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Corresponding Author:	Hitomi Fujisaki Nippi Inc Ibaraki, JAPAN
Corresponding Author's Institution:	Nippi Inc
Corresponding Author E-Mail:	fujisaki@nippi-inc.co.jp
Order of Authors:	Hitomi Fujisaki
	Sugiko Futaki
	Shunji Hattori
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TITLE:

Evaluation of Keratinocyte Proliferation on Two- and Three-Dimensional Type I Collagen Substrates

AUTHORS AND AFFILIATIONS:

Hitomi Fujisaki[1], Sugiko Futaki[2], Shunji Hattori[1]

[1]Nippi Research Institute of Biomatrix, Toride, Ibaraki, Japan
[2]Department of Anatomy and Cell Biology, Osaka Medical College, Osaka, Japan

Corresponding Author:

Hitomi Fujisaki, Ph.D. (fujisaki@nippi-inc.co.jp)

Email Addresses of Co-Authors:

Sugiko Futak (an1029@osaka-med.ac.jp)
Shunji Hattori (shunhatt@nippi-inc.co.jp)

KEYWORDS:

type I collagen, fibril/non-fibrous form, two-/three-dimensional culture substrates, on-gel culture, keratinocyte culture, proliferation

SUMMARY:

Here, we present a protocol for the preparation of two different forms of culture substrates utilizing type I collagen. Depending on how collagen is handled, collagen molecules either maintain two-dimensional, non-fibrous form or reassemble into three-dimensional, fibril form. Cell proliferation on type I collagen is drastically affected by fibril formation.

ABSTRACT:

Type I collagen, useful as a substrate for cell culture, exists in two forms: the two-dimensional, non-fibrous form and three-dimensional, fibril form. Both forms can be prepared with the same type I collagen. In general, the non-fibrous form promotes cell adhesion and proliferation. The fibril form (gels) provides more physiological conditions in many types of cells; therefore, gel culture is useful for examining physiological behaviors of cells, such as drug efficacy.

Researchers can select the appropriate form according to the purpose of its use. For example, in the case of keratinocytes, on-gel culture has been used as a wound healing model. FEPE1L-8, a keratinocyte cell line cultured on the non-fibrous form of type I collagen, promote cell adhesion. Notably, keratinocyte proliferation is slower on the fibril form than the non-fibrous form. Protocols for the preparation of type I collagen for cell culture are simple and have wide applications depending on the experimental needs.

INTRODUCTION:

Interstitial connective tissues comprise a three-dimensional protein meshwork of heterogeneous composition, primarily composed of type I collagen fibrils[1]. Collagen fibrils play a key role as a

scaffold for cells[1-3] and interact with other extracellular matrix (ECM) proteins[3]. In vitro, different forms of type I collagen can be used as substrates for cell culture depending on the handling method[1-4]. Under acidic conditions, type I collagen maintains the non-fibrous form[5]. Coating the surface of culture dishes with the non-fibrous form promotes cell adhesion and proliferation[6, 7]. At physiological pH and temperature, type I collagen molecules reassemble into fibrils that form gels possessing a three-dimensional structure[1-8]. There are several important differences between the fibril and non-fibrous forms of type I collagen, including matrix stiffness and efficiencies of reconstruction of ECM components by the cells during the culture[1]. Matrix stiffness is one of the most studied regulatory factors of cell culture[1, 9]. However, the complex interactions between substrates and cells remain to be clarified. To examine the complex interactions between cells and environmental factors, a simple system is useful. Comparison of the cellular behavior on the two different forms of collagen may help to simplify the effect of environmental factors. Depending on the purpose of their use, different forms of type I collagen can be selectively used. Normally, keratinocytes are in contact with the basement membrane but not with type I collagen. However, during wound healing, keratinocytes move to the dermal connective tissue, proliferate, and heal the wound[10].

Recently, we demonstrated that the concentration of extracellular calcium is important for proliferation of keratinocyte line cells by using the culture system on the fibril form of type I collagen mimicking the dermal connective tissue[11]. When the keratinocyte cell line FEPE1L-8 was cultured on the fibril form of type I collagen, the shape of the cells was round and their proliferations were stopped at an extracellular calcium concentration of 30 μM[11]. When the calcium concentration was increased to 1.8 mM, cell growth was recovered[11]. The cells grew under both calcium concentrations (30 μM and 1.8 mM) when cultured on the non-fibrous form[11], whereas they were more sensitive to the exogenous calcium concentration when cultured on the fibril form. FEPE1L-8 was generated through transfection with the papillomavirus type 16 transforming genes E6 and E7 from human cervical carcinoma, non-tumorigenic, inhibit unlimited proliferation with limited differentiation potential like normal keratinocytes[12, 13]. FEPE1L-8 cells can be maintained by using some kinds of keratinocyte specific medium, including K110 Type-II with additive supplement K-1 (K110)[6]. Here, we describe the culture protocol of the human keratinocyte cell line on the non-fibrous and fibril forms of type I collagen.

PROTOCOL:

1. Preparation of keratinocyte culture medium

NOTE: Perform all procedures under aseptic conditions.

1.1) Add 5 mL of penicillin-streptomycin and 10 mL of additive supplement K-1 to 500 mL of K110 Type-II medium (K110) using a pipette.

2. **Preparation of the fibril form of type I collagen**

NOTE: Perform procedures until step 2.6 under aseptic conditions.

2.1) Keep 10x phosphate-buffered saline (PBS (-)), deionized water, collagen, a 96-well culture plate, and an empty 2-mL tube on ice.

NOTE: Do not use the same plate to prepare the fibril and non-fibrous forms.

2.2) Add 1.12 mL of deionized water to an empty 2 mL tube using a pipette. Add 200 µL of 10x PBS (-) to the deionized water in the 2 mL tube using a pipette. Gently shake the tube several times.

2.3) Add 0.66 mL of collagen to the 2 mL tube using a pipette. Gently and quickly shake the tube several times.

NOTE: Avoid bubble formation in the solution as much as possible. Prepare collagen solution immediately prior to use.

2.4) Pour 100 µL of the collagen solution from step 2.3 into each well of the 96-well culture plate. Gently shake the culture plate in a left to right motion.

NOTE: Cover the entire surface of the wells with the collagen solution. If the solution does not cover the entire surface, spread the solution using a pipette tip. Avoid bubble formation in the solution as much as possible.

2.5) Place the culture plate in a CO_2 incubator and incubate at 37 °C for 1 h. Check the gelation of collagen and move the culture plate to a clean bench.

NOTE: Confirm gelation by tilting the culture plate.

2.6) Gently pour 150 µL of K110 on the gels using a pipette along the wall of the well, place the culture plate in a CO_2 incubator and incubate at 37 °C for 1 h. Move the culture plate to a clean bench. Prior to cell culture, gently discard the K110 using a pipette.

NOTE: To protect the gels, the tip of the pipette should touch the wall of the well.

3. Preparation of the non-fibrous form of type I collagen

NOTE: Perform all procedures under aseptic conditions.

3.1) Add 4 µL of collagen to 1.2 mL of 1 mM hydrochloric acid (HCl) in a 1.5 mL tube and gently mix using a pipette.

NOTE: Prepare collagen immediately prior to use. Keep collagen and HCl chilled until the time of use.

3.2) Pour 100 µL of collagen into each well of a 96-well culture plate using a pipette. Gently shake the culture plate in a left to right motion and incubate at room temperature for 1 h.

NOTE: Ensure that the entire surface of the wells is covered with solution.

3.3) After the incubation, discard the collagen solution and wash the wells with PBS (-) twice using a pipette.

3.4) Pour 150 µL of 1% bovine serum albumin/PBS (-) (1% BSA) to a well of the 96-well culture plate and incubate at room temperature for 1 h. Prior to cell culture, discard the 1% BSA.

NOTE: Ensure that the entire surface of the wells is covered with solution.

4. Culture of FEPE1L-8 cells

NOTE: Perform all procedures under aseptic conditions.

4.1) Maintain FEPE1L-8 cells in K110 in a 100 mm culture dish in a CO_2 incubator and incubated at 37 °C and 5% CO_2, to semi-confluency.

4.2) Prepare K110, trypsin, and trypsin inhibitor in a water bath at 37 °C.

4.3) Carefully remove the medium from the culture dish, add 3 mL of 0.05% trypsin using a pipette, place the dish in a CO_2 incubator and incubate at 37 °C for 5 min. After incubation, check cell detachment from the surface of the culture dish using phase-contrast microscopy at 10x magnification.

NOTE: The morphology of the detached cells becomes round. If the cells spread, incubate for an additional period of 5 min.

4.4) Add 3 mL of trypsin inhibitor and collect the detached cells in a 15 mL centrifuge tube using a pipette. Centrifuge the cells in a 15 mL centrifuge tube at 200 x g for 5 min.

4.5) Discard the supernatant and re-suspend the pellet in 10 mL of K110 using a pipette. Count the cells using phase-contrast microscopy at 10x magnification and prepare cell concentration at 5.0 x 10^4 cells/mL by appropriate dilution with K110.

4.6) Gently seed 0.1 mL of K110 with cells in each well of the culture plate using a pipette along the wall of the well. Place the culture plate in a CO_2 incubator and incubate at 37 °C for the indicated time (2 h, 1 day, 3 days).

NOTE: To protect the gels, the tip of the pipette should touch the wall of the well.

5. Estimation of the number of viable cells

5.1) Incubate K110 in a water bath at 37 °C. Mix 130 µL of tetrazolium salt, 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H tetrazolium, monosodium salt (WST-8), and 1.3 mL of K110 in a 2-mL tube using a pipette.

5.2) Move the culture plate to a clean bench and gently discard the K110 using a pipette. Gently wash away non-adherent cells with K110 by using a pipette

5.3) Add 110 µL of K110 mixed with WST-8 in each well using a pipette, place the culture plate in a CO_2 incubator and incubate at 37 °C for 2 h.

5.4) Move the culture plate to a clean bench, collect 100 µL of the conditioned medium from each well, and move it into a well of another 96-well culture plate. Measure absorbance at a wavelength of 450 nm (OD_{450}) using a microplate reader and estimate the number of viable cells.

REPRESENTATIVE RESULTS:

A schematic representation of the treatment of the surfaces of culture dishes using type I collagen is depicted in **Figure 1**. Cell morphologies observed on the non-fibrous and fibril forms are presented in the left- and right-side panels of **Figure 2**, respectively. FEPE1L-8 cells were cultured for 2 h (upper panels) and 3 days (lower panels). In the initial 2 h of culturing, the cells adhered and spread on both forms of collagen (**Figure 2**, upper panels). Three days after seeding, cells on the non-fibrous form continued to spread and cell numbers increased (**Figure 2**, lower left panel). In contrast, the cells on the fibril form showed limited spreading (**Figure 2**, lower right panel). FEPE1L-8 cells continued to proliferate on the non-fibrous form of type I collagen (**Figure 3**, solid black line, closed black circles) and on the untreated dish surfaces (**Figure 3**, dotted gray line, closed gray circles). In contrast, cells did not proliferate on the fibril form (**Figure 3**, dotted line, open circles). **Figure 2** and **Figure 3** have been modified from Fujisaki et al[11].

FIGURE AND TABLE LEGENDS:

Figure 1: Schematic representations of culture dish surfaces treated with type I collagen. Under acidic conditions, type I collagen molecules are adsorbed on the surface of a dish in the non-fibrous form (left panel). Under neutral conditions at 37 °C, type I collagen molecules are reassembled into fibrils and adsorbed on the surface of dishes in the gel form (right panel).

Figure 2: Morphology of FEPE1L-8 cells. FEPE1L-8 cells in K110 were cultured using the non-fibrous form (10 µg/mL; left panels) or fibril form (1 mg/mL; right panels) of type I collagen for 2 h (upper panels) or 3 days (lower panels). White bars indicate 100 µm. Figure 2 has been modified from Fujisaki et al. in Figure 1E–H[11].

Figure 3: Proliferation of FEPE1L-8 cell. The number of viable cells were estimated on the non-fibrous form (black solid line, black filled circles) or on the fibril form (dotted line, open circle) of type I collagen, or untreated dish surfaces (gray dotted line, gray filled circles) for 2 h, 1 day, and 3 days. Experiments were performed in triplicates and values are shown as means + SD. This

figure has been modified from Fujisaki et al. in Figure 1J[11].

DISCUSSION:

Some ECM components, including type I collagen, form three-dimensional structures in vivo[1]. Culturing on such a three-dimensional, gel substrate provides more physiological conditions in vitro than on a two-dimensional, plastic surface[1-4]. Numerous protocols regarding the gel culture method have been reported, such as using type I collagen[1-4,6,7,11], type IV collagen[14,15], and Matrigel[16]. Type I collagen is a well-defined and widely used material because of its abundance and ease of handling. The features of purified type I collagen depend on the animal species, age, and purification methods[5,17]. Type I collagen can be purified using acetic acid and/or proteases, such as pepsin, papain, and proctase[5,17]. Acid-soluble collagen maintains amino-telopeptides and protease-soluble collagen are cleaved amino-telopeptides[5,17]. The reserved length of amino-telopeptides depends on the type of proteases, and the presence of telopeptides affects fibril morphology and gel strength[5,17]. The viscosity of acid-soluble collagen fibrils is greater than that of protease-treated collagens[17]. In this study, we used acid-soluble bovine type I collagen. Pepsin-solubilized collagen can also be used in this gel culture protocol; however, the gel strength is weaker[17]. These differences in materials can cause cell behavioral differences, but they are currently not well understood.

The gel culture protocol described in this study is very simple. Many modifications of this method have been reported. One possible modification for the culture of keratinocytes is to mimic the basement membrane. Type IV collagen gels may be better to keep a basement membrane-like substrate structure in vitro[15,18]. However, a long incubation period is required for the preparation of type IV collagen gels[14, 15, 18]. Instead, mixing type IV collagen with type I collagen gels can produce novel culture substrates (type I/type IV collagen hybrid gels)[19]. These hybrid gels are easy to handle, require a short time for gelation, and yield more basement membrane-like conditions for keratinocytes. On type I/type IV collagen hybrid gels, keratinocytes survive, form colonies, and induce terminal differentiation[19]. This hybrid method has versatile applications.

On-gel culture using type I collagen affects cancer cells. On the fibril form of type I collagen, Akt activation and growth of Caco-2 cells (a colon cancer cell line) are suppressed[7]. In addition, the growth of human melanoma cells (M24met) on the fibril form is arrested at the G1/S checkpoint[20]. Moreover, markedly increased levels of reactive oxygen species are observed in murine 3T3-L1 preadipocytes cultured on the fibril form. Furthermore, cell proliferation and migration are stimulated in opposite directions by the non-fibrous and fibril forms of type I collagen[21].

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DISCLOSURES:

The authors declare that they have no competing financial interests.

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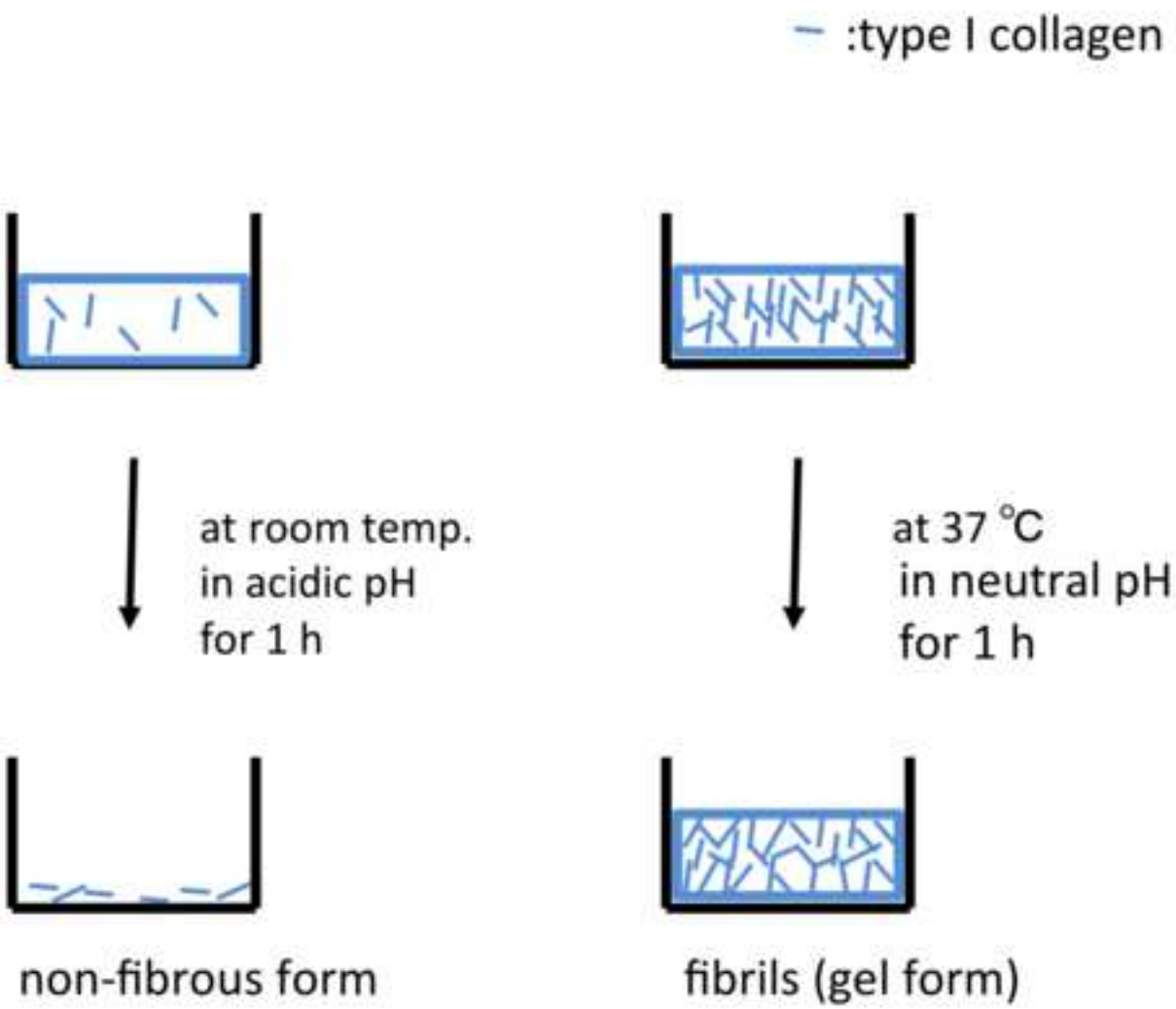
Figure



Figure

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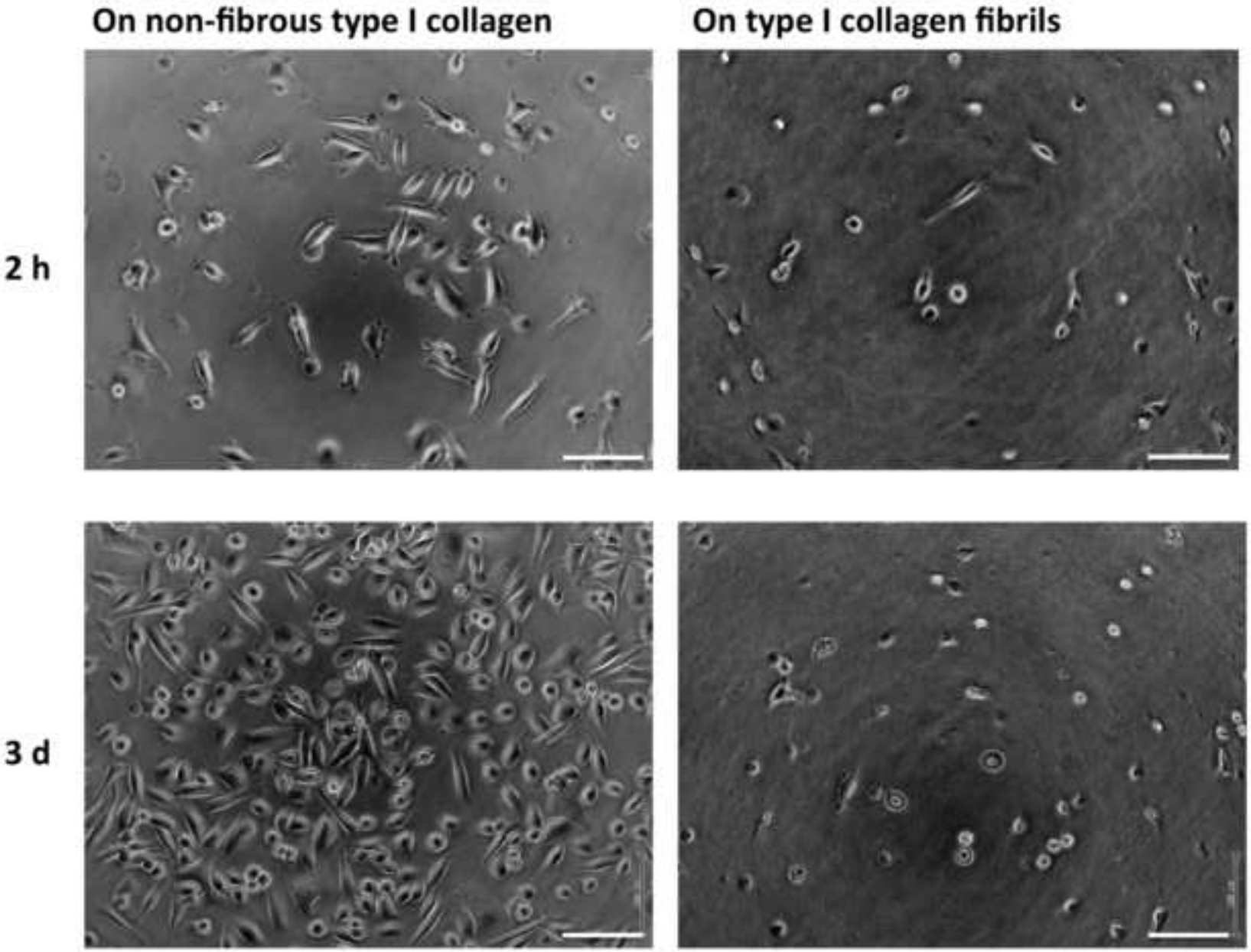

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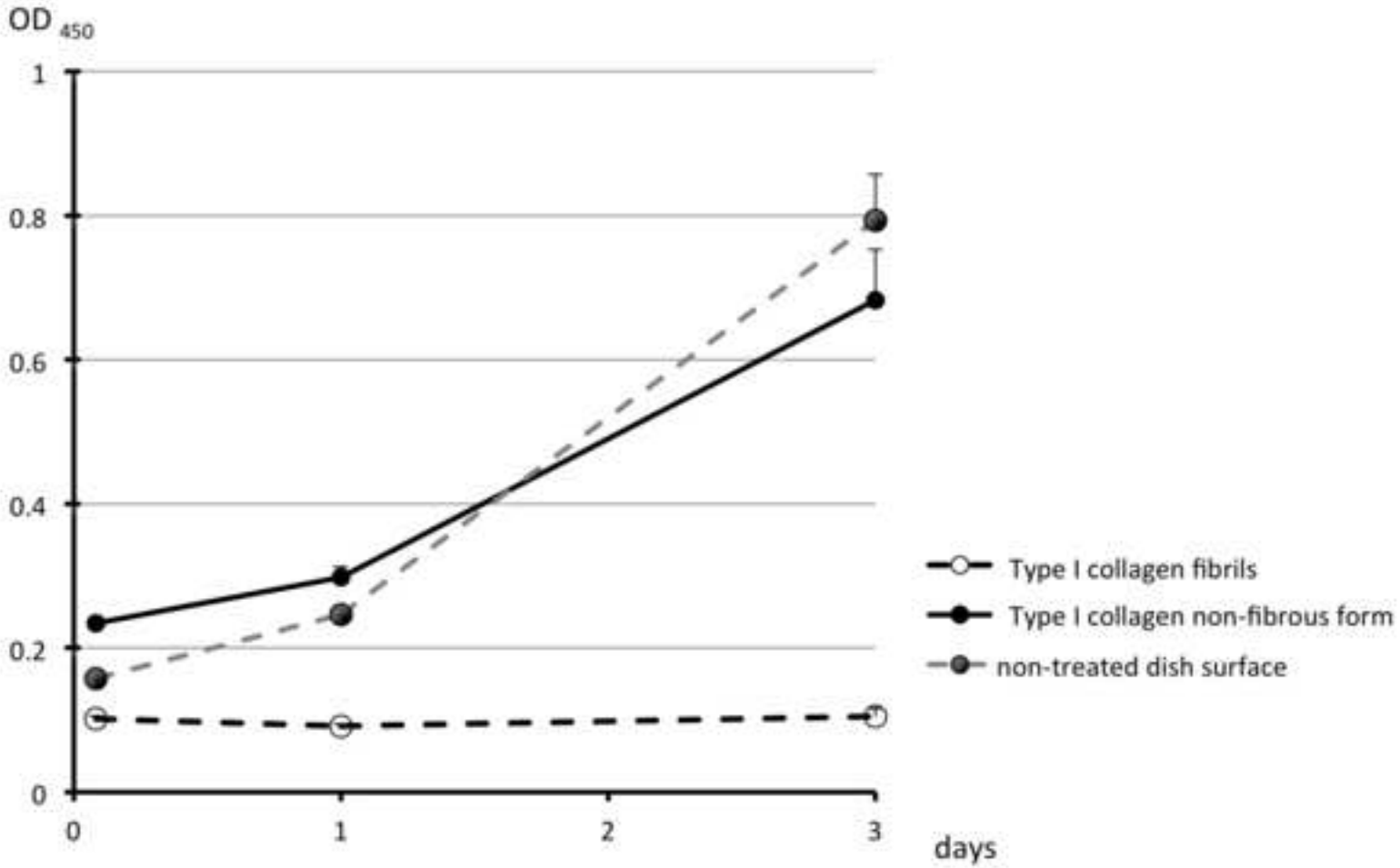

Table of Materials

Name of Reagent/ Equipment	Company	Catalog Number
Albumin, from bovine serum (BSA)	Merck KGaA	A4503
1 % BSA	Merck KGaA	A4503
Cell Counting Kit-8	Dojindo Molecular Technologies, Inc.	347-07621
Collagen type I (Acid soluble collgen)	Nippi Inc.	ASC-1-100-20
disposable membrane filter unit DISMIC cellulose acetate	ADVANTEC Co., LTD.	25CS020AS
human keratinocyte line cell FEPE1L-8		
K-1	Kyokuto Seiyaku Inc.	28204
K110 Type-II medium	Kyokuto Seiyaku Inc.	28204
K110 Type-II medium with K-1 and Penicillin-Streptomycin (K110)	Kyokuto Seiyaku Inc.	28204
PBS (-)	Merck KGaA	P-5368
10x PBS (-)	Merck KGaA	P-5368
Penicillin-Streptomycin	MP Biomedicals, LLC	1670049
Phosphate bufferred saline BioPerformance CertiCertified, pH 7.4	Merck KGaA	P-5368-10 pack
Trypsin from porcine pancreas	Merck KGaA	T4799
0.05 % trypsin	Merck KGaA	T4799
Trypsin inhibitor from soybean	FUJIFILM Wako Pure Chemical corporatio	202-20123
Trypsin inhibitor	FUJIFILM Wako Pure Chemical corporatio	202-20123

Comments/Description

Dissolve 1 g of BSA powder in 100 mL of PBS (-) and filtrate
tetrazolium salt, 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H* tetrazolium, monosodium salt (WST-8)
from bovine (any other species available, concentration is 3.0 mg/mL
0.2 µm pore size
Donated Dr.W.G. Carter (Fred Hutchinson Cancer Research Center, Seattle, WA)
additive supplement with 500 mg of BSA, 500 mg of bovine pituitarybody extracts, 2.5 mg of insulin, 0.05 µg of h-EGF and 5 mg of he
keratinocyte basal culture medium

Add 5 mL of Penicillin-Streptomycin and 10 mL of additive supplement (K-1) in 500 mL of K110 type-II keratinocyte basal culture medi
Dissolve 1 pouch of PBS (-) powder in 1000 mL of deionized water and filtrate
Dissolve 1 pouch of PBS (-) powder in 100 mL of deionized water and filtrate
10000 units/mL of penisillin G and 10,000µg/mL of streptmycin sulfate in physiological saline

Dissolve 0.25 g of trypsin and 0.186 g of EDTA.2Na in 500 mL of PBS (-) and filtrate

Dissolve 50 mg of trypsin inhibitor and 500 mg of BSA in 500 mL of PBS (-) and filtrate

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CORRESPONDING AUTHOR:

Name: Hitomi Fujisaki

Department: Nippi Research Institute of Biomatrix

Institution: Nippi, inc.

Article Title: On-gel-culture using type I collagen fibrils down-regulates cell proliferation in keratinocyte line cells

Signature: Hitomi Fujisaki Date: Oct. 30. 2018

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January 25th, 2019

Dear Dr. Alisha DSouza,

We thank Editors and Reviewers for careful reading our manuscript. We read them carefully, thought about well.

We think that experimental comments of Reviewer 2 make sense in terms of cell biology. But some comments are not pointed out in terms of methodology. We think one of the reasons of this confusion is the title. We renamed the title“On-gel-culture using type I collagen fibrils down-regulates cell proliferation in keratinocyte line cells” to “**Evaluation of keratinocyte proliferation on two- and three-dimensional type I collagen substrates**”. We have revised the manuscript and figures and got professional English proofreading. We believe these changes are contributed improvement of the manuscript. Please find enclosed our manuscript.

Our responses to each comment are as follows in blue letters:

Sincerely,

Hitomi Fujisaki

Nippi Research Institute of Biomatrix

520-11 Kuwabara, Toride, Ibaraki 302-0017, Japan

Phone No: +81(297)71-3047

Fax No: +81(297)71-3041

Email Address: fujisaki@nippi-inc.co.jp

Editorial comments:

Changes to be made by the author(s) regarding the manuscript:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We rewrote the manuscript accordingly to the comments and got professional English proofreading.

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We uploaded each Figure individually as a tiff file.

4. Figures: Please include a space between the numbers and their corresponding units (37 °C, 2 h, 3 d, etc.).
We added a space between the numbers and their corresponding units.

5. Figure 3: Please make the number 450 a subscript and add minor ticks to the y-axis.
We made the number 450 a subscript and added minor ticks to the y-axis.

6. Please revise lines 51-53 to avoid previously published text.
We rewrote lines 51-53.

7. Please rephrase the Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: “Here, we present a protocol to …”
We rewrote the Summary according the advice.

8. Please revise the Introduction to include all of the following:
a) A clear statement of the overall goal of this method
b) The rationale behind the development and/or use of this technique
c) The advantages over alternative techniques with applicable references to previous studies
d) A description of the context of the technique in the wider body of literature
e) Information to help readers to determine whether the method is appropriate for their application
We rewrote the Introduction according the advice.

9. Please define all abbreviations before use.
We defined abbreviations.

10. Please revise the protocol to contain only action items that direct the reader to do something (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible. Please move the discussion about the protocol to the Discussion.
We revised the protocol. The actions were described in the imperative tense in complete sentences wherever possible.

11. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See examples below.
We made efforts according your advice and rewrote protocol.

12. 2.1: Please specify the reagents and instruments used in this step. Please describe how to keep the instruments cold on ice.

We added the description.

13. 2.5: Please specify the volume of supplemental K-1 solution and 1% Penicillin-Streptomycin added.

We added the description.

14. 4.1: Please specify the indicated times.

We added the description.

15. 4.2 and 4.3: Please reference the Table of Materials for the composition of cell proliferation kits solution and cell counting kit solution.

We unified the name of solution.

16. 4.4: Please provide some guidance on the appropriate color tone.

In this case, the color became appropriate level for 2 h. So we rewrote the description.

17. Discussion: As we are a methods journal, please also discuss critical steps within the protocol, any modifications and troubleshooting of the technique, and any limitations of the technique.

We rewrote the Discussion.

18. References: Please do not abbreviate journal titles.

We rewrote the References.

19. Table of Materials: Please sort the items in alphabetical order according to the name of material/equipment.

We sorted the items alphabetically.

December 27th, 2018

Reviewer #1:

Dear Reviewer

Thank you for your kind comments.

Major Concerns:

- Please characterize Nippi collagen. How was it isolated (tissue, acid and its concentrations, conditions of the extraction. Was pepsin used?)

Collagen solution which used in this paper (ASC-1-100-20) is extracted from bovine skin with 0.5 M acetic acid and purified by salt fractionation. Pepsin or other proteases were not used. Describes are written in Materials file.

- Fig.3 shows that there is no difference between the effect of uncoated plastic and the "non-fibrous form" of collagen. Any protein may have the same (probably negligible) influence on the cells. Is there any difference between the growth of the cells on non-fibrous collagen (Fig. 2, 3d) and on uncoated plastic?

At the early stage of culture, living cell numbers and morphology are different between on non-fibrous form of collagen and on uncoated plastic. On non-fibrous collagen for 2 h, cells spread well (Fig. 2, 2 h) and living cell number was 1.5 times that on non-treated dish surface (Fig. 3, 2 h). On plastic surface, cells did not spread yet for 2 h and spread well for 3 days (data not shown).

During culture term, keratinocytes produce their appropriate ECM for survival and proteases. Cell scaffold is reconstructed between the balance of produced and degraded ECM. After keratinocyte specific ECM deposited on dish surface (for 3 days), the gap of morphology and proliferation ratios has been narrowed.

Minor Concerns:

English needs to be revised.

We rewrote the manuscript accordingly to the comments and got professional English proofreading.

January 25th, 2019

Reviewer #2:

Manuscript Summary:

The manuscript describes a quite simple approach for differential behavior of epidermal cell cultures on type I collagen (collagen I) depending on its supramolecular structure, i.e. being in an unordered mono- or oligomeric state ('non-fibrous') versus a scaffold of fibrillar assembly ('fibrils') mimicking the physiological arrangement in genuine connective tissue. However, it is well understood that, though ECM turnover and degradation may be stimulatory, native collagen I is not the genuine matrix substrate for keratinocytes. Even in healing wounds keratinocytes migrate on a different provisional ECM composed mainly of fibrin and fibronectin. So collagen I matrix may be but rather a 'forbidden territory' which is changing during tumorigenesis. Accordingly, on the fibrillar collagen I matrix the growth of the applied keratinocyte line FEPE1L-8 growth rate and migration are markedly reduced. The proposed protocols appear straightforward and simple to reproduce.

Nevertheless, it should be clearly indicated in the beginning that the data (including Fig. 2) have been presented already in a very recent paper.

Dear Reviewer

Thank you for your useful and kind comments.

We think that experimental comments about keratinocytes of Reviewer make sense in terms of cell biology, but this manuscript is written in terms of methodology. Considering this point and according Reviewer's comments, we rewrote the manuscript and renamed the title "On-gel-culture using type I collagen fibrils down-regulates cell proliferation in keratinocyte line cells" to "**Evaluation of keratinocyte proliferation on two- and three-dimensional type I collagen substrates**" We think these changes are clarifying the purpose of this work.

In this paper, we would like to describe the usefulness of type I collagen as culture substrate. As is well known, type I collagen solution change the texture by handling. In

acidic condition, collagen solution keeps soluble (non-fibrous) form and in neutral pH forms gels (fibrils). As you said, the protocols presented in this paper are very simple and easy but we think it is in valid to visualize the methods on movie for beginners.

Our responses to each comment are as follows in blue letters:

Major Concerns:

Some principal questions.

The keratinocyte line FEPE1L-8 must be defined, providing also the source and general features. At least one or two references should be given, as well for K1/K110.

We added the description about FEPE1L-8 culture by using K1/K110 medium (L80~L82).

The experiment shown is in deed very simple. There are several points that should be questioned or proven. It may just reflect mainly the plating efficiency or cloning capacity. Maybe, when compensating for that, i.e. a comparable cell density, may reveal similar growth. The local concentration of juxta- or paracrine factors could be important.

As Reviewer described, culture conditions, such as cell density, effect on proliferation drastically. We have tested in some conditions (non-published data). However in this paper, we focus the protocol of collagen handling, so we quoted the one results presented in a recent paper and did not discuss the culture conditions (including cell density) in more details.

Keratinocytes produce their own ECM, a major component in this regard is laminin 332, binding also to integrin alpha6beta 4. The cell line may express less of these components which could be easily demonstrated on slides by immunofluorescence. Also, apoptotic cells could be detected this way.

About integrin expression and synthesis of laminin 332, fibronectin and laminin 111 in FEPE1L-8 cells has been already reported in reference [13]. We examined the expression of laminin 332 preliminary (unpublished data).

We have analyzed the apoptotic cell ratios of FEPE1L-8 previously in reference [11]. In this paper, we focus the protocol of collagen handling so we omitted the keratinocyte specific properties.

Collagen density in vivo is much higher than in vitro. Molecular crowding certainly influences cell behavior. One likely effect is the changing of cell receptor patterns (clustering) and this way altering also intracellular signaling and migratory behavior.

The point that Reviewer described about collagen density *in vivo* is widely accepted. It is important. But to make high dense collagen fibrils for culture substrates is difficult and complicated process technically and remains unsolved mechanism. We think to analysis the mechanism, to adopt easier protocols (like presented here) is still useful.

194, Discussion, 'In the body...plastic dishes.' - This statement is trivial.

We deleted the sentence.

200-203, The triple-helical type I collagen molecules (monomers) are cross-linked via their telopeptides which controls and promotes the alignment of collagen I fibrils and higher ordered structures. Pepsin cleaves and removes the telopeptides and those cross-links, which allows complete collagen solubilization. This should be explained more clearly.

We rewrote the sentences and explained more clearly.

212, concerning cancer cells, EMT (epithelial mesenchymal transition) should increase adhesion and promote migration of those cells on collagen I fibrils, facilitating invasion

of connective tissue.
Some of these points have been addressed elsewhere, which should be referred to.

We are so sorry we could not find the sentence “212, concerning cancer cells, EMT (epithelial mesenchymal transition) should increase adhesion and promote migration of those cells on collagen I fibrils, facilitating invasion of connective tissue.”.

Minor Concerns:
In general, the text could be a bit condensed, eliminating some redundancies.
Throughout it needs thorough revision in terms of style, proper terms and statements - a few suggestions below.

We tried to condense the sentences and rewrote.

23-25, Keywords, 'fibrillar/non-fibrous form, keratinocyte line,...'. - keratinocytes are cells.

We changed the key words; type I collagen, fibril/non-fibrous form, two-/three-dimensional culture substrates, on-gel culture, keratinocyte culture, proliferation.
.

We rewrote Summary and Abstract.

28-31, Summary, '...systems using type I collagen (collagen I; later on?) can be applied in non-fibrous or fibrillar form. Under physiological conditions (neutral pH) collagen I molecules assemble to fibrils forming a fibrous scaffold. On such a matrix keratinocyte proliferation was suppressed.'

34, Abstract, '...in vertebrates, forming fibrils and as such the three-dimensional...'. Extracting collagen from tissue, usually with diluted acetic acid, it is dissociated and can be kept in ...non-...'. When coated in that non-fibrous form collagen I promotes...'.

40, '...(gel form) which assemble to three-...structures.'
41, 'For many cell types collagen I in its native fibrillar form provides more ...conditions, influencing cell fates...'
43, '...present simple methods to examine the different cell behavior allowing wide application for many cell types accordingly.'
51, Introduction, '...collagen I forming fibrils of high mechanical strength, representing 90% of total ECM proteins.'
For further careful revision the authors should proceed this way.

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