demonstrated that ECM components are necessary and sufficient for in vitro myogenesis.

Typically, myoblast cultures are utilized in investigating conditions that contribute to skeletal muscle pathophysiology. For this purpose, various myoblast cell lines such as C2C12, H2K, and L6, as well as primary myoblast cultures from humans or different animals, can be established [13-15]. In order to mimic muscle fibers in skeletal muscles, the differentiation of myoblast cells is stimulated to fuse with each other and form myotubes. Since myoblasts and myotubes are adhesion-dependent cells, they need to adhere to culture surfaces [16, 17]. In order to mimic the adhesive environment of adhesion-dependent cells *in vivo*, the culture surface is usually coated with ECM components or synthetic polymers [18, 19]. Although there is no generally accepted culture surface coating material for myogenic cells, culture surfaces are commonly coated with various materials in the myogenic differentiation process due to their low capacity to adhere only to physically treated culture surfaces. Within the scope of the study, the differentiation potential of H2K myoblasts, which have a very low capacity to adhere to the uncoated culture surface like primary myoblasts, on the culture surface coated with various ECM components and synthetic materials was evaluated comparatively by morphological analysis.

MATERIAL AND METHODS

Coating Procedures and Cell Culture Conditions

Before seeding cells, the well surfaces of 12-well plates were coated with 0.1 mg/ml poly-l-lysine (PLL) (Sigma, P5899), 1.5 µg/cm² laminin (Sigma, L2020), 1.5 µg/cm² fibronectin (Sigma, F2006), 0.1% gelatin (Sigma, G1393), and 100 µg/ml matrigel (Corning, CLS354234). To ensure adherence of the materials to the well surfaces, plates coated with laminin and gelatin were incubated for 2 hours and 45 minutes at 37°C, respectively; matrigel-coated plates were incubated for 1 hour at room temperature; and plates coated with fibronectin and PLL were air-dried for 2 hours. Additionally, to coat PLL-coated plates with laminin, after air-drying PLL-coated plates for 2 hours, 1.5 µg/cm² laminin was applied and incubated for 2 hours at 37°C.

The conditionally immortal mouse H2K myogenic cell line [20] was used for the experiments. A seeding density of 1 ×

10^5 myoblasts per well was used with growth medium (GM) containing high glucose DMEM (Dulbecco's modified Eagle's medium; Invitrogen, Gibco), 20% FBS (Fetal Bovine Serum; Biochrom), 0.5% chick embryo extracts (CEEs; US Biological), and 20 U/mL interferon (IFN)-γ (Roche). Cells were initially incubated in GM in a humidified incubator (33°C, 10% CO_2) for one day and then induced to differentiate using differentiation medium (DM) containing high glucose DMEM (Gibco) and 5% HS (Horse Serum, Biochrom) in a humidified incubator (37°C, 5% CO_2). Cells were allowed to differentiate for 4 days and were visualized using phase contrast brightfield microscopy. Images were captured at 100X magnification. At least 100 myotubes were randomly selected from 12 fields in triplicate wells for each experimental condition. The diameter of each myotube was measured at three approximately equidistant points along their length using Image J software (NIH) to calculate the mean diameter.

RESULTS

To evaluate the extent to which coating the culture surface with various biological and synthetic molecules affects the myogenesis performance of myoblasts, the H2K cell line was utilized. Since H2K cells are conditionally immortal, they are the cell line most similar to primary myoblasts directly cultured from animals, unlike cell lines such as C2C12 or L6 [20, 21]. Therefore, just as they need to adhere to ECM proteins under *in vivo* conditions, their need for attachment in the culture environment is very high for proliferation and differentiation. In order to make a comprehensive comparison, culture surfaces were coated with fibronectin and laminin proteins, which are the main components of the ECM; gelatin, a molecular derivative of collagen; matrigel, a direct ECM extract; and PLL, a synthetic poly-amino acid. PLL+laminin coating was also performed in order to see the effectiveness of synthetic and biological coating materials together.

The cells were left to differentiate in the culture environment for 4 days. It was observed that in the uncoated culture surface environment, cell density remained limited to 30-50%, and dead cells were found floating on the medium surface in clumped forms due to their inability to adhere to the culture surface. It was also observed that the limited number of developing myotubes were not aligned with each other and that the lengths were shorter and the diameters were narrower than the myotubes developing on the surface coated with ECM components. In the culture medium whose surface was coated only with PLL, cell density remained around 30-40%, there were dead cells clumped on the surface of the medium and no myotube development was observed. On all other surfaces coated with ECM components, it was observed that the cell density covering the surface and the development of myotubes were more effective. On the culture surface coated with gelatin, the number, diameter, length, and alignment of the formed myotubes showed more limited development compared to other ECM components, and the cells did not cover the entire culture surface. The culture surfaces coated with fibronectin, laminin, PLL+laminin, and matrigel were completely covered by the cells. Additionally, on these surfaces, it was determined that myotube development was more effective compared to the remaining surfaces, with myotube length, diameter, and alignment being similar to each other (Figure 1). Although it can be observed under the microscope that myotube development is more efficient on surfaces coated with ECM components than other coatings, myotube diameter analysis was performed to reveal which ECM component supports myotube maturation more efficiently. For this purpose, analyses of measuring the average myotube diameter and determining myotube contents based on their diameters were utilized. The mean myotube diameters of fibronectin, laminin, matrigel, PLL+laminin and gelatin were 49.71µm (±16.3µm), 52.31µm (±15.7µm), 51.9µm (±15.3µm), 53.06µm (±14.2µm) and 35.25µm (±11.4µm), respectively (Figure 2A). While the diameters of myotubes growing on culture surfaces coated with fibronectin, laminin, matrigel and PLL+laminin showed a similar distribution, the distribution of myotubes on surfaces coated with gelatin differed from the others, with a higher percentage of small diameter myotubes and a lower percentage of large diameter myotubes compared to other coatings (Figure 2B).

DISCUSSION

Skeletal muscle development and repair involve the differentiation of myoblasts originating from progenitor cells, which fuse with each other to form muscle fibers [4]. During this process, myoblasts need to migrate to reach other myoblasts if a new muscle fiber is being formed, or to reach the existing muscle fiber if a damaged muscle fiber is being repaired. For myoblasts to both migrate and differentiate, interaction with cell adhesion molecules on the cell surface and the ECM is necessary. The connections established by integrin proteins on the surfaces of myoblasts and muscle fibers with the major ECM proteins laminin and fibronectin regulate the processes involved in skeletal muscle development and repair [22-26]. Laminin, basal lamina's primary component, consists of three polypeptide

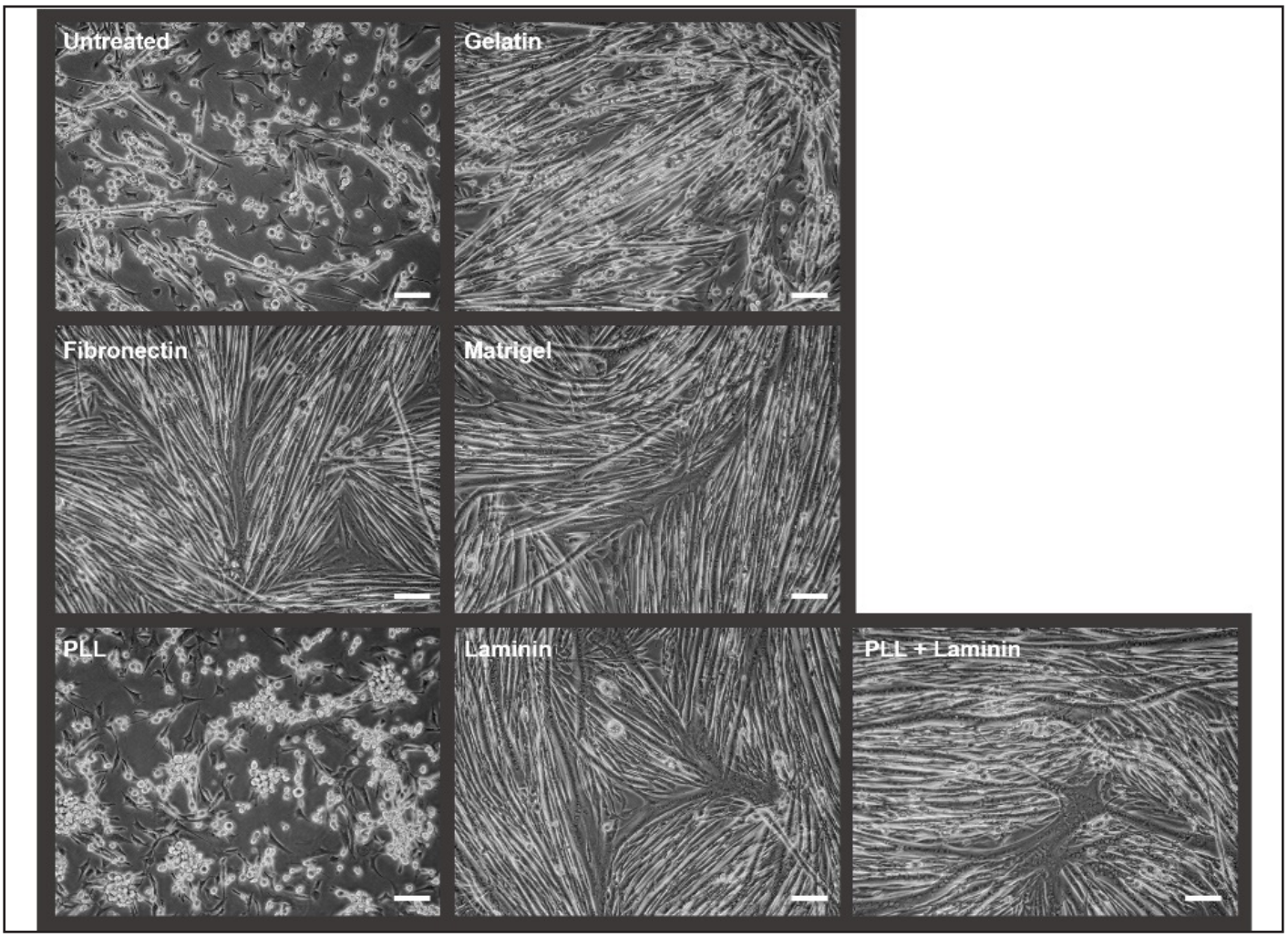

Figure 1. Morphological appearance of differentiated H2K myoblasts on different culture surfaces. Representative images showing morphological features of myogenic changes of H2K myoblasts differentiated on culture surfaces coated with gelatin, fibronectin, matrigel, PLL, laminin, and PLL+laminin after four days. The scale bar is 100 µm.

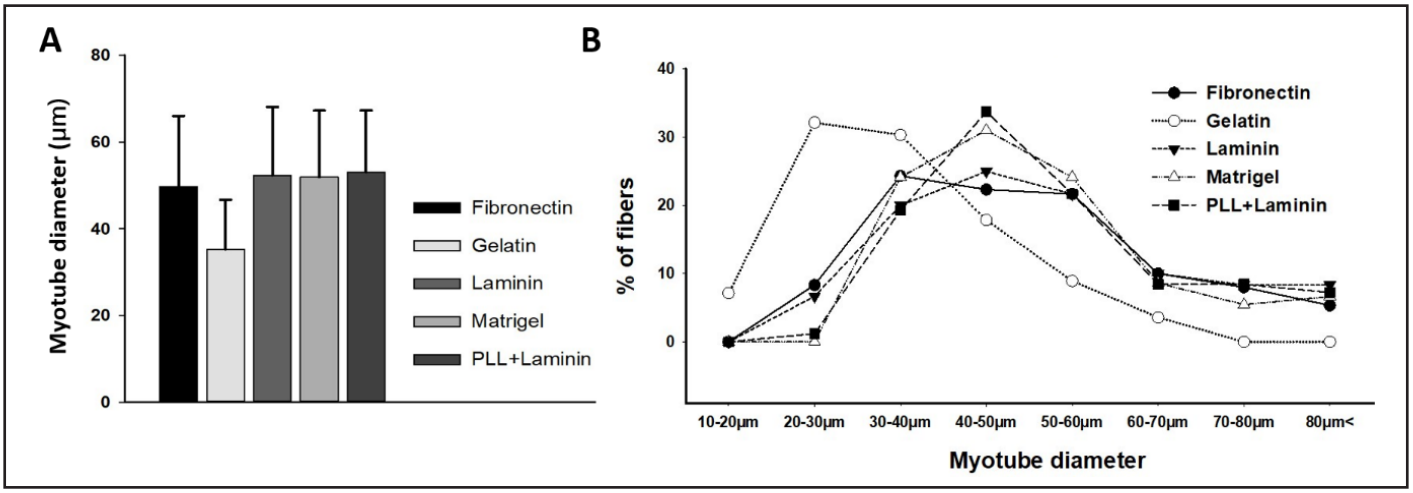

Figure 2. Myotube diameter analysis. (A) Mean myotube diameters. (B) Distribution of myotube diameters within each group. Data are means ± SEM.

chains held together by disulfide bonds and plays a major role in cell adhesion to the ECM [27-29]. Fibronectin, rich in RGD (Arg-Gly-Asp) motifs which serve as ligands for integrins, is an important ECM protein that facilitates cell binding to collagen and proteoglycans found in the ECM [30]. The presentation of these proteins, which support myoblast migration and muscle fiber differentiation *in vivo*, to H2K cells *in vitro* supported myoblast differentiation and myotube maturation. A similar differentiation profile was found on surfaces coated with matrigel. Matrigel is a basement membrane extract purified from murine Engelbreth-Holm-Swarm (EHS) tumors. It is high in laminin, entactin, collagen and proteoglycan levels but very low in fibronectin. Matrigel also contains many soluble factors that promote cell proliferation and growth such as transforming growth factor (TGF-beta), epidermal growth factor (EGF), insulin-like growth factor (IGF-1) and fibroblast growth factor (bFGF) [31]. Therefore, matrigel's ability to promote myoblast differentiation may be attributed to the presence of laminin and growth factors and partly to the presence of collagen. Gelatin is a hydrolyzed derivative of collagen. Myoblasts bind to collagen molecules via fibronectin, although there are some studies showing that muscle fibers can bind directly to collagen [32, 33]. Although the coating of the culture surface with gelatin supports myoblast differentiation, myogenesis efficiency seems to be limited compared to other ECM components included in the study. The PLL tested in this study did not promote differentiation of myoblasts into myotubes. PLL, a synthetic lysine polymer, mediates the cationic coating of the culture surface and thus the binding of cells to the culture surface due to their anionic membrane. The results of the study suggest that a cationic surface alone is not sufficient for myogenesis to occur.

CONCLUSIONS

As the largest tissue in the human body, skeletal muscle supports numerous vital activities, both mechanical and metabolic. Aging, cancer, metabolic diseases, and various genetic conditions often impair skeletal muscle function, significantly reducing quality of life. Therefore, the development and function of skeletal muscle are subjects of extensive research interest. *In vitro* skeletal muscle models, created for this purpose, particularly rely on primary myoblast cells that require adhesive conditions similar to those *in vivo*. Within this study, using H2K cells akin to primary myoblasts, it was demonstrated that merely coating the culture surface with a cationic material does not support myogenesis. Instead, it was shown that ECM components are necessary to maintain cell viability and enable differentiation, highlighting their critical role in this process.

Conflict of interest: The author declares that there is no conflicts of interest.

Funding: No funding was received to assist with the preparation of this manuscript

REFERENCES

[1] Brooks SV, Guzman SD, Ruiz LP (2023) Skeletal muscle structure, physiology, and function. Handb Clin Neurol 195:3-16. https://doi.org/10.1016/B978-0-323-98818-6.00013-3

[2] Frontera WR, Ochala J (2015) Skeletal muscle: a brief review of structure and function. Calcif Tissue Int 96:183-195. https://doi.org/10.1007/s00223-014-9915-y

[3] Lieber RL, Friden J (2000) Functional and clinical significance of skeletal muscle architecture. Muscle Nerve 23:1647-1666. https://doi.org/10.1002/1097-4598(200011)23:11<1647::aid-mus1>3.0.co;2-m

[4] Mukund K, Subramaniam S (2020) Skeletal muscle: A review of molecular structure and function, in health and disease. Wiley Interdiscip Rev Syst Biol Med 12:e1462. https://doi.org/10.1002/wsbm.1462

[5] Argiles JM, Campos N, Lopez-Pedrosa JM, Rueda R, Rodriguez-Manas L (2016) Skeletal Muscle Regulates Metabolism via Interorgan Crosstalk: Roles in Health and Disease. J Am Med Dir Assoc 17:789-796. https://doi.org/10.1016/j.jamda.2016.04.019

[6] Westerblad H, Bruton JD, Katz A (2010) Skeletal muscle: energy metabolism, fiber types, fatigue and adaptability. Exp Cell Res 316:3093-3099. https://doi.org/10.1016/j.yexcr.2010.05.019

[7] Lieber RL, Ward SR (2011) Skeletal muscle design to meet functional demands. Philos Trans R Soc Lond B Biol Sci 366:1466-1476. https://doi.org/10.1098/rstb.2010.0316

[8] Chang M, Cai Y, Gao Z, Chen X, Liu B, Zhang C, Yu W, Cao Q, Shen Y, Yao X, Chen X, Sun H (2023) Duchenne muscular dystrophy: pathogenesis and promising therapies. J Neurol 270:3733-3749. https://doi.org/10.1007/s00415-

023-11796-x

[9] Shefner JM, Musaro A, Ngo ST, Lunetta C, Steyn FJ, Robitaille R, De Carvalho M, Rutkove S, Ludolph AC, Dupuis L (2023) Skeletal muscle in amyotrophic lateral sclerosis. Brain 146:4425-4436. https://doi.org/10.1093/brain/awad202

[10] Cruz-Jentoft AJ, Bahat G, Bauer J, Boirie Y, Bruyere O, Cederholm T, Cooper C, Landi F, Rolland Y, Sayer AA, Schneider SM, Sieber CC, Topinkova E, Vandewoude M, Visser M, Zamboni M, Writing Group for the European Working Group on Sarcopenia in Older P, the Extended Group for E (2019) Sarcopenia: revised European consensus on definition and diagnosis. Age Ageing 48:16-31. https://doi.org/10.1093/ageing/afy169

[11] Martin A, Gallot YS, Freyssenet D (2023) Molecular mechanisms of cancer cachexia-related loss of skeletal muscle mass: data analysis from preclinical and clinical studies. J Cachexia Sarcopenia Muscle 14:1150-1167. https://doi.org/10.1002/jcsm.13073

[12] Espino-Gonzalez E, Dalbram E, Mounier R, Gondin J, Farup J, Jessen N, Treebak JT (2024) Impaired skeletal muscle regeneration in diabetes: From cellular and molecular mechanisms to novel treatments. Cell Metab 36:1204-1236. https://doi.org/10.1016/j.cmet.2024.02.014

[13] Romagnoli C, Iantomasi T, Brandi ML (2021) Available In Vitro Models for Human Satellite Cells from Skeletal Muscle. Int J Mol Sci 22. https://doi.org/10.3390/ijms222413221

[14] Carter S, Solomon TPJ (2019) In vitro experimental models for examining the skeletal muscle cell biology of exercise: the possibilities, challenges and future developments. Pflugers Arch 471:413-429. https://doi.org/10.1007/s00424-018-2210-4

[15] Chal J, Pourquie O (2017) Making muscle: skeletal myogenesis in vivo and in vitro. Development 144:2104-2122. https://doi.org/10.1242/dev.151035

[16] Taylor L, Wankell M, Saxena P, McFarlane C, Hebbard L (2022) Cell adhesion an important determinant of myogenesis and satellite cell activity. Biochim Biophys Acta Mol Cell Res 1869:119170. https://doi.org/10.1016/j.bbamcr.2021.119170

[17] Burkin DJ, Kaufman SJ (1999) The alpha7beta1 integrin in muscle development and disease. Cell Tissue Res 296:183-190. https://doi.org/10.1007/s004410051279

[18] Cooke MJ, Phillips SR, Shah DS, Athey D, Lakey JH, Przyborski SA (2008) Enhanced cell attachment using a novel cell culture surface presenting functional domains from extracellular matrix proteins. Cytotechnology 56:71-79. https://doi.org/10.1007/s10616-007-9119-7

[19] Wysotzki P, Gimsa J (2019) Surface Coatings Modulate the Differences in the Adhesion Forces of Eukaryotic and Prokaryotic Cells as Detected by Single Cell Force Microscopy. Int J Biomater 2019:7024259. https://doi.org/10.1155/2019/7024259

[20] Morgan JE, Beauchamp JR, Pagel CN, Peckham M, Ataliotis P, Jat PS, Noble MD, Farmer K, Partridge TA (1994) Myogenic cell lines derived from transgenic mice carrying a thermolabile T antigen: a model system for the derivation of tissue-specific and mutation-specific cell lines. Dev Biol 162:486-498. https://doi.org/10.1006/dbio.1994.1103

[21] Jat PS, Noble MD, Ataliotis P, Tanaka Y, Yannoutsos N, Larsen L, Kioussis D (1991) Direct derivation of conditionally immortal cell lines from an H-2Kb-tsA58 transgenic mouse. Proc Natl Acad Sci U S A 88:5096-5100. https://doi.org/10.1073/pnas.88.12.5096

[22] Thorsteinsdottir S, Deries M, Cachaco AS, Bajanca F (2011) The extracellular matrix dimension of skeletal muscle development. Dev Biol 354:191-207. https://doi.org/10.1016/j.ydbio.2011.03.015

[23] Mayer U (2003) Integrins: redundant or important players in skeletal muscle? J Biol Chem 278:14587-14590. https://doi.org/10.1074/jbc.R200022200

[24] Boppart MD, Mahmassani ZS (2019) Integrin signaling: linking mechanical stimulation to skeletal muscle hypertrophy. Am J Physiol Cell Physiol 317:C629-C641. https://doi.org/10.1152/ajpcell.00009.2019

[25] Crawley S, Farrell EM, Wang W, Gu M, Huang HY, Huynh V, Hodges BL, Cooper DN, Kaufman SJ (1997) The alpha7beta1 integrin mediates adhesion and migration of skeletal myoblasts on laminin. Exp Cell Res 235:274-286. https://doi.org/10.1006/excr.1997.3671

[26] Cachaco AS, Pereira CS, Pardal RG, Bajanca F, Thorsteinsdottir S (2005) Integrin repertoire on myogenic cells changes during the course of primary myogenesis in the mouse. Dev Dyn 232:1069-1078. https://doi.org/10.1002/dvdy.20280

[27] Gullberg D, Tiger CF, Velling T (1999) Laminins during muscle development and in muscular dystrophies. Cell Mol Life Sci 56:442-460. https://doi.org/10.1007/pl00000616

[28] Shibuya S, Wakayama Y, Inoue M, Kojima H, Oniki H (2003) Merosin (laminin-2) localization in basal lamina of normal skeletal muscle fibers and changes in plasma membrane of merosin-deficient skeletal muscle fibers. Med Electron Microsc 36:213-220. https://doi.org/10.1007/s00795-003-0227-y

[29] Yurchenco PD, McKee KK, Reinhard JR, Ruegg MA (2018) Laminin-deficient muscular dystrophy: Molecular pathogenesis and structural repair strategies. Matrix Biol 71-72:174-187. https://doi.org/10.1016/j.matbio.2017.11.009

[30] Snow CJ, Peterson MT, Khalil A, Henry CA (2008) Muscle development is disrupted in zebrafish embryos deficient for fibronectin. Dev Dyn 237:2542-2553. https://doi.org/10.1002/dvdy.21670

[31] Hughes CS, Postovit LM, Lajoie GA (2010) Matrigel: a complex protein mixture required for optimal growth of cell culture. Proteomics 10:1886-1890. https://doi.org/10.1002/pmic.200900758

[32] Wohlgemuth RP, Brashear SE, Smith LR (2023) Alignment, cross linking, and beyond: a collagen architect's guide to the skeletal muscle extracellular matrix. Am J Physiol Cell Physiol 325:C1017-C1030. https://doi.org/10.1152/ajpcell.00287.2023

[33] Kiss AA, Somlyai-Popovics N, Kiss M, Boldogkoi Z, Csiszar K, Mink M (2019) Type IV Collagen Is Essential for Proper Function of Integrin-Mediated Adhesion in Drosophila Muscle Fibers. Int J Mol Sci 20. https://doi.org/10.3390/ijms20205124

How to Cite;

Akpulat U (2024) ECM Components are Essential for Proper in Vitro Myogenesis. Eur J Ther. 30(5):675-681. https://doi.org/10.58600/eurjther2260