2011 International Nuclear Atlantic Conference - INAC 2011
Belo Horizonte,MG, Brazil, October 24-28, 2011
ASSOCIAÇÃO BRASILEIRA DE ENERGIA NUCLEAR - ABEN
ISBN: **978-85-99141-04-5**

REPLACEMENT OF MURINE FIBROBLASTS BY HUMAN FIBROBLASTS IRRADIATED IN OBTAINING FEEDER LAYER FOR THE CULTURE OF HUMAN KERATINOCYTES

Daniele Yoshito[1], Bianca S. Sufi[1], Silvana C. Altran[2], César Isaac[2], Stefany P. Santin[1], Natália M. Esteves-Pedro[3] Marisa R. Herson[4] and Monica B. Mathor[1]

[1] Instituto de Pesquisas Energéticas e Nucleares (IPEN / CNEN - SP)
Av. Professor Lineu Prestes 2242
05508-000 São Paulo, SP
daniele.yoshito@usp.br

[2] Laboratório de Microcirurgia Plástica - Faculdade de Medicina
Universidade São Paulo
Av. Dr. Arnaldo 455, 1º andar sala 1363
01246-903 São Paulo, SP

[3] Laboratório de Controle Biológico - Faculdade de Ciências Farmacêutica
Universidade São Paulo
Av. Professor Lineu Prestes 580
05508-000 São Paulo, SP

[4] DonorTissue Bank of Victoria
57 - 83 Kavanagh St.
Southbank - Victoria 3006 Australia

ABSTRACT

Human autologous epithelia cultivated in vitro, have been used successfully in treating damage to skin integrity. The methodology allowed the cultivation of these epithelia was described by Rheinwald and Green in 1975, this methodology consisted in seeding keratinocytes onto a feeder layer composed of lineage 3T3 murine fibroblasts, the proliferation rate is controlled through the action of ionizing radiation. However, currently there is a growing concern about the possibility of transmitting prions and murine viruses to transplanted patients. Taking into account this concern, in this present work, we replaced the feeder layer originally composed of murine fibroblasts by human fibroblasts. To obtain this new feeder layer was necessary to standardize the enough irradiation dose to inhibit the replication of human fibroblasts and the verification of effectiveness of the development of keratinocytes culture on a feeder layer thus obtained. According to the obtained results we can verify that the human fibroblasts irradiated at various tested doses (60, 70, 100, 200, 250 and 300 Gy) had their mitotic activity inactivated by irradiation, allowing the use of any of these doses to confection of the feeder layer, since these fibroblasts irradiated still showed viable until fourteen days of cultivation. In the test of colony formation efficiency was observed that keratinocytes seeded on irradiated human fibroblasts were able to develop satisfactorily, preserving their clonogenic potential. Therefore it was possible the replacement of murine fibroblasts by human fibroblasts in confection of the feeder layer, in order to eliminate this xenobiotic component of the keratinocytes culture.

1. INTRODUCTION

Skin is considered the largest human organ, representing 16% of body weight [1], being an important barrier to the environment by hindering the penetration of microorganisms into our bodies, preventing several problems and diseases, besides participating in homeostatic and physiological regulation processes [1, 2]. For such reasons, when this protection is lost or severely compromised it may have an impact in the patient's survival rate, as the case of people that suffered serious burns or extensive, who are susceptible to infections and septicemia due to depression of the immune response [3]. Therefore, the challenge of finding the means to substitute skin has been translated into an important field of research. Being the skin mainly constituted by confluent keratinocytes [2, 3, 4], one of the possibilities to be explored was the feasibility to establish keratinocyte cell cultures in vitro [2, 4].

In 1975, Rheinwald and Green published a methodology for cultivating human keratinocytes, where they used irradiated fibroblasts to form a sustentation layer called feeder layer, making possible the lifespan extension of keratinocytes in culture [4, 5, 6]. For the use of these cells as sustentation layer, they modified murine fibroblasts cell line (NIH-3T3), arriving at called 3T3-J2 line cell [5, 6, 7], later deposited in the ATCC under acronym CCL-92.

The importance of using irradiated fibroblasts as feeder-layer is due to these cells' capacity to synthesize fibronectin, small amounts of Type IV collagen, and laminin [8, 9]. These cells also release growth factors [9, 10] and extracellular matrix proteins into the culture medium, facilitating keratinocytes adhesion and growth [8, 9].

Irradiation of murine fibroblasts is necessary to control their growth so that it is not opposed to the growth of key cells to be cultivated. A dose of 60 Grays (Gy) was set up by Rheinwald and Green as enough to inhibit fibroblast duplication [5, 6].

With the formation of a feeder layers, formed by irradiated murine fibroblasts, it was possible to develop keratinocytes in colonies and propagate the cells in further cultures, allowing confection of transplantable epithelium [3, 4, 7, 11, 12].

For over 30 years, the use of murines fibroblasts, with the rate of proliferation controlled by irradiation or per share anticarcinogenic drugs, has been playing successfully its role in assisting the development of keratinocytes in culture, for clinical purposes. However, currently there is a growing concern about the possibility of transmitting prions and animals viruses to transplant patients [13].

Taking into account this concern, the present work, we replaced the feeder layer originally composed of murine fibroblasts by human fibroblasts. To obtain this new feeder layer was necessary to standardize the enough irradiation dose to inhibit the replication of human fibroblasts and the verification of effectiveness of the development of keratinocytes culture on a feeder layer thus obtained

2. MATERIAL AND METHODS

2.1. Medium Preparation

Culture medium D10: Dulbecco's Modified Eagle's Médium (DMEM), 10% of bovine calf serum, penicilin (100 U/mL)/streptomicin (100 μg/mL), amphotericin B (2,5 μg/mL) and L-glutamin (4 mM)
Culture medium K-: Dulbecco's Modified Eagle's Medium (DMEM)/Ham's F12 (2:1) supplemented with 10% of fetal bovine serum, penicilin (100 U/mL), streptomycin (100 μg/mL), amphotericin B (2.5 μg/mL), glutamine (4 mM), adenin (0,18 mM), insulin (5 μg/mL) , hydrocortisone (0,4μg/mL), cholera toxin (0,1 nM), triiodothyironin(20 pM).
Culture medium K+: For make K+ it's add Epidermal Growth factor (10 ng/mL).

2.2. Murine Fibroblast Culture

Approximately $1x10^6$ murine fibroblasts (CCL-92, ATCC) were defrosted and plated in cell culture flasks, at the density of $2.8x10^3$ cells/cm^2, in culture medium D10, at 37°C, in a humidified atmosphere of 5% of CO_2. When cells reached a 70% confluence rate, were detached from the flask with trypsin (0,05%)/EDTA(0,02%) and amplified to new cultures.

2.3 Human Fibroblast Culture

Human fibroblasts were obtained from skin samples donated by the Tissue Bank of the FMUSP Clinical Hospital' Central Institute. The cells were isolated *in vitro* according to the explants technique proposed by Carrel & Burrows [14], namely, the tissue samples were cut into fragments of 1 mm2 and placed in flasks of 25 cm2 containing culture medium D10. By the time, the fibroblasts migrated from skin fragment and reached around the same subconfluent, they were removed from bottles with trypsin (0.05%)/EDTA (0.02%) and amplified in new cultures. Subsequent amplifications were carried out to achieve the required number of cells for the experiments.

2.4 Keratinocytes Culture

Human keratinocytes were obtained from skin samples donated by the Tissue Bank of the FMUSP Clinical Hospital Central Institute. The samples were submitted to a cellular dissociation treatment with enzymatic action (Tripsin), in periods of 30 minutes at 37 ºC. Obtained keratinocytes were seeded at $2x10^4$ cells/cm^2 density over a feeder layer formed by previously irradiated murine fibroblasts (60 Gy). The keratinocytes/feeder layer set was nourished through a K+ medium, and kept in a humid incubator adjusted to 37 ºC, with 5% CO_2 atmosphere. At the sub-confluence of the primary culture, cells were trypsinized with

tripsin (0.05%)/EDTA (0.02%) and seeded in a new feeder layer, which became the secondary culture. At the sub-confluence of the secondary culture, cells were trypsinized, counted, and employed for experiments.

2.5 Standardization of irradiation dose

The human fibroblasts were exposed to irradiation of ^{60}Co source, type Gammacell-220, with a dose rate of 567 Gy/irradiation at doses of 60, 70, 100, 200, 250 and 300 Gy. After irradiation, cells were seeded in culture plates with 24 wells at a concentration of 5 x 10^4 cells/well in specific medium to keratinocytes K-. As a control we used two groups: one composed of human fibroblasts non-irradiated and other of murine fibroblasts irradiated at 60 Gy. The cells were incubated at 37 ºC and 5% CO_2, with change of medium every two days, being that from the first medium change, was added epidermal growth factor (EGF) at a concentration of 10 ng/mL. The counting of number of cells per well was carried out on zero, first, second, third, seventh, tenth and fourteeth days. The cell vitality test was performed using the dye transmembrane non-vital trypan blue.

2.6 Colony Formation Efficiency Test

The colony formation efficiency test (CFE) was to evaluate the growth of keratinocytes in different feeder layers: murine fibroblasts (CCL-92) irradiated at 60 Gy and human fibroblasts were irradiated at doses of 70, 100, 150 and 200 Gy. The lineage CCL-92 and human fibroblasts were irradiated and seeded in the respective densities of 0.26 and 0.2 x 10^5 cells/cm^2. After adhesion of feeder layer, were added 100 keratinocytes per well. The culture keratinocyte/feeder layer was maintained in culture medium K+, the change of medium was performed every 48 hours for fourteen days. After this period, the cultures were stained with Rhodamine B and the keratinocytes colonies were morphologically analyzed and counted, taking into account the number of colonies in development and formation of abortive colonies.

2.7 Statistical Analysis

The results were obtained and expressed as mean and standard deviation of the averages. The statistical significances of differences were determined by the analysis if variance ANOVA (ONE WAY) post hoc Tukey. Values of $p < 0.05$ were considered significant. All calculations were performed using the statistical software *GraphPad Prism for Windows*, version 5.0.

3. RESULTS AND DISCUSSION

Seeking an inactivation of the proliferation and not cell death, whereas these cells are used as feeder layer and sustenance to keratinocytes growth, the fibroblasts were irradiated with different doses (ranging from 60 to 300 Gy), in order to determine the optimal dose to inactivate their proliferation.

According to Figure 1, it was observed that until the third day, the non-irradiated and irradiated cells showed the same number of living cells as the day of sowing (day zero). Changes were only observed on the seventh day, where we could see that human fibroblasts non-irradiated continued to multiply, while the same irradiated remained about the same number of viable cells. The same behavior was not observed for murine fibroblasts which was observed a decrease in the number of viable cells over the the 14 days. These results corroborate data reported by Almeida et al., 2007.

Although Szabowski-Maas et al. (1999) [16] claim that at dose of 70 Gy human fibroblasts interrupt their duplication, this fact was not confirmed in our conditions, where we observed a slight increase between the third and seventh days, and subsequent maintenance of the number of viable cells. We obtained a count of about $3 \times 10^4 \pm 0.2 \times 10^4$ cells at day zero to 70 Gy in the 7 day the number of cells was counted for $4 \pm 0.26 \times 10^4$. The same was observed to the others doses. However, after the seventh day, was not observed increase nor decrease in the number of viable cells in cultures of fibroblasts irradiated at doses ranging from 60 to 300 Gy, during the period in which the test was performed. Noting the possibility of use any of these doses for inactivation of human fibroblasts.

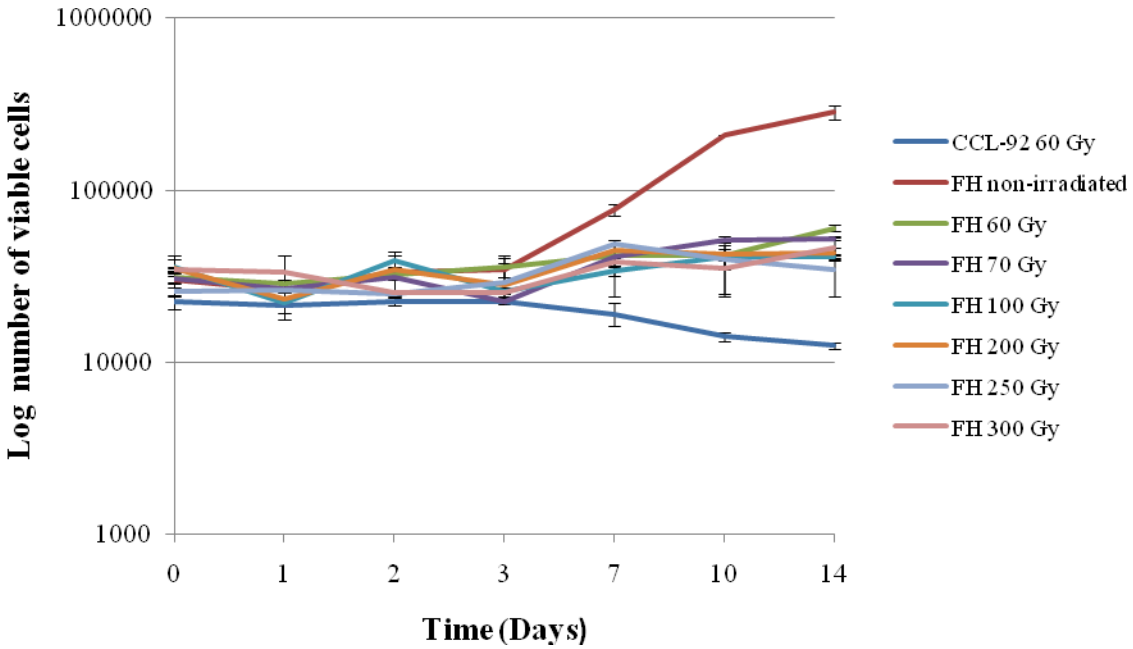

Figure 1. Graph of the survival curve of human fibroblasts irradiated with various doses of radiation compared to control (CCL-92, 60 Gy). The control of the assay was determinate by the non-irradiated human fibroblasts (HF, control non-irradiated)

Despite the possibility of irradiating human fibroblasts at different doses, we should verify if this variation may affect the development of keratinocytes. Therefore, was performed the colony formation efficiency test of keratinocytes cultured on feeder-layer obtained as shown in the previous item. Using this test, it's possible to analyze the clonogenic potential of these cells, by counting the different types of colonies formed, as the colonies in development and abortive colonies, besides the macroscopic visualization of these colonies.

In Figure 2, observes that the culture of keratinocytes over the feeder layers obtained with doses of 100, 150 and 200 Gy, showed similarity in the percentage of colonies, both in development and abortion, with the types corresponding on control consisted of feeder layer of murine origin irradiated to 60 Gy. However, when the cultures observed at a dose of 70 Gy, there is an increase in the percentage of colonies in development, already about abortive colonies the percentage remains similar to control and other doses. The above results diverge with those reported by Rheinwald and Green (1975) [5], where these authors verified that keratinocytes cultured with human fibroblasts grow slower than when culured with murine fibroblastes, but the main difference between both assays is that in our case was used a standard type of FBS, fetal clone III serum, thus enabling a better development of keratinocytes cultured with irradiated human fibroblasts.

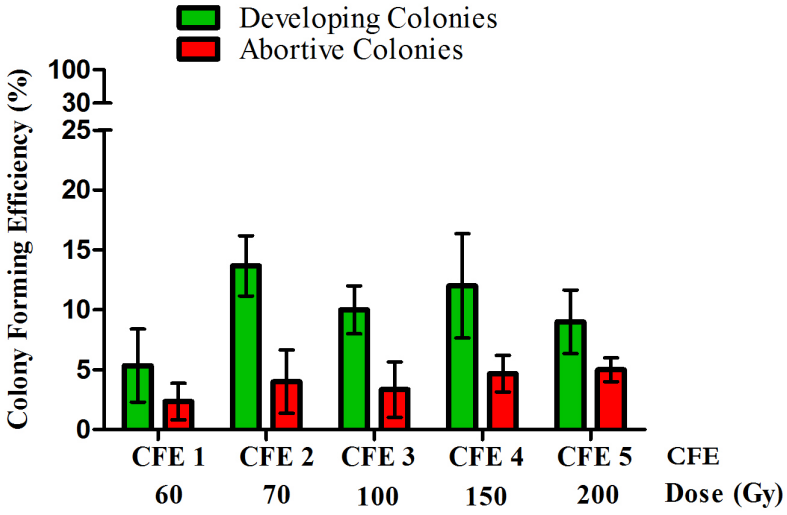

Figure 2. Percentage of colonies in development and abortives obtained in CFE of keratinocytes, with human fibroblasts feeder layer, at different irradiation doses (70, 100, 150 and 200 Gy), respectively EFC2, EFC3, EFC4 and EFC5. As a control, we used CCL-92 irradiated at 60 Gy (EFC1).

Still talking about these cells cultures, we can visualize in Figure 3, in the Colony Formation Efficiency (CFE) with feeder layer in various doses, especially at doses from 150 to 200 Gy, the emergence of larger colonies of keratinocytes in relation to other doses including the control CCL-92 irradiated at 60 Gy. In microscopic images, at doses of 150 and 200 Gy, observes a decrease in the amount of human fibroblasts which allowed better development of the keratinocytes, this is due to the influence of density on feeder layer. According to Rheinwald and Green [5], this is a primordial factor to the development of keratinocytes in colony.

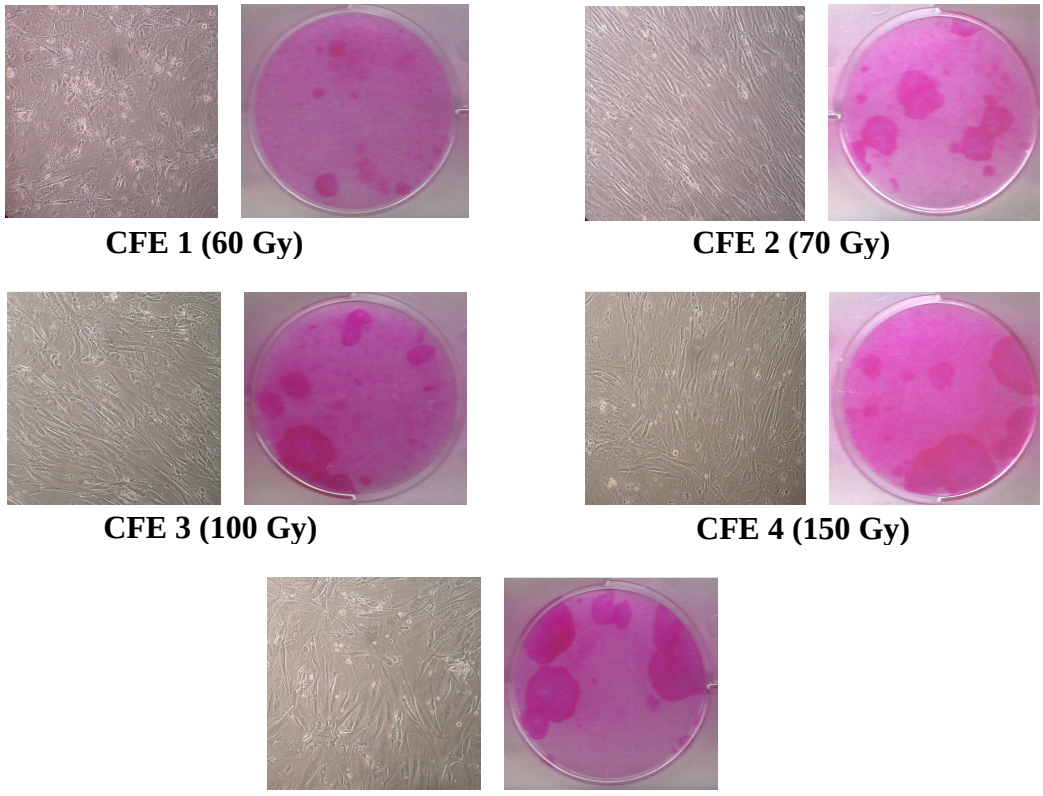

Figure 3: Photographs of optic microscopic observations (left) of the fibroblasts irradiated at doses of 60, 70, 100, 150 and 200 Gy, and macroscopic visualization (right) of the respective CFE of the keratinocytes (CFE 1, CFE 2, CFE 3, CFE 4 and CFE 5) stained with Rhodamine B.

4. CONCLUSIONS

According to the obtained results we can verify that the human fibroblasts irradiated at various tested doses (60, 70, 100, 200, 250 and 300 Gy) had their mitotic activity inactivated by irradiation, allowing the use of any of these doses to confection of the feeder layer, since these fibroblasts irradiated still showed viable until fourteen days of cultivation. In the test of colony formation efficiency was observed that keratinocytes seeded on irradiated human fibroblasts were able to develop satisfactorily, preserving their clonogenic potential. Then, we recommend the doses of 70 and 100 Gy to confection of the human feeder layer, because at these doses it's possible to obtain the same results from the others using lower doses. Therefore it was possible the replacement of murine fibroblasts by human fibroblasts in confection of the feeder layer, in order to eliminate this xenobiotic component of the keratinocytes culture.

ACKNOWLEDGMENTS

We are grateful to the Biotechnology Center – CB, IPEN-CNEN/SP, for equipment and Laboratories, IPEN-CNEN/SP for the fellowship and FAPESP (Fundação de Amparo à Pesquisa do Estado de São Paulo) through the project number 2010/05824-3, for the financial support.

REFERENCES

1. L. C. Junqueira, J. Carneiro, *Histologia Básica*, Rio de Janeiro, Guabadara Koogan (1999).
2. M. Regnier, D. Caron, U. Reichert, H. Schaefer, "Barrier function of human skin and human reconstructed epidermis", *J. Pharm. Sci,*. **v. 82**, p 404-407, (1993).
3. M. R. Herson, *Estudo da composição in vitro de substituto cutâneo dermo-epidérmico constituído por epitélio de queratinócitos cultivados sobre base dérmica alógena*, Tese (Doutorado), Faculdade de Medicina da Universidade de São Paulo, São Paulo (1999).
4. L. Andreassi, "History of keratinocyte cultivation", *Burns*, **v. 18**, pp. 2-4 (1992).
5. J. G. Rheinwald, H. Green, "Serial cultivation of human epidermal keratinizing colonies from single cells", *Cell,* **v. 6**, pp. 331-334 (1975).
6. J. G. Rheinwald, H. Green, "Formation of a keratinizing epithelium in culture by a clone cell line derived from teratoma", *Cell,* **v. 6**, pp. 317-330 (1975).
7. M. B. Mathor, *Estudos da expressão gênica mediante utilização de queratinócitos humanos normais transduzidos com o gene do hormônio de crescimento humano. "Possível utilização em terapia gênica*", Tese (Doutorado), Instituto de Pesquisas Energéticas e Nucleares, São Paulo (1994).
8. K. Alitalo, E. Kuismanen, R. Myllya, U. Kiistala, S. Asko-Seljavaara, A. Vaheri, "Extracellular matrix proteins of human epidermal keratinocytes and feeder 3T3 cells". *J. Cell Biol*., **v. 9**, pp. 497-505 (1982).
9. P. C. Cristovam, M. A. Gloria, G. B. Melo, J. A. P. Gomes,"Importância do co-cultivo com fibroblastos de camundongo 3T3 para estabelecer cultura de suspensão de células epiteliais do limbo humano", *Arq. Bras. Oftalmol*., **v. 71**, pp. 689-694 (2008).
10. A. Barreca, M. De Luca, P. Del Monte, S. Bondanza, G. Damonte, G. Cariola, E. Di Marco, G. Giordano, R. Cancedda, F. Minuto, "In vitro paracrine regulation of human keratinocyte growth by fibroblast-derived insulin-like growth factors", *J. Cell Physiol.*, **v. 151**, pp. 262-268 (1992).
11. M. De Luca, R. Cancedda, "Culture of human epithelium", *Burns*, **v. 18**, pp. 5-10 (1992).
12. H. Green, O. Kehinde, J. Thomas, "Growth of cultured human epidermal cells into multiple epithelia suitable for grafting", *Proc. Natl. Acad. Sci. USA*, **v. 76**, pp.5665-5668 (1979).
13. A. J. Bullock, M. C. Higham, S. Macneil, "Use of human fibroblasts in the development of a xenobiotic-free culture and delivery system for human keratinocytes", *Tissue Engineering*, **v. 12,** pp. 245-255 (2006).
14. A. Carrel, M. T. Burrows, "Cultivation of adult tissues and organs outside the body", *JAMA.*, **v. 55**, pp. 1379-1381 (1910).

15. T. L. Almeida, M. F. G. Klingbeil, D. Yoshito, M. R. Herson, P. Caproni, M. B. Mathor, “Irradiated murine fibroblasts feeder layer used in human cell cultured”. *International Nuclear Atlantic Conference*, Santos, September 30 to October (2007).
16. N. Maas-szabowski, A. Shimotoyodone, N. E. Fusenig, “Keratinocyte growth regulation in fibroblast cocultures via a double paracrine mechanism”, *J. Cell Sci.*, **v. 112**, pp. 1843-1853 (1999).