Acta Biomaterialia 31 (2016) 144–155

Contents lists available at ScienceDirect

Acta Biomaterialia

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

Full length article

Preservation of human limbal epithelial progenitor cells on carbodiimide cross-linked amniotic membrane via integrin-linked kinase-mediated Wnt activation

David Hui-Kang Ma [a,b,c,*,1], Hung-Chi Chen [a,c,d,1], Kevin Sheng-Kai Ma [e], Jui-Yang Lai [c,f,g,*], Unique Yang [h], Lung-Kung Yeh [a], Yi-Jen Hsueh [a,c], Wing-Keung Chu [i], Chyong-Huey Lai [j], Jan-Kan Chen [i]

[a] Limbal Stem Cell Laboratory, Department of Ophthalmology, Chang Gung Memorial Hospital, No. 5, Fushing Street, Kweishan District, Taoyuan City 33305, Taiwan
[b] Department of Chinese Medicine, College of Medicine, Chang Gung University, No. 259 Wen-Hwa 1st Road, Kweishan District, Taoyuan City 33305, Taiwan
[c] Center for Tissue Engineering, Chang Gung Memorial Hospital, No. 5, Fushing Street, Kweishan District, Taoyuan City 33305, Taiwan
[d] Department of Medicine, College of Medicine, Chang Gung University, Taoyuan, No. 259 Wen-Hwa 1st Road, Kweishan District, Taoyuan City 33305, Taiwan
[e] Department of Dentistry, College of Oral Medicine, Chung Shan Medical University, No. 110, Section 1 Jianguo N. Road, South District, Taichung City 40201, Taiwan
[f] Institute of Biochemical and Biomedical Engineering, Chang Gung University, No. 259 Wen-Hwa 1st Road, Kweishan District, Taoyuan City 33305, Taiwan
[g] Biomedical Engineering Research Center, Chang Gung University, No. 259 Wen-Hwa 1st Road, Kweishan District, Taoyuan City 33305, Taiwan
[h] Department of Biology, University of California, 142 LSA #3200, Berkeley, CA 94720-3200, USA
[i] Department of Physiology, College of Medicine, Chang Gung University, No. 259 Wen-Hwa 1st Road, Kweishan District, Taoyuan City 33305, Taiwan
[j] Department of Obstetrics and Gynecology, Chang Gung Memorial Hospital, No. 5, Fushing Street, Kweishan District, Taoyuan City 33305, Taiwan

ARTICLE INFO

Article history:
Received 9 July 2015
Received in revised form 17 November 2015
Accepted 19 November 2015
Available online 26 November 2015

Keywords:
Limbal epithelial stem cells
Carbodiimide cross-linked amniotic membranes
Integrins
Wnt signaling pathways
Stem cell–microenvironment interactions

ABSTRACT

The Wnt pathway is a major signaling pathway that regulates corneal epithelial stem cells. However, little is known about how the ultrastructure of the limbal epithelial basement membrane (EBM) affects Wnt activity. Due to its enhanced matrix stability, the cross-linked amniotic membrane (AM) has gained increasing interest in the field of regenerative medicine. For the first time, we used EDC/NHS cross-linked denuded AM (CLDAM) as a simulated EBM substrate to investigate this mechanism. Human limbal epithelial (HLE) cells were cultured on dishes (HLE/dish), denuded AM (HLE/DAM) or CLDAM (HLE/CLDAM). Compared with HLE/dish or HLE/DAM cultures, HLE/CLDAM cultures showed greater BrdU retention and colony formation efficiency and expressed higher levels of p63, ABCG2, integrin β1, and integrin-linked kinase (ILK). Nuclear β-catenin and TCF-4 levels were higher in HLE/CLDAM cultures compared with HLE cells cultured on collagen IV, laminin, Matrigel, or DAM. Silencing of ILK in HLE/CLDAM cultures resulted in decreased levels of nuclear β-catenin, TCF-4 and deltaNp63α, whereas cytokeratin 12 expression increased. Over-expression of ILK in HLE/dish cultures had the opposite effects. Accordingly, we proposed that the CLDAM matrix, with its higher rigidity and rougher ultrastructure, better preserved HLE progenitor cells in vitro, possibly by activating integrin β1/ILK, which indirectly activated Wnt/β-catenin and subsequently deltaNp63α. Crosstalk between the integrin β1/ILK and Wnt/β-catenin pathways appears to play a crucial role in limbal progenitor cell survival on EBM.

Statement of significance

We demonstrated the superior capability of carbodiimide cross-linked denuded amniotic membrane (CLDAM) than natural DAM to preserve limbo-corneal epithelial progenitor cells in vitro, then we used CLDAM as a simulated epithelial basement membrane (EBM) to study how EBM maintains limbal epithelial stem cells (LESCs). We found that integrin-linked kinase (ILK) is an important mediator that transfers survival signals detected by integrin β1 to the Wnt/β-catenin pathway, which in turn up-regulates

* Corresponding authors at: Limbal Stem Cell Laboratory, Department of Ophthalmology, Chang Gung Memorial Hospital, No. 5, Fushing Street, Kweishan District, Taoyuan City 33305, Taiwan (D.H.-K. Ma). Institute of Biochemical and Biomedical Engineering, Chang Gung University, No. 259 Wen-Hwa 1st Road, Kweishan District, Taoyuan City 33305, Taiwan (J.-Y. Lai).
E-mail addresses: davidhkma@yahoo.com (D.H.-K. Ma), jylai@mail.cgu.edu.tw (J.-Y. Lai).
[1] Both authors contributed equally to the manuscript.

http://dx.doi.org/10.1016/j.actbio.2015.11.042

deltaNp63α, a master gene that regulates LESC function. The rougher surface of the limbal EBM suggests that the surface complexity of the LESC niche may be important in regulating LESC function, which is triggered by the recognition of topographic cues by integrin β1, followed by activation of the ILK/Wnt/β-catenin/p63 cascade.

1. Introduction

The limbus is a junctional zone between the cornea and conjunctiva. It has a specialized composition of epithelial basement membrane (EBM) proteins and cytokines produced by the resident cells. The limbal ultrastructure is referred to as the limbal stem cell "niche", which preferentially maintains limbal epithelial stem cells (LSCs) in an undifferentiated state [1,2]. The expression of stem cell markers and the unique ECM components in the limbal region have been previously summarized [3–5]. However, little is known about how the limbal EBM preserves the stemness properties of LSCs. Recently, Wnt signaling was implicated in the regulation of LSC activity [6–8]. In addition, TCF-4, the key transcription factor for Wnt signaling, is crucial for LSC maintenance [9,10]. Several Wnt ligands are expressed in the limbus, and Wnt can activate the transcription factor deltaNp63α, which is the master regulator of LSC stemness [7,11,12]. Nonetheless, little is known about how the limbal niche ultrastructure influences Wnt activity. Because integrins are the cell surface receptors for EBM components, such as laminin and collagen IV, it is likely that the activation of a downstream kinase in the integrin pathway (i.e., focal adhesion kinase or integrin-linked kinase) interacts with the Wnt pathway to affect LSC stemness.

The human amniotic membrane (AM) has been successfully used for LSC cultivation and transplantation [13–15]. Because the AM can preserve human limbo-corneal epithelial (HLE) progenitor cells in vitro, it is often considered a "surrogate" LSC niche [16,17]. The human AM is composed of three layers: a single epithelial layer, a thick basement membrane, and an avascular stroma that contains stromal cells. The AM EBM shares many similarities with the limbal EBM; four of the six EBM components that show increased expression in the limbus (i.e., the laminin α1, α2, and β1 chains; agrin; collagen type XVI; and fibulin-2) are also expressed in the AM [18].

However, the EBM of the AM cannot maintain long during cell culture due to digestion by proteases secreted from HLE cells [19]. Therefore, to prolong AM function, we previously cross-linked the AM with 1-ethyl-3-(3-dimethylaminopropyl)carbodii mide hydrochloride (EDC)/N-hydroxysuccinimide (NHS) [20,21]. Carbodiimide cross-linking significantly enhanced the mechanical and thermal stability, the optical transparency, and the resistance to collagenase digestion [21]. The processed AM was non-toxic to cultured HLE cells and retained anti-inflammatory activity, indicating the tolerability and safety of the carbodiimide treatment [20]. Following culture on cross-linked de-epithelialized AM (CLDAM), BrdU label-retaining rabbit limbo-corneal epithelial cells were observed after short-term transplantation [21]. In addition, cell growth and p63 and ABCG2 gene expression were proportional to the degree of AM cross-linking, indicating that the matrix rigidity and nanoroughness of carbodiimide cross-linked AM may control LSC-microenvironment interactions [20].

Because the EBM structure of CLDAM endures longer than natural DAM during cell culture, CLDAM was used in this study as a cell culture substrate to investigate how the EBM ultrastructure affects the differentiation and longevity of HLE cells. We found that, compared with dish or DAM cultures, cultivation on CLDAM better preserved HLE progenitor cells in vitro. More importantly, using CLDAM as a model of EBM, we determined that integrin-linked kinase (ILK)-mediated Wnt (ILK/Wnt) signaling activation might be responsible for the LSC preservation on EBM. This observation is supported by the report by Novak et al., who demonstrated that the modest over-expression of ILK in intestinal epithelial cells mediated cross-talk between cell–matrix interactions and cell–cell adhesion as well as components of the Wnt signaling pathway [22]. Our current findings further illustrate the importance of chemically cross-linked biological scaffolds in the determination of stem cell states.

2. Materials and methods

2.1. Preparation of EDC/NHS cross-linked CLDAM

Human tissues were handled following the tenets of the Declaration of Helsinki, and the research was approved by the Committee of Medical Ethics and Human Experiment of Chang Gung Memorial Hospital, Taoyuan, Taiwan. The epithelial cells on intact AM were removed by EDTA treatment and scraping to expose the underlying EBM [23]. Procedures to cross-link human AM have been described previously [21]. Briefly, before cross-linking, the AM was thawed and incubated with MES buffer for 1 h. The membrane was then immersed in 30 mL of MES buffer containing 5:1 EDC to NHS with gentle shaking at 25 °C for 24 h. Based on a previous study, we used the optimal cross-linker concentration of 0.05 mmol of EDC per mg of AM. Afterwards, the AM was thoroughly washed with double-distilled water to remove excess byproducts. The physical characterization of CLDAM has been described previously [21].

2.2. Cell culture

HLE cells were grown from limbal explants after removing the central cornea for transplantation. Three 2 × 1 mm limbal explants were placed on a 35-mm dish (HLE/dish), on natural de-epithelialized AM (HLE/DAM), or on CLDAM (HLE/CLDAM). The cultures were maintained in SHEM containing 5% fetal bovine serum (FBS). The cultures were harvested at 70–80% confluency for clonal analysis, immunostaining, quantitative real-time reverse transcription polymerase chain reaction (QRT-PCR), and Western blot. SB216763 and SB415286 are maleimide compounds that selectively inhibit glycogen synthase kinase-3 (GSK-3) in an ATP-competitive manner, which subsequently stabilizes and increases cytoplasmic β-catenin levels, thereby facilitating the nuclear translocation of β-catenin and the activation of Wnt signaling [24]. To study the effect of Wnt activation on LSC preservation, SB216763 and SB415286 (Sigma; 10 μM/mL) were added to the HLE/dish cultures for 5 days. The cells were then subjected to colony formation efficiency assays and Western blot. Finally, to compare HLE cell differentiation on purified basement membrane proteins or on CLDAM, limbal explants were grown on dishes coated with human collagen IV (0.5 μg/cm^2, Sigma), laminin (2 μg/cm^2, Sigma), Matrigel (growth factor-reduced BD Matrigel™ Matrix, 500 μL per 35 mm dish), and on dishes overlaid with DAM or CLDAM. Cells from 70–80% confluent cultures were collected for Western blot.