EXPERIMENTAL CELL RESEARCH 330 (2015) 222–232

Available online at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/yexcr

Research Article

Interactive relationship between basement-membrane development and sarcomerogenesis in single cardiomyocytes

Huaxiao Yang[a], *Thomas K. Borg*[b], *Honghai Liu*[c], *Bruce Z. Gao*[a,*]

[a]*Department of Bioengineering, Clemson University, SC, United States*
[b]*Department of Regenerative Medicine, Medical University of South Carolina, SC, United States*
[c]*Department of Pathology, University of Cincinnati, OH, United States*

ARTICLE INFORMATION

Article Chronology:
Received 13 June 2014
Received in revised form
10 August 2014
Accepted 13 August 2014
Available online 21 August 2014

Keywords:
Basement membrane
Laminin
Sarcomerogenesis
Z-lines
Integrin

ABSTRACT

The cardiac basement membrane (BM), the highly organized layer of the extracellular matrix (ECM) on the external side of the sarcolemma, is mainly composed of laminin and collagen IV, which assemble a dense, well-organized network to surround the surface of each adult cardiomyocyte. The development of the cardiac BM plays a key role in organogenesis of the myocardium through interactions between sarcomeres and integrins. Because of the complicated structure of cardiac muscle fibers and lack of a proper investigation method, the detailed interactions among BM development, sarcomeric growth, and integrin expression remain unclear. In this study, freshly isolated 3-day neonatal cardiomyocytes (CMs) were cultured on aligned collagen, which mimics the in vivo ECM structure and induces neonatal CMs to grow into rod-like shapes. Then double fluorescence-immunostained laminin and α-actinin or integrin β1 on neonatal CMs cultured 4–72 h were imaged using a confocal microscope, and the spatial relationship between laminin deposition and α-actinin expression was evaluated by colocalization analysis. At 4 h, laminin was deposited around Z-bodies (dot-shaped α-actinin) and integrins; from 18-to-72 h, its gradual colocalization with Z-lines (line-shaped α-actinin) and integrins increased Pearson's coefficient; this indicates that development of the BM network from the neonatal stage to adulthood is closely related to sarcomeric formation via integrin-mediated interactions.

Introduction

The basement membrane (BM), the highly organized layer of the extracellular matrix (ECM) on the external side of the sarcolemma, is composed of numerous glycoproteins and proteoglycans, such as type IV collagens, laminins, entactins, perlecan, and chondroitin sulfate proteoglycans [6,35]. The BM is structurally or functionally associated with various cell types, such as

Abbreviations: BM, basement membrane; ECM, extracellular matrix; neonatal CMs, neonatal cardiomyocytes; CMs, cardiomyocytes; GBM, glomerular BM; SD, Sprague–Dawley; DPBS, phosphate buffered saline; KRB, Kreb's Ringer Bicarbonate Buffer; DMEM, Dulbecco's Modified Eagle's Medium; RT, room temperature; JACoP, Just Another Colocalization Plugin; PCs, Pearson's coefficients; EB, embryoid body

*Corresponding author. Fax: +1 864 656 4466.
E-mail address: zgao@clemson.edu (B.Z. Gao).

http://dx.doi.org/10.1016/j.yexcr.2014.08.020
0014-4827/

endothelial cells, podocytes [19], aortic smooth muscle cells [29], tracheal epithelial cells [8], Schwann cells [17], and cardiomyocytes (CMs) [15]; it first appears during the blastocyst stage between the primitive endoderm and the inner cell mass and is the first ECM produced during embryogenesis [13]. In different organs and tissues, the BM network takes different paths of development and maturation. For example, during glomerulogenesis in the kidney, laminin α1β1γ1 and α4β1γ1 are both present from the comma-shape through the S-shape stages. They are rapidly removed during the capillary-loop stage, and laminin α5β2γ1 is deposited in the glomerular BM (GBM) throughout adulthood [1]. In the human heart, several subunits of laminin have been detected; for example, at Gestational Week 8, laminin β1 and β2 were found in the ECM that surrounds CMs and in the BM zone of the endo/pericardium [22]. After birth, collagen IV is increasingly deposited in the endomysium from Day 0-Day 3, and collagen fibers form a densely woven network that surrounds groups of CMs [3]. Observation of adult CMs with transmission electron microscopy shows that the BM forms a dense network around each adult CM [12]. During dedifferentiation and redifferentiation of adult CMs cultured in vitro, the BM forms a fine network radiating from the central area near the nucleus to the pseudopods [15].

The BM's unique structural relationship with its associated tissues makes it critical in organogenesis of a variety of organs and tissues. For example, GBM is instrumental in glomerulogenesis; without GBM, a glomerular cell is unable to maintain its position, leading to a disorganized glomerulus [18]. In the circulatory system, blood-vessel maturation requires the presence of laminin α4β1γ1 [8]. In skeletal muscle, the BM contributes to differentiation of the myotube and is crucial in development of the neuromuscular junction [20]. In heart muscle, the BM network is influential in sarcomeric formation and remodeling because of its interaction with integrins, which serve as receptors on the plasma membrane and orchestrate multiple myocardial functions, including organogenesis [24]. For example, integrins α1β1 and α3β1 can directly bind with laminin to sense outside-in signals [24] and transfer them to the sarcomeres near the Z-line [4]. Although the BM-integrin–sarcomere complex is known to mediate growth and differentiation of neonatal cardiomyocytes (CMs) [23], BM involvement in sarcomerogenesis has not been studied. Particularly, fundamental knowledge of the spatial correlation between distribution of the BM network and the Z-lines during development of a single neonatal CM is poorly understood. The network formation around a single CM during its development from a neonate to an adult is largely unknown because of the complicated structure of cardiac-muscle fibers and lack of a comprehensive in vitro culture model.

A substrate coated with an aligned collagen using the method used by Ross and coworkers [27] was found to promote in vitro neonatal CMs to form in vivo-like cardiac muscle-fiber structures. This type of substrate was utilized to study sarcomerogenesis of cardiac cells [26] and was demonstrated to maintain neonatal CMs in the rod-like phenotype that is typical of Week 4.

In this study, confocal images of single neonatal CMs cultured from 4–72 h on aligned collagen were analyzed sequentially. The purpose of this study was to examine the sequential formation of the sarcomere in relation to expression of basement membrane (laminin), α-actinin, and integrin β1. According to our unpublished data, BM-collagen IV, another major component of the BM, shows a different pattern of formation around the neonatal CMs in comparison to BM-laminin; its expression may be closely related to cardiomyocyte–fibroblast interactions. The study of CM BM-collagen IV will be reported in our future publications.

Methods and materials

Cell harvest and culture

Adult Sprague–Dawley (SD) rats and 3-day-old neonatal SD rats were euthanized according to a procedure approved by the Clemson University Institutional Animal Care and Use Committee (Protocol number AUP2013-035). The procedure conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication, 8th Edition, 2011). The methods of euthanasia for neonatal and adult animals are consistent with the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association.

Neonatal CM harvest

Neonatal CMs were isolated from 3-day-old SD rats using the two-day protocol we previously reported [16]. In brief, ten neonatal rats were dissected, and the hearts were collected and minced in Moscona's saline. The tissue was transferred to 50 ml Dulbecco's Phosphate Buffered Saline (DPBS) with 4 mg trypsin and 50 mg neutral protease and stored in a 4 °C refrigerator overnight. The next day, the tissue was transferred into 50 ml Kreb's Ringers Bicarbonate Buffer (KRB) with 10 mg collagenase type I and 30 mg collagenase type II and then shaken in a water bath at 50 rpm for 45–60 min. The cell suspension was washed twice using cardiomyocyte culture medium (high glucose Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin streptomycin) to remove enzyme residue. The isolated cells were transferred into a 150 cm^2 flask for a cell-adhesive assay to remove cardiac fibroblasts. After two hours, the unattached neonatal CMs were collected for use. Our immunofluorescence staining data and data from other groups that used the same neonatal CM purification procedure have demonstrated that 95% neonatal CM purification can be achieved.

Adult CM harvest

Adult CMs were harvested from the hearts of one-month-old adult SD rats [33]. In brief, after a heparin injection (45 mg/kg) and the intraperitoneal sodium pentobarbital injection (0.5 ml/100 g) that followed, the heart with at least 5 mm of the aortic arch was removed and placed in the perfusion buffer. The heart was then perfused through the aortic arch with the perfusion buffer for 5 min at a flow rate of 12 ml/min. The perfusion buffer was replaced with a digestive buffer, and the heart was perfused for 60 min at a flow rate of 6 ml/min. Once enzymatic digestion of the heart was complete, the ventricle was placed in a 100 mm dish containing 5 ml of digestion buffer and gently minced into small pieces (~1 mm^3) with fine forceps. The cell suspension was transferred to a 50 ml conical polypropylene tube. Approximately 5 ml of perfusion buffer (37 °C) was added to the tube to create a final cell-suspension volume of 40 ml. After agitation, a stop buffer (10 ml) was added and mixed well using a 50 ml plastic transfer pipette; this was followed by ten gentle agitations of the solution with the pipette. The cell clumps were then allowed to settle by gravity sedimentation for 2 min, and the supernatant was transferred into a new 50 ml tube. Another 40 ml