Cell Transplantation, Vol. 14, pp. 683–693, 2005
Printed in the USA.

0963-6897/05 $20.00 + .00
E-ISSN 1555-3892
www.cognizantcommunication.com

Synergistic Effects of FGF-2 With Insulin or IGF-I on the Proliferation of Human Auricular Chondrocytes

Tsuguharu Takahashi,*† Toru Ogasawara,*‡ Junji Kishimoto,§ Guangyao Liu,*¶ Hirotaka Asato,# Takashi Nakatsuka,** Eijyu Uchinuma,†† Kozo Nakamura,§ Hiroshi Kawaguchi,§ Tsuyoshi Takato,†‡ and Kazuto Hoshi*†

*Department of MENICON Cartilage & Bone Regeneration, Graduate School of Medicine and Faculty of Medicine, The University of Tokyo, Hongo 7-3-1, Bunkyo-Ku, Tokyo 113-0033, Japan
†Division of Tissue Engineering, Graduate School of Medicine and Faculty of Medicine, The University of Tokyo, Hongo 7-3-1, Bunkyo-Ku, Tokyo 113-0033, Japan
‡Department of Oral & Maxillofacial Surgery, Graduate School of Medicine and Faculty of Medicine, The University of Tokyo, Hongo 7-3-1, Bunkyo-Ku, Tokyo 113-0033, Japan
§Department of Clinical Bioinformatics, Graduate School of Medicine and Faculty of Medicine, The University of Tokyo, Hongo 7-3-1, Bunkyo-Ku, Tokyo 113-0033, Japan
¶Department of Orthopaedics Surgery, Graduate School of Medicine and Faculty of Medicine, The University of Tokyo, Hongo 7-3-1, Bunkyo-Ku, Tokyo 113-0033, Japan
#Department of Plastic & Reconstructive Surgery, Graduate School of Medicine and Faculty of Medicine, The University of Tokyo, Hongo 7-3-1, Bunkyo-Ku, Tokyo 113-0033, Japan
**Department of Plastic & Reconstructive Surgery, Saitama Medical School, Kerohongo 38, keroyama-cho, Iruma, Saitama 350-0495, Japan
††Department of Plastic & Reconstructive Surgery, Kitasato University, Kitasato 1-15-1, Sagamihara, Kanagawa 228-8555, Japan

Chondrocyte preparation with the safety and efficiency is the first step in cartilage regenerative medicine. To prepare a chondrocyte proliferation medium that does not contain fetal bovine serum (FBS) and that provides more than a 1000-fold increase in cell numbers within approximately 1 month, we attempted to use the medium containing 5% human serum (HS), but it exerted no more than twofold increase in 2 weeks. To compensate for the limited proliferation ability in HS, we investigated the combinational effects of 12 factors [i.e., fibroblast growth factor(FGF)-2, insulin-like growth factor(IGF)-I, insulin, bone morphogenetic protein-2, parathyroid hormone, growth hormone, dexamethasone, 1α25-dihydroxy vitamin D_3, L-3,3′,5′-triodothyronine, interleukine-1 receptor antagonist, 17β-estradiol, and testosterone] on the proliferation of human auricular chondrocytes by analysis of variance in fractional factorial design. As a result, FGF-2, dexamethasone, insulin, and IGF-I possessed promotional effects on proliferation, while the combination of FGF-2 with insulin or IGF-I synergistically enhanced the proliferation. Actually, the chondrocytes increased 7.5-fold in number in 2 weeks in a medium containing 5% HS with 10 ng/ml FGF-2 , while the cell number synergistically gained a 10–12-fold increase with 5 μg/ml insulin or 100 ng/ml IGF-I in the same period. The proliferation effects were more enhanced at a concentration of 100 ng/ml for FGF-2, and especially for the combination of 100 ng/ml FGF-2 and 5 μg/ml insulin (approximately 16-fold within 2 weeks). In the long-term culture with repeated passaging, this combination provided more than 10,000-fold within 8 weeks (i.e., passage 4). Thus, we concluded that such a combination of FGF-2 with insulin or IGF-I may be useful for promotion of auricular chondrocyte proliferation in a clinical application for cartilage regeneration.

Key words: Chondrocyte; Proliferation; Regenerative medicine; Medium; Soluble factor; Fractional factorial design

INTRODUCTION

Tissue engineering is a challenging technology in which the tissues or the cells are cultured in the laboratory and are used for replacement or support of the function of defective or injured body parts. This new approach is anticipated to overcome the difficulties or problems in the present clinical treatment. Recently, the studies on tissue engineering have endeavored to grow every type of human tissue: liver, bone, muscle, carti-

Address correspondence to Kazuto Hoshi, M.D., Ph.D., Department of Fujisoft ABC Cartilage & Bone Regeneration, Graduate School of Medicine, The University of Tokyo, Hongo 7-3-1, Bunkyo, Tokyo 113-8655, Japan. Tel: 81-3-3815-5411, ext. 37386; Fax: 81-3-5800-9891; E-mail: pochitky@umin.ac.jp

lage, blood vessels, heart muscles, nerves, pancreatic islets, and more. Among them, cartilage regenerative medicine has progressed well. Tissue-engineered cartilage has already been available for clinical use in the treatment of patients with joint defects (5,22,23) or for the correction of vesicoureteral reflux (6).

However, to broaden the indication range of cartilage regenerative medicine (e.g., to microtia, which is a congenital anomaly of the ear, cleft lip and palate, or osteoarthritis), some improvement would be necessary. One of issues to be improved is cell preparation with safety and efficiency. We should obtain a sufficient cell number from a small volume of specimen within a limited period, for clinical use. In a previous report on autologous chondrocyte transplantation for joint defects, a culture medium with 10% autologous human serum provided approximately 5×10^6 cells within 3 weeks, corresponding to 0.3–0.4 ml of regenerative cartilage material (23). However, because the volume of the regenerative cartilage needed in the treatment of microtia or end-stage osteoarthritis of knees may be some tens of milliliters, more than a 100-fold increase in cell numbers would need to be prepared for such cases, compared with that in an autologous transplantation.

To improve the efficiency of cell preparation, many researchers have attempted to use growth factors with fetal bovine serum (FBS). Quatela and coworkers examined the proliferation of human auricular chondrocytes in DMEM containing 5% FBS with 10 ng/ml fibroblast growth factor (FGF)-2 and 3 ng/ml transforming growth factor (TGF)-β, which exerted a maximal synergistic effect on thymidine uptake (25).

Although FBS contains various factors that can increase chondrocyte proliferation (7,10), the use of FBS may be restricted for clinical application because it includes the risk for transmission of viral and other pathogens. In addition, problems of possible immune reaction against bovine protein in the serum should be considered when the regenerated tissues cultured in the FBS-contained medium are transplanted into humans. The previous studies had shown immune response by antibody detection against bovine serum proteins in burn patients receiving keratinocyte grafts cultured from FBS (12,19).

Under clinical conditions, we may use human serum (HS) obtained from the patients by autologous blood transfusion, which would help the proliferation of their own cells. With HS, we statistically examined the additional effectiveness of soluble factors with regard to their synergy in chondrocytes obtained from the patients. For this experiment, we chose 12 kinds of soluble factors, all of which possess some effect on chondrocyte proliferation or differentiation and are clinically available because their safety has already been secured. Using the statistical method that is termed "analysis of variance by fractional factorial design" (8), the effects of the individual factors and the synergy of combinations were evaluated. This statistical method is useful when the number of potential factors is large, because it can minimize the total numbers of runs required. According to this method, we selected highly effective combinations of soluble factors for proliferation of human auricular chondrocytes, which might be immediately applied in the clinical field.

MATERIALS AND METHODS

Growth Factors and Reagents

Dulbecco's modified Eagle's medium (DMEM), DMEM nutrient mixture F-12 HAM (DMEM/F12), penicillin-streptomycin solution, trypsin-EDTA solution, fetal bovine serum (FBS), and human serum (HS, lot# 043K0500, 043K0501, and 043K0502) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Dexamethasone (Dex), 1α25-dihydroxy vitamin D_3 (vitD), L-3,3′,5′-triiodothyronine (T3), and 17β-estradiol (E_2) were from EMD Bioscience (San Diego, CA, USA). Collagenase from *Clostridium histolyticum* and ISOGEN were from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Other reagents were: atelocollagen (Kawaken Fine Chemicals Co., Ltd., Tokyo, Japan), bullet kit chondrocyte growth medium (CGM, Cambrex Bioscience Walkersville, Inc., Walkersville, MD, USA), FGF-2 (kindly provided by Kaken Pharmaceutical Corporation, Ltd., Tokyo, Japan), recombinant human insulin-like growth factor-I (IGF-I, former Genzyme-Techne, Minneapolis, MN), insulin (MP Biomedicals Inc., Irvine, CA, USA), recombinant human bone morphogenetic protein-2 (BMP-2, kindly provided by Yamanouchi Pharmaceutical Co., Ltd., Tokyo, Japan), human parathyroid hormone [PTH (1–34), Anaspec, Inc., San Jose, CA], human growth hormone (GH, Biogenesis, Ltd., Poole, UK), recombinant human interleukin-1 receptor antagonist (IL-1 RA, Strathmann Biotech GmbH, Hamburg, Germany), and testosterone (Ultrafine Chemicals, Manchester, UK).

Cell Isolation

All procedures of the present experiments were approved by the ethic committee of the University of Tokyo Hospital (ethic permission #622). Remnant auricular cartilage and surgical debris of the costal cartilage were obtained from five children (age range 10–15 years) who underwent microtia surgery at the University of Tokyo Hospital, with informed consent. The perichondrium was separated from the auricular cartilage under sterile conditions, while that of the costal cartilage had been already removed during the operation. The isolated cartilage was minced into 1-mm^3 pieces and digested with 0.15% collagenase in DMEM containing penicillin and streptomycin at 37°C for 24 h. The digested suspen-

sion was filtered using a sterile 100-μm nylon cell strainer (BD Falcon, Bedford, MA, USA) and centrifuged at 430 × *g* for 5 min. The resulting pellet of cells was washed twice with DMEM containing the antibiotics and then resuspended in the medium. The number of cells was calculated using a hemocytometer, and the viability of the cells was determined using trypan blue vital dye.

Chondrocyte Culture and Evaluation of Proliferation

Chondrocytes were suspended in 0.24% atelocollagen solution (pH 7). The mixture was placed in each well of a six-well plate at 2 ml, or a 96-well plate for 0.1 ml, at a density of 2×10^4 cells/ml. The atelocollagen formed a gel in 1-h incubation at 37°C, embedding the cells in a 3D condition. The commercial medium, CGM containing 5% FBS with an undisclosed concentration of FGF-2, IGF-I, and insulin, or the medium containing DMEM/F-12 with or without serum and/or soluble factors was gently poured on the gel at a volume of 2 ml or 0.1 ml, respectively, in a 37°C/5% CO_2 incubator. Throughout the experiment, the medium was changed three times per week. To release the cells, the gel was incubated in 0.3% collagenase at 37°C for 2 h.

To evaluate the cell proliferation by cell count, the auricular chondrocytes (cultured in CGM, passage 4) from the five patients were individually incubated in five wells of a six-well plate for each medium. After a 2-week incubation, the cell numbers were counted by a hematocytometer, while the viability of cells was checked by trypan blue staining. For the long-term culture with repeated passaging of the auricular or costal chondrocytes, we cultured the cells in three wells of six-well plates with the CGM or the DMEM/F-12 with 5% HS and soluble factors. Passage was performed every week or every other week, while the mean cell numbers in the three wells were counted during every passage.

To evaluate the samples on the 96-well plates, we used a colorimetric assay for cell proliferation, Cell Proliferation Kit II (Roche Molecular Biochemicals, Mannheim, Germany). Briefly, after the release of chondrocytes from the gel in each well of the 96-well plates, the cells were centrifuged for 5 min at 430 × *g* and separated from the supernatant, which was then removed. The labeling mixture was then added to the cells, which were incubated for 4 h in a 37°C/5% CO_2 incubator. The spectrophotometric absorbance of the samples using a microtiter plate reader was measured at a wavelength of 450 nm. The reference wavelength was 650 nm.

Fractional Factorial Design

Twelve factors (i.e., FGF-2, IGF-I, insulin, BMP-2, PTH, GH, Dex, vitD, T3, IL-1RA, E_2, and testosterone) were examined for their combinational effects on proliferation. The doses of each factor were used from previous papers or were determined as their modifications due to our own preliminary data and/or economical reasons (Table 1). With the usual factorial design, $2^{12} = 4096$ treatment combinations would be needed, but this is practically impossible. We adopted fractional factorial design (8) to reduce the experimental units to a practical number of 256 combinations. This design retains good statistical properties and has found its greatest use in industrial research. We used the software JMP-5.1.1J (SAS Institute, Inc., Cary, NC, USA) to generate the 256 kinds of randomized combinations in which those 12 factors independently appeared on the incidence of 50% (Table 2). We then made the same number of media containing DMEM/F-12 with those combinations of factors and 5% HS. Three kinds of auricular chondrocytes obtained from different patients were incubated in each well of 96-well plates with those media for 2 weeks. The effects of each medium on proliferation were examined in the colorimetric assay for cell proliferation. Values for three kinds of auricular chondrocytes in each media were processed by analysis of variance using the software JMP, providing an *F*-value, meaning the effects of the individual factors or the interaction terms of two factors, as well as a parameter estimate indicating expected changes produced by one factor or two. Total effect of two factors on proliferation was compared with each other, as the sum of parameter estimates of two main effects and interaction.

Total RNA Extraction and Real-Time PCR

The chondrocytes were released from the atelocollagen gel by a 30-min incubation in 0.3% collagenase. The total RNA was isolated from the chondrocytes with ISOGEN following the supplier's protocol. Complementary DNA (cDNA) was synthesized from 1 μg of total

Table 1. Twelve Soluble Factors Affecting Chondrocyte Proliferation and Differentiation

Soluble Factors	Doses	References
FGF-2	10 ng/ml	13
IGF-I	100 ng/ml	30
Insulin	5 μg/ml	14
BMP-2	200 ng/ml	27
PTH	5×10^{-8} M	32
GH	100 ng/ml	20
Dex	10^{-7} M	21
VitD	10^{-7} M	11
T3	10^{-7} M	17
IL-1 RA	20 ng/ml	31
E_2	10^{-7} M	26
Testosterone	10^{-6} M	18

Table 2. Combinations of Soluble Factors Determined by Fractional Factorial Design F, FGF-2; I, IGF-I; In, insulin; B, BMP-2; P, PTH; G, GH; D, Dex; V, vitD; T, T3; IL, IL-1 RA; E, E_2; Te, testosterone.

	F	I	in	B	P	G	D	V	T	IL	E	Te
1	+	+	+	+	+	+	+	+	+	+	+	+
2	+	+	+	+	+	+	+	−	−	−	−	−
3	+	+	+	+	+	+	−	+	−	+	+	−
4	+	+	+	+	+	+	−	+	−	+	−	+
5	+	+	+	+	+	+	−	−	−	−	+	+
6	+	+	+	+	+	−	+	+	−	−	−	+
7	+	+	+	+	+	−	+	−	+	−	+	−
8	+	+	+	+	+	−	−	−	+	+	−	+
9	+	+	+	+	−	+	+	+	+	−	−	−
10	+	+	+	+	−	+	+	+	−	+	+	+
11	+	+	+	+	−	+	−	+	+	+	+	−
12	+	+	+	+	−	+	−	−	+	+	−	−
13	+	+	+	+	−	+	−	−	−	−	+	−
14	+	+	+	+	−	−	+	+	+	+	−	−
15	+	+	+	+	−	−	+	+	+	−	+	+
16	+	+	+	+	−	−	+	−	−	+	+	+
17	+	+	+	+	−	−	+	−	−	−	−	+
18	+	+	+	−	+	+	+	+	+	+	+	−
19	+	+	+	−	+	+	+	+	+	−	−	−
20	+	+	+	−	+	+	−	+	−	−	+	+
21	+	+	+	−	+	+	−	−	+	+	+	+
22	+	+	+	−	+	−	+	+	−	+	+	+
23	+	+	+	−	+	−	−	+	+	−	+	−
24	+	+	+	−	+	−	−	+	−	−	−	−
25	+	+	+	−	+	−	−	−	+	−	+	−
26	+	+	+	−	+	−	−	−	−	+	−	+
27	+	+	+	−	−	+	+	−	+	+	+	−
28	+	+	+	−	−	+	+	−	+	−	−	+
29	+	+	+	−	−	+	+	−	−	+	−	+
30	+	+	+	−	−	+	−	+	+	−	−	+
31	+	+	+	−	−	−	+	+	−	−	+	−
32	+	+	+	−	−	−	+	−	−	+	−	−
33	+	+	+	−	−	−	−	+	+	−	−	+
34	+	+	+	−	−	−	−	−	−	+	+	+
35	+	+	−	+	+	+	+	+	+	+	−	−
36	+	+	−	+	+	+	+	+	+	−	+	+
37	+	+	−	+	+	+	+	−	−	+	+	−
38	+	+	−	+	+	+	−	+	+	−	−	−
39	+	+	−	+	+	+	−	−	+	−	−	−
40	+	+	−	+	+	−	+	+	−	+	−	−
41	+	+	−	+	+	−	−	−	+	−	+	+
42	+	+	−	+	+	−	−	−	−	+	+	−
43	+	+	−	+	−	+	+	−	+	−	+	−
44	+	+	−	+	−	+	+	−	−	+	−	+
45	+	+	−	+	−	+	−	+	−	−	−	+
46	+	+	−	+	−	−	+	−	−	−	−	−
47	+	+	−	+	−	−	−	+	+	+	−	+
48	+	+	−	+	−	−	−	+	−	−	+	+
49	+	+	−	−	+	+	+	+	−	+	−	−
50	+	+	−	−	+	+	−	−	+	+	−	+
51	+	+	−	−	+	+	−	−	−	−	+	+
52	+	+	−	−	+	−	+	+	+	+	+	+
53	+	+	−	−	+	−	+	−	−	+	+	−
54	+	+	−	−	+	−	+	−	−	−	−	+
55	+	+	−	−	+	−	−	+	+	+	−	−
56	+	+	−	−	+	−	−	−	+	−	−	+
57	+	+	−	−	−	+	+	+	+	+	+	+
58	+	+	−	−	−	+	+	+	−	−	+	−
59	+	+	−	−	−	+	−	−	+	−	+	+
60	+	+	−	−	−	+	−	−	−	+	−	−
61	+	+	−	−	−	−	+	−	+	+	−	+
62	+	+	−	−	−	−	+	−	+	−	−	−
63	+	+	−	−	−	−	−	+	+	+	+	−
64	+	+	−	−	−	−	−	+	−	−	−	−

	F	I	in	B	P	G	D	V	T	IL	E	Te
65	+	−	+	+	+	+	+	+	+	+	+	−
66	+	−	+	+	+	+	+	−	+	−	−	+
67	+	−	+	+	+	+	+	−	−	+	−	−
68	+	−	+	+	+	+	−	+	+	−	+	+
69	+	−	+	+	+	−	+	−	−	−	+	+
70	+	−	+	+	+	−	−	+	+	+	+	+
71	+	−	+	+	+	−	−	+	−	+	−	−
72	+	−	+	+	+	−	−	+	−	−	+	−
73	+	−	+	+	−	+	+	+	−	−	−	+
74	+	−	+	+	−	+	+	−	+	+	+	+
75	+	−	+	+	−	+	−	+	−	+	−	−
76	+	−	+	+	−	+	−	−	+	−	+	−
77	+	−	+	+	−	−	+	+	−	+	+	−
78	+	−	+	+	−	−	−	−	+	−	−	+
79	+	−	+	+	−	−	−	−	+	−	−	−
80	+	−	+	+	−	−	−	−	−	+	−	+
81	+	−	+	−	+	+	+	+	+	+	−	+
82	+	−	+	−	+	+	+	−	−	−	+	+
83	+	−	+	−	+	+	−	−	+	+	−	−
84	+	−	+	−	+	+	−	−	−	+	+	−
85	+	−	+	−	+	+	−	−	−	−	−	−
86	+	−	+	−	+	−	+	−	+	+	+	+
87	+	−	+	−	+	−	+	−	+	−	−	−
88	+	−	+	−	+	−	−	+	+	−	−	+
89	+	−	+	−	−	+	+	+	−	−	+	−
90	+	−	+	−	−	+	−	+	+	+	+	+
91	+	−	+	−	−	−	+	+	+	+	−	+
92	+	−	+	−	−	−	+	+	+	+	−	−
93	+	−	+	−	−	−	−	+	−	−	+	+
94	+	−	+	−	−	−	−	−	+	+	+	−
95	+	−	+	−	−	−	−	−	−	−	+	−
96	+	−	−	+	+	+	+	+	−	−	+	+
97	+	−	−	+	+	+	+	−	+	+	−	−
98	+	−	−	+	+	+	−	+	+	+	−	+
99	+	−	−	+	+	+	−	+	−	−	+	−
100	+	−	−	+	+	+	−	−	−	+	+	+
101	+	−	−	+	+	−	+	+	+	−	−	−
102	+	−	−	+	+	−	+	+	−	+	−	+
103	+	−	−	+	+	−	−	−	+	+	+	−
104	+	−	−	+	+	−	−	−	−	−	−	+
105	+	−	−	+	−	+	+	+	+	+	+	−
106	+	−	−	+	−	+	+	+	−	+	−	−
107	+	−	−	+	−	+	−	−	+	+	−	+
108	+	−	−	+	−	+	−	−	−	+	+	+
109	+	−	−	+	−	−	+	+	+	−	−	+
110	+	−	−	+	−	−	+	−	+	+	+	−
111	+	−	−	+	−	−	+	−	−	−	+	+
112	+	−	−	+	−	−	−	+	+	−	+	−
113	+	−	−	+	−	−	−	+	−	−	−	−
114	+	−	−	−	+	+	+	−	+	−	+	−
115	+	−	−	−	+	+	−	+	+	+	+	−
116	+	−	−	−	+	+	−	+	−	−	−	+
117	+	−	−	−	+	−	+	+	−	−	+	−
118	+	−	−	−	+	−	+	−	+	+	−	−
119	+	−	−	−	+	−	−	+	+	−	+	+
120	+	−	−	−	+	−	−	−	−	−	−	−
121	+	−	−	−	−	+	+	+	+	−	−	−
122	+	−	−	−	−	+	+	−	−	−	−	+
123	+	−	−	−	−	+	−	+	+	−	−	−
124	+	−	−	−	−	+	−	+	−	+	+	−
125	+	−	−	−	−	−	+	+	−	+	+	+
126	+	−	−	−	−	−	+	−	+	−	+	+
127	+	−	−	−	−	−	+	−	−	+	−	−
128	+	−	−	−	−	−	−	+	−	+	−	+

	F	I	in	B	P	G	D	V	T	IL	E	Te
129	−	+	+	+	+	+	+	+	−	+	−	+
130	−	+	+	+	+	+	+	+	−	−	−	−
131	−	+	+	+	+	+	−	−	+	−	−	+
132	−	+	+	+	+	−	+	+	+	+	+	+
133	−	+	+	+	+	−	+	−	+	+	−	−
134	−	+	+	+	+	−	+	−	−	+	−	−
135	−	+	+	+	+	−	−	+	+	−	−	+
136	−	+	+	+	+	−	−	−	−	−	+	+
137	−	+	+	+	−	+	+	−	−	+	+	+
138	−	+	+	+	−	+	−	+	+	−	+	+
139	−	+	+	+	−	+	−	−	+	+	−	−
140	−	+	+	+	−	+	−	−	−	−	−	+
141	−	+	+	+	−	−	+	+	+	−	+	−
142	−	+	+	+	−	−	+	−	+	−	−	−
143	−	+	+	+	−	−	−	+	−	+	+	−
144	−	+	+	+	−	−	−	+	−	−	−	−
145	−	+	+	−	+	+	+	+	−	−	+	+
146	−	+	+	−	+	+	+	−	+	−	−	+
147	−	+	+	−	+	+	−	+	+	−	+	−
148	−	+	+	−	+	+	−	−	−	+	+	−
149	−	+	+	−	+	−	+	+	−	+	+	−
150	−	+	+	−	+	−	+	−	−	+	−	+
151	−	+	+	−	+	−	−	+	+	+	−	+
152	−	+	+	−	−	+	+	+	+	+	−	−
153	−	+	+	−	−	+	+	−	−	−	+	−
154	−	+	+	−	−	+	−	+	−	+	−	+
155	−	+	+	−	−	+	−	+	−	+	−	−
156	−	+	+	−	−	−	+	+	+	+	+	+
157	−	+	+	−	−	−	+	−	+	−	+	+
158	−	+	+	−	−	−	−	+	+	+	+	−
159	−	+	+	−	−	−	−	−	+	+	+	+
160	−	+	+	−	−	−	−	−	−	−	−	−
161	−	+	−	+	+	+	+	−	−	−	+	+
162	−	+	−	+	+	+	−	+	+	+	+	−
163	−	+	−	+	+	+	−	+	−	−	−	−
164	−	+	−	+	+	−	+	+	+	−	+	−
165	−	+	−	+	+	−	+	−	+	+	+	+
166	−	+	−	+	+	−	−	+	−	+	+	+
167	−	+	−	+	+	−	−	+	−	+	−	−
168	−	+	−	+	−	+	+	+	+	+	−	−
169	−	+	−	+	−	+	+	+	−	−	+	−
170	−	+	−	+	−	+	+	−	+	−	−	+
171	−	+	−	+	−	+	−	−	+	+	+	+
172	−	+	−	+	−	+	−	−	−	+	+	−
173	−	+	−	+	−	−	+	+	−	+	+	−
174	−	+	−	+	−	−	−	+	+	−	−	+
175	−	+	−	+	−	−	−	−	+	−	+	−
176	−	+	−	+	−	−	−	−	−	+	−	+
177	−	+	−	−	+	+	+	−	+	+	+	−
178	−	+	−	−	+	+	+	−	−	−	−	−
179	−	+	−	−	+	+	−	+	+	+	+	+
180	−	+	−	−	+	+	−	−	−	+	−	+
181	−	+	−	−	+	−	+	+	+	−	−	+
182	−	+	−	−	+	−	+	+	+	−	−	−
183	−	+	−	−	+	−	−	+	−	−	+	+
184	−	+	−	−	+	−	−	−	+	+	−	−
185	−	+	−	−	+	−	−	−	−	−	+	−
186	−	+	−	−	−	+	+	+	−	−	−	+
187	−	+	−	−	−	+	−	+	+	−	+	−
188	−	+	−	−	−	+	−	+	−	+	+	+
189	−	+	−	−	−	+	−	−	+	−	−	−
190	−	+	−	−	−	−	+	+	−	+	−	+
191	−	+	−	−	−	−	+	−	+	+	+	−
192	−	+	−	−	−	−	+	−	−	−	+	+

	F	I	in	B	P	G	D	V	T	IL	E	Te
193	−	−	+	+	+	+	+	+	+	+	−	+
194	−	−	+	+	+	+	+	+	+	−	−	−
195	−	−	+	+	+	+	+	+	−	−	+	−
196	−	−	+	+	+	+	−	−	+	+	+	−
197	−	−	+	+	+	+	−	−	−	+	−	+
198	−	−	+	+	+	−	+	+	−	−	+	+
199	−	−	+	+	+	−	−	+	+	+	−	−
200	−	−	+	+	+	−	−	+	−	−	−	+
201	−	−	+	+	+	−	−	−	−	−	+	−
202	−	−	+	+	−	+	+	+	+	−	+	+
203	−	−	+	+	−	+	+	−	+	−	+	−
204	−	−	+	+	−	+	−	+	+	−	−	−
205	−	−	+	+	−	+	−	+	−	+	+	+
206	−	−	+	+	−	−	+	+	−	+	−	+
207	−	−	+	+	−	−	+	−	+	+	−	+
208	−	−	+	+	−	−	+	−	−	+	+	−
209	−	−	+	+	−	−	−	−	+	+	+	+
210	−	−	+	−	+	+	+	+	−	+	+	+
211	−	−	+	−	+	+	−	+	+	−	−	−
212	−	−	+	−	+	+	−	−	+	−	+	+
213	−	−	+	−	+	−	+	+	+	+	+	−
214	−	−	+	−	+	−	+	−	+	−	+	+
215	−	−	+	−	+	−	+	−	−	−	−	−
216	−	−	+	−	+	−	−	+	−	+	−	−
217	−	−	+	−	+	−	−	−	−	+	+	+
218	−	−	+	−	−	+	+	−	+	+	+	−
219	−	−	+	−	−	+	+	−	−	+	−	−
220	−	−	+	−	−	+	−	+	−	−	+	−
221	−	−	+	−	−	+	−	+	+	+	−	+
222	−	−	+	−	−	+	−	−	−	−	−	+
223	−	−	+	−	−	−	+	+	−	−	−	+
224	−	−	+	−	−	−	+	−	+	−	−	−
225	−	−	+	−	−	−	−	+	+	−	+	−
226	−	−	−	+	+	+	+	+	−	+	+	+
227	−	−	−	+	+	+	−	−	+	−	+	+
228	−	−	−	+	+	+	−	−	−	+	−	−
229	−	−	−	+	+	−	+	+	+	+	−	−
230	−	−	−	+	+	−	+	−	+	−	−	+
231	−	−	−	+	+	−	+	−	−	−	+	−
232	−	−	−	+	+	−	−	−	+	−	−	−
233	−	−	−	+	−	+	+	−	−	−	−	−
234	−	−	−	+	−	+	−	+	+	+	+	−
235	−	−	−	+	−	+	−	+	+	+	−	+
236	−	−	−	+	−	+	−	−	−	−	+	+
237	−	−	−	+	−	−	+	+	+	+	+	+
238	−	−	−	+	−	−	−	+	−	−	+	+
239	−	−	−	+	−	−	−	−	−	+	−	−
240	−	−	−	−	+	+	+	+	+	−	−	+
241	−	−	−	−	+	+	+	+	−	+	−	−
242	−	−	−	−	+	+	+	−	+	+	−	+
243	−	−	−	−	+	+	+	−	+	−	+	−
244	−	−	−	−	+	+	−	+	−	−	−	+
245	−	−	−	−	+	−	+	−	−	+	−	+
246	−	−	−	−	+	−	−	+	−	+	+	−
247	−	−	−	−	+	−	−	−	+	+	+	+
248	−	−	−	−	−	+	+	+	+	−	+	+
249	−	−	−	−	−	+	+	−	−	+	+	+
250	−	−	−	−	−	+	−	−	+	+	−	−
251	−	−	−	−	−	+	−	−	−	−	+	−
252	−	−	−	−	−	−	+	+	+	−	+	−
253	−	−	−	−	−	−	+	+	−	−	−	−
254	−	−	−	−	−	−	−	+	+	+	−	+
255	−	−	−	−	−	−	−	−	+	−	−	+
256	−	−	−	−	−	−	−	−	−	+	+	−

RNA with the Superscript II reverse transcriptase kit (Invitrogen, Carlsbad, CA, USA). For the real-time PCR, the ABI Prism Sequence Detection System 7000 was used. Primers were designed based on the sequences obtained from the GenBank, and amplicons of 50–250 base pairs with a melting temperature of between 55°C and 60°C were selected. Aliquots of the first-strand cDNA (1 μg) were amplified with the QuantiTect SYBER Green PCR Kit (Qiagen, Osaka, Japan) under the following conditions: initial denaturation for 10 min at 94°C followed by 40 cycles consisting of 15 s at 94°C and 1 min at 60°C. The data analysis consisted of a fold induction, and the expression ratio was calculated from the differences in the threshold cycles at which an increase in the reporter fluorescence above a baseline signal could first be detected among the three samples and was averaged for duplicate experiments. The sequence of primers we utilized in the real-time PCR to detect human Col2A1 and human GAPDH were: hCol2A1 forward 5′-GAGT CAAGGGTGATCGTGGT-3′, reverse 5′-CACCTTGG TCT CCAGAAGGA-3′; hGAPDH forward 5′-GAAGG TGAAGGTCGGAGTCA-3′, reverse 5′-GAAGATGGT GATGGGATTTC-3′. hGAPDH was used as a housekeeping gene.

RESULTS

Proliferation of Auricular or Costal Chondrocytes With or Without FBS

In a microtia operation, both remnant auricular cartilage and surgical debris of the costal cartilage, neither of which is used for reconstructive surgery, were obtained from individual patients. The mean weight was 1.18 g (1.00–1.42, $n = 5$) or 3.60 g (2.2–5.0, $n = 5$), respectively, while the mean cell number of the isolated chondrocytes was 3.8×10^6 (1.5×10^6–7.0×10^6) or 2.5×10^6 (1.0×10^6–4.0×10^6). We first examined the proliferation of the auricular and costal chondrocytes taken from these tissues. When the commercial medium, CGM containing 5% FBS with an undisclosed concentration of FGF-2, IGF-I, and insulin, was used for chondrocyte proliferation and passage was performed every week, both kinds of chondrocytes showed exponential growth after some lag phase in all five patients. The growth rate was higher in the auricular chondrocytes than in costal ones. Figure 1 shows the typical growth curve of two patients. The auricular chondrocytes increased their number by 1000-fold within passages 3–4 (3–4 weeks), while it took more than five passages (5 weeks) for the costal ones (Fig. 1).

Next, we attempted to substitute FBS for HS and/or some other factors. When we examined the proliferation of the auricular chondrocytes in the medium without any serum or soluble factors, they did not undergo proliferation at all (Fig. 2, open bar). When we added FGF-2 and IGF-I, whose combination had been known to increase the proliferation in a previous paper (42), little proliferation was noted (Fig. 2, open bar). In contrast, HS may have some effects on the proliferation of auricular chondrocytes, but it provided no more than twofold in 2 weeks (Fig. 2, filled bar). FBS showed better proliferation of approximately threefold within 2 weeks (Fig. 2, filled bar). When FGF-2 and IGF-I were added to the medium containing HS or FBS, the proliferation was increased to approximately seven- to ninefold in both (Fig 2, slanted striped bars), suggesting the possibility that HS may promote proliferation to the same extent as FBS, with some soluble factors. However, none of them were over that of CGM (Fig. 2, horizontal striped bar).

Statistical Analysis for Combinational Effects of Soluble Factors

We selected 12 kinds of soluble factors to be examined (Table 1). We used a fractional factorial design to determine an optimal combination with a minimal number of experiments. Using 256 kinds of media determined by the software JMP-5.1.1.J (Table 2), we examined the proliferation effects in a colorimetric assay. The results obtained for the 256 kinds of media were also analyzed by the software JMP-5.1.1.J.

In this analysis, FGF-2, Dex, insulin, and IGF-I showed parameter estimates of 0.192 ($F = 676$), 0.0943 ($F = 166$), 0.0626 ($F = 75.1$), and 0.0524 ($F = 52.6$), respectively, and they showed significant effects on proliferation alone ($p < 0.001$). Eight other factors did not prominently promote proliferation of the auricular chondrocytes in the presence of 5% HS, and especially BMP-2 worked rather negatively in the chondrocyte proliferation (parameter estimate −0.194, $F = 720$). Regarding synergy, the interaction term of FGF-2 and insulin and that of FGF-2 and IGF-I exhibited statistically positive effects (parameter estimates 0.0582 and 0.0565, $F = 64.3$ and 60.7, $p < 0.001$), although IGF-I and insulin decreased the effects of synergy (parameter estimate −0.0601, $F = 68.7$, $p < 0.001$). Therefore, we chose the combinations of FGF-2 with insulin or IGF-I as ideal ones that showed high F-values in both individual factors and interaction terms of two factors. Actually, the combinations of FGF-2 and insulin or FGF-2 and IGF-I showed high values of parameter estimates, suggesting strong effects on proliferation, when the chondrocytes were cultured together with 5% HS (Fig. 3).

Effects of Doses of FGF-2, Insulin, and IGF-I on the Synergy

To confirm the effects of FGF-2, insulin, IGF-I, or their combinations on chondrocyte proliferation, the increase in cell numbers was counted, following the culture in the media containing 5% HS with those factors

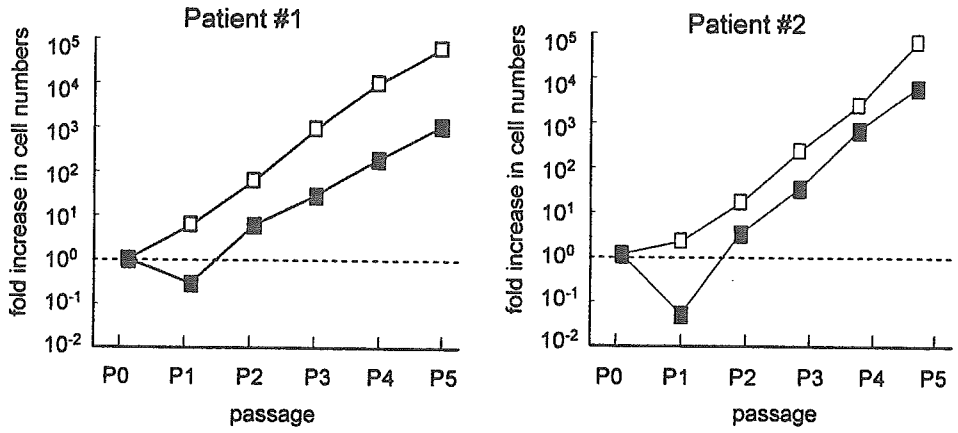

Figure 1. Growth curves of human chondrocytes. Each kind of cell was cultured in a six-well plate with CGM. Every week, the cells were harvested, counted in numbers, and reseeded in a six-well plate until the cell numbers increased more than 1000-fold. The number of auricular chondrocytes (open square) arrived at a 1000-fold increase more quickly than that of the costal cartilage (filled square) in all patients ($n = 5$).

or their combination in various doses. FGF-2 showed a dose dependency for proliferation in the range of 10–500 ng/ml, although more than 1 μg /ml seemed to be the top limit. Insulin and IGF-I did not exhibit increasing effects on proliferation in the range of 5–500 μg/ml or 100 ng–10 μg/ml, respectively (Fig. 4).

The synergistic effects of insulin or IGF-I with FGF-2 seemed to be maintained until the dose of FGF-2 increased to 100 ng/ml (Fig. 5). In the combination of 10 ng/ml FGF-2 with 5 μg/ml insulin or 100 ng/ml IGF-I, the cell number increased to approximately 12- or 10-fold, respectively, within 2 weeks, while the increase was limited to eightfold with FGF-2 alone (Fig. 5, FGF-2 10 ng/ml). The addition of both insulin and IGF-I to FGF-2 showed an 11-fold increase, which was somewhat lower, compared with the combination of FGF-2 with insulin (12-fold) (Fig. 5, FGF-2 10 ng/ml). The same tendency was observed in the combination of 100 ng/ml FGF-2 with insulin, IGF-I, or both. Especially the combination of 100 ng/ml FGF-2 and insulin showed prominent proliferation (16-fold) (Fig. 5, FGF-2 100 ng/ml). When the dose of FGF-2 was 500 ng/ml, the addition of insulin, IGF-I, or both made little difference compared with FGF-2 alone, suggesting that synergistic effects of insulin and IGF-I were not found at this dose of FGF-2 (Fig. 5, FGF-2 500 ng/ml). Although 500 ng/

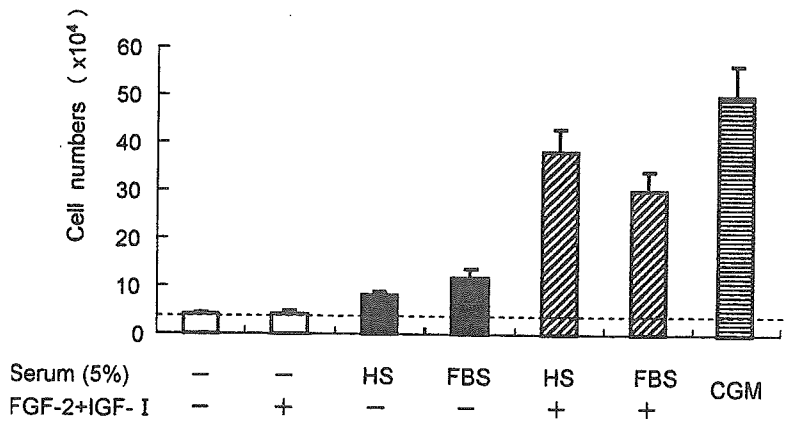

Figure 2. Effects of FBS, HS, or additional soluble factors on the chondrocyte proliferation. Human auricular chondrocytes (2×10^4 cells corresponding to the broken line) obtained from five patients were encapsulated in 2 ml of atelocollagen gel and incubated with each kind of medium in a six-well plate for 2 weeks ($n = 5$ for each medium). HS, human serum; FBS, fetal bovine serum; FGF-2, 10 ng/ml; IGF-I, 100 ng/ml. All values are presented as mean + SD.

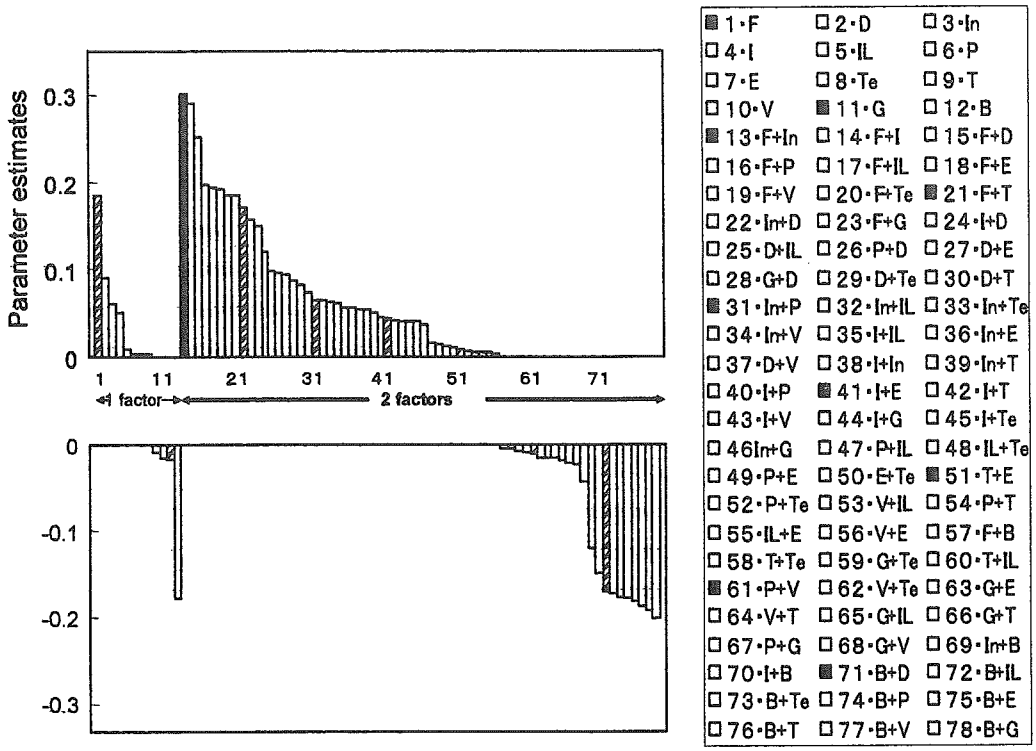

Figure 3. Results of analysis of variance by fractional factorial design. FGF-2 (column #1), Dex (column #2), insulin (column #3), and IGF-I (column #4) showed high values of parameter estimates, suggesting the promotion of proliferation. The combination of FGF-2 and insulin (column #13) and that of FGF-2 and IGF-I (column #14) were strongly effective for proliferation. However, the simultaneous use of insulin and IGF-I (column #38) somewhat decreased the effects of synergy, compared with a single use of insulin (column #3) or IGF-I (column #4). F, FGF-2; D, Dex; In, insulin; I, IGF-I; IL, IL-1RA; P, PTH; E, E_2; Te, testosterone; T, T3; V, vitD; G, GH; B, BMP-2.

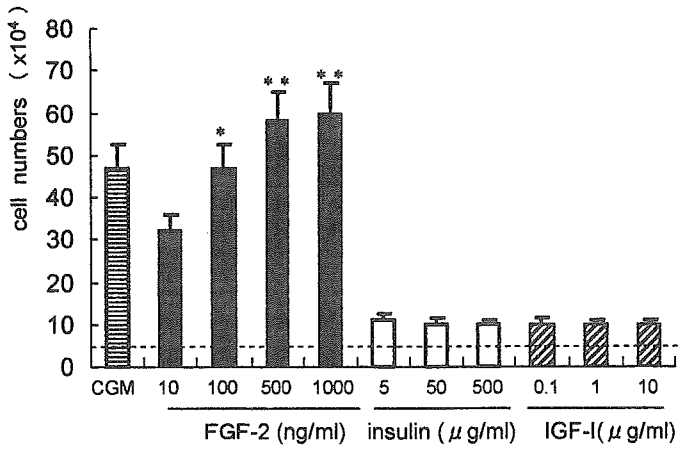

Figure 4. Dose dependency of FGF-2, insulin, and IGF-I in the chondrocyte proliferation. Human auricular chondrocytes were 3D cultured in the media containing DMEM/F-12 with 5% HS and FGF-2, insulin, or IGF-I at various kinds of doses for 2 weeks ($n = 5$ for each medium). All values are presented as mean + SD. Statistics were assessed using the Student's t-test (*$p < 0.05$ vs. FGF-2 10 ng/ml, **$p < 0.05$ vs. FGF-2 100 ng/ml). The broken line indicates the number of chondrocytes at the starting point of incubation (2×10^4 cells).

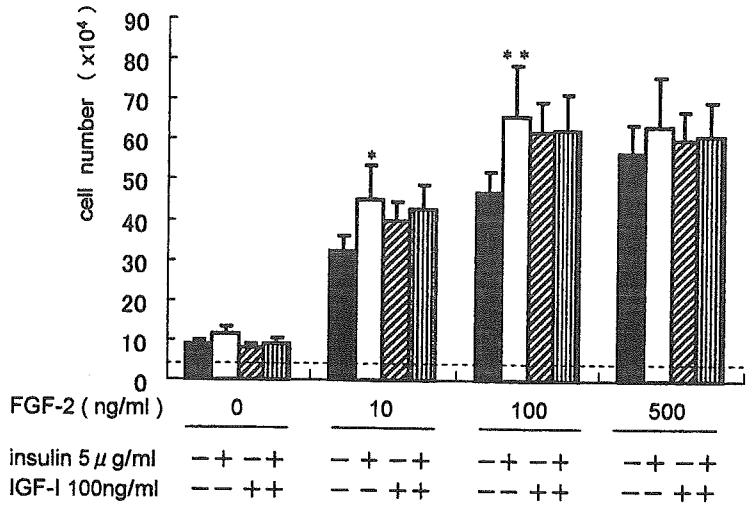

Figure 5. Synergistic effects of FGF-2 with insulin or IGF-I on the chondrocyte proliferation. The cell numbers of human auricular chondrocytes from the different patients were counted after a 2-week incubation ($n = 5$ for each medium). The media contained DMEM/F-12 with 5% HS and the soluble factors. The broken line indicates the initial number of chondrocytes at the starting point (2×10^4 cells). All values are presented as mean + SD. Statistics were assessed using the Student's t-test (*$p < 0.05$ vs. the group using FGF-2 10 ng/ml alone, **$p < 0.05$ vs. the group using FGF-2 100 ng/ml alone).

ml of FGF-2 alone showed higher proliferation effects than did 100 ng/ml, the combinations of 500 ng/ml FGF-2 with insulin, IGF-I, or both showed almost the same proliferation ability as those combinations with 100 ng/ml FGF-2 (Fig. 5). These results were reproducible in every lot of HS (data not shown).

To confirm the actual 1000-fold increase in the cell numbers, we examined a long-term culture with repeated passaging of the auricular chondrocytes in 5% HS-DMEM/F-12 containing FGF-2 (100 ng/ml) with or without insulin (5 μg/ml) or IGF-I (100 ng/ml). A passage was performed every other week, an interval that corresponded to the period used in the above experiments for determining the kinds and doses of the soluble factors. In the growth curves, all kinds of media as well as the CGM provided good proliferation after some lag phase, and realized a 1000-fold increase during passage 4 (Fig. 6). Especially, the combination of FGF-2 and insulin exhibited the highest growth rate, arriving at 10,000-fold within passage 4.

Finally, we checked the gene expression pattern of the chondrocytes proliferated in these four kinds of media. All of them showed an absolutely lower expression in Col2A1, compared with chondrocytes of the primary culture (passage 0). The expression ratio was 7470 ± 2450 in the primary culture, while those of a long-term culture in CGM, the medium containing FGF-2, and that in the combination of FGF-2 with insulin or IGF-I were 2.96 ± 1.82, 7.26 ± 4.59, 11.3 ± 7.11, and 15.5 ± 3.50, respectively.

DISCUSSION

The first issue that we must achieve is to gain sufficient cell numbers of cultured chondrocytes for the cartilage regenerative medicine. A previous report described that fetal elastic chondrocytes proliferated more quickly than fetal hyaline cartilage (9). In the present study, the auricular chondrocytes of school children age patients also had more favorable proliferation, compared with the costal ones. In addition, we could determine an effective proliferation medium containing human serum or several soluble factors, showing high responsiveness to the auricular chondrocytes. Also because the auricular cartilage can be accessed easily in minimally invasive fashion, through a simple auricle biopsy, this kind of chondrocyte would be a good candidate for a cell source for cartilage regenerative medicine.

In the present study, we determined the combinations of two factors, which promote the proliferation of human auricular chondrocyte in the presence of 5% HS, among 12 soluble factors, by analysis of variance in fractional factorial design. For the analysis, we did not confine the factors to be examined to those that had been previously published to be effective for the proliferation of the human auricular chondrocytes, but used many factors that are reported to possess some effects on either

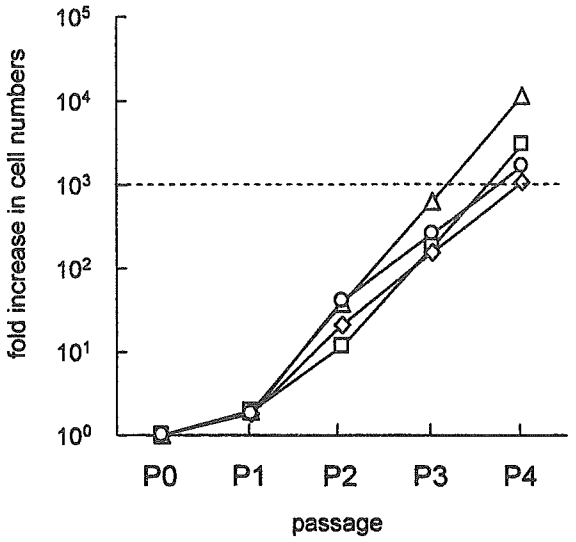

Figure 6. Growth curves of human auricular chondrocytes cultured in various kinds of media. Passage was performed every other week. DMEM/F-12 containing 5% HS with 100 ng/ml FGF-2 and 5 μg/ml insulin (triangles) provided more than a 10,000-fold increase during passage 4, while CGM (squares) and DMEM/F-12 containing 5% HS with FGF-2 alone (diamonds) or the combination of FGF-2 and IGF-I (circles) also arrived at 1000-fold during passage 4.

the proliferation or differentiation of chondrocytes, including those derived from animals and cell lines in order to widen the screening size. The doses of each soluble factor were those used in previous studies (Table 1).

We confirmed the synergistic effects of FGF-2 with insulin or IGF-I in the human auricular chondrocytes and proved their usefulness in combination with HS. Although it is well known that FGF-2, insulinm and IGF-I are mitogenic to chondrocytes (13,14,30), the mechanisms that promote chondrocyte proliferation seem to be different among such mitogens. Bohme and collaborators reported that insulin or IGF-1 could trigger chondrocyte proliferation under strictly serum-free conditions, although FGF-2 did not promote cell division in serum-free cultures of chondrocytes (3). Quarto and coworkers also described that FGF-2 alone does not induce cell proliferation of chick embryonic chondrocytes without FBS (24). In contrast, FGF-2 could bolster the mitogenic activity of FBS in cultures, indicating that the cells became conditioned by FBS so that they could respond to FGF-2 (3).

This difference in the conditions in which each factor expresses mitogenic activity may be explained by the general concept of competence and progression factors, both of which are required to trigger cell division. The former include FGF-2, EGF, or PDGF, allowing the passage from G_0 to G_1 at the entry in the cell cycle, while the latter correspond to insulin or IGF-I, which commit the cells to DNA synthesis (1). The synergy of FGF-2 with insulin or IGF-I seemed to be associated with the cooperation of these competence and progression factors. In contrast, because insulin and IGF-I, which possess a common role as progression factors, did not compensate each other and could not accelerate the cell cycle effectively, the simultaneous use of both insulin and IGF-I did not help the promotion of proliferation, as seen in the present study. Further studies on signal pathways downstream of FGF-2 and insulin or IGF-I in chondrocytes are anticipated to disclose not only the detailed mechanisms of the synergistic effects of those factors but also to provide the optimal promotion of chondrocyte proliferation.

In contrast, BMP-2 worked rather negatively in the chondrocyte proliferation. In the experiment using the murine chondrocytic cell line ATDC5, BMP-2 upregulated the expression of the type II and type X collagen mRNA and stimulated the sequential progression of the early and late phase differentiation, although it had little effect on the proliferation of the cells (27). Also, for human auricular chondrocytes, BMP-2 is speculated to drive the direction to produce the cartilaginous matrix, but to arrest the progression of the cell cycles.

Regarding the doses of FGF-2, less than 10 ng/ml was used for the promotion of proliferation in chondrocytes and MSC in many previous reports (13,25,28). Quatela and collaborators did not show that doses of FGF-2 greater than 100 ng/ml provide increasing effects on proliferation of human auricular chondrocytes in monolayer culture (25). Because the capacity of a 3D culture system for cells is higher than that in a monolayer culture, even more than 100 ng/ml FGF-2 continues to promote chondrocyte proliferation and maintain the synergistic effects with insulin or IGF-I, as seen in the present data.

After the long-term culture, which provided more than a 1000-fold increase in cell numbers, any kind of media we examined equivalently brought dedifferentiation of the chondrocytes. Chondrocytes embedded in native tissue synthesize type II collagen and proteoglycan, although the same cells cultured in a monolayer lose their typical morphology from round to a spindle fibroblast-like shape and protein synthesis, a phenomenon termed dedifferentiation (29). In order to reverse this inevitable phenomenon, many researchers have used 3D culture embedded in agarose gel (2), alginate beads (4), collagen type I gel (15), or collagen type II gel (16). However, when we applied the 3D embedded culture for chondrocyte proliferation, mRNA expression of the proliferated chondrocytes showed a dedifferentiation

pattern that the Col2a1 expression decreased, compared with those of the primary culture of the chondoryctes. It suggested that dedifferentiation inevitably occurs, even if it may be less in the 3D-embedded culture than in the monolayer culture, when the period of cell culture becomes long. Therefore, when we obtain a sufficient number of cells after a long proliferation culture, we should change the culture system, including the medium contents or cell density, in order to reverse the dedifferentitaion. At present, we have investigated the optimal medium inducing redifferentiation of the chondoryctes, and realized the production of regenerated cartilage possessing mature and abundant cartilaginous matrix in vitro.

ACKNOWLEDGMENTS: Funding for this research was provided by Grants-in-Aid for Scientific Research from the Japanese Ministry of Education, Culture, Sports, Science and Technology (#15390539, #16390431, and #16659546), Daiwa Securities Health Foundation, and Research Society for Metabolic Bone Diseases.

REFERENCES

1. Aaronson, S. A. Growth factors and cancer. Science 254: 1146–1153; 1991.
2. Benya, P. D.; Shaffer, J. D. Dedifferentiated chondrocytes reexpress the differentiated collagen phenotype when cultured in agarose gels. Cell 30:215–224; 1982.
3. Bohme, K.; Conscience-Egli, M.; Tschan, T.; Winterhalter, K. H.; Bruckner, P. Induction of proliferation or hypertrophy of chondrocytes in serum-free culture: The role of insulin-like growth factor-I, insulin, or thyroxine. J. Cell Biol. 116:1035–1042; 1992.
4. Bonaventure, J.; Kadhom, N.; Cohen-Solal, L.; Ng, K. H.; Bourguignon, J.; Lasselin, C.; Freisinger, P. Reexpression of cartilage-specific genes by dedifferentiated human articular chondrocytes cultured in alginate beads. Exp. Cell Res. 212:97–104; 1994.
5. Brittberg, M.; Lindahl, A.; Nilsson, A.; Ohlsson, C.; Isaksson, O.; Peterson, L. Treatment of deep cartilage defects in the knee with autologous chondrocyte transplantation. N. Engl. J. Med. 331:889–895; 1994.
6. Caldamone, A. A.; Diamond, D. A. Long-term results of the endoscopic correction of vesicoureteral reflux in children using autologous chondrocytes. J. Urol. 165:2224–2227; 2001.
7. Choi, Y. C.; Morris, G. M.; Lee, F. S.; Sokoloff, L. The effect of serum on monolayer cell culture of mammalian articular chondrocytes. Connect. Tissue Res. 7:105–112; 1980.
8. Finney, D. J. The fractional replication of factorial arrangements. Ann. Eugenics 12:291–301; 1945.
9. Fuchs, J. R.; Terada, S.; Hannouche, D.; Ochoa, E. R.; Vacanti, J. P.; Fauza, D. O. Engineered fetal cartilage: Structural and functional analysis in vitro. J. Pediatr. Surg. 37:1720–1725; 2002.
10. Glaser, J. H.; Conrad, H. E. Properties of chick embryo chondrocytes grown in serum-free medium. J. Biol. Chem. 259:6766–6772; 1984.
11. Grigoriadis, A. E.; Aubin, J. E.; Heersche, J. N. Effects of dexamethasone and vitamin D3 on cartilage differentiation in a clonal chondrogenic cell population. Endocrinology 125:2103–2110; 1989.
12. Johnson, L. F.; deSerres, S.; Herzog, S. R.; Peterson, H. D.; Meyer, A. A. Antigenic cross-reactivity between media supplements for cultured keratinocyte grafts. J. Burn Care Rehabil. 12:306–312; 1991.
13. Kamil, S. H.; Aminuddin, B. S.; Bonassar, L. J.; Silva, C. A.; Weng, Y.; Woda, M.; Vacanti, C. A.; Eavey, R. D.; Vacanti, M. P. Tissue-engineered human auricular cartilage demonstrates euploidy by flow cytometry. Tissue Eng. 8:85–92; 2002.
14. Kato, Y.; Gospodarowicz, D. Growth requirements of low-density rabbit costal chondrocyte cultures maintained in serum-free medium. J. Cell Physiol. 120:354–363; 1984.
15. Kimura, T.; Yasui, N.; Ohsawa, S.; Ono, K. Chondrocytes embedded in collagen gels maintain cartilage phenotype during long-term cultures. Clin. Orthop. 186:231–239; 1984.
16. Malemud, C. J.; Stevenson, S.; Mehraban, F.; Papay, R. S.; Purchio, A. F.; Goldberg, V. M. The proteoglycan synthesis repertoire of rabbit chondrocytes maintained in type II collagen gels. Osteoarthritis Cartilage 2:29–41; 1994.
17. Malinin, T. I.; Hornicek, F. J. Response of human chondrocytes cultured in vitro to human somatotropin, triiodothyronine, and thyroxine. Transplant. Proc. 29:2037–2039; 1997.
18. Maor, G.; Segev, Y.; Phillip, M. Testosterone stimulates insulin-like growth factor-I and insulin-like growth factor-I-receptor gene expression in the mandibular condyle—a model of endochondral ossification. Endocrinology 140: 1901–1910; 1999.
19. Meyer, A. A.; Manktelow, A.; Johnson, M.; deSerres, S.; Herzog, S.; Peterson, H. D. Antibody response to xenogeneic proteins in burned patients receiving cultured keratinocyte grafts. J. Trauma 28:1054–1059; 1988.
20. Monsonego, E.; Halevy, O.; Gertler, A.; Hurwitz, S.; Pines, M. Growth hormone inhibits differentiation of avian epiphyseal growth-plate chondrocytes. Mol. Cell. Endocrinol. 114:35–42; 1995.
21. Mushtaq, T.; Farquharson, C.; Seawright, E.; Ahmed, S. F. Glucocorticoid effects on chondrogenesis, differentiation and apoptosis in the murine ATDC5 chondrocyte cell line. J. Endocrinol. 175:705–713; 2002.
22. Ochi, M.; Uchio, Y.; Kawasaki, K.; Wakitani, S.; Iwasa, J. Transplantation of cartilage-like tissue made by tissue engineering in the treatment of cartilage defects of the knee. J. Bone Joint Surg. Br. 84:571–578; 2002.
23. Peterson, L.; Minas, T.; Brittberg, M.; Lindahl, A. Treatment of osteochondritis dissecans of the knee with autologous chondrocyte transplantation: Results at two to ten years. J. Bone Joint Surg. Am. 85A:17–24; 2003.
24. Quarto, R.; Campanile, G.; Cancedda, R.; Dozin, B. Modulation of commitment, proliferation, and differentiation of chondrogenic cells in defined culture medium. Endocrinology 138:4966–4976; 1997.
25. Quatela, V. C.; Sherris, D. A.; Rosier, R. N. The human auricular chondrocyte. responses to growth factors. Arch. Otolaryngol. Head Neck Surg. 119:32–37; 1993.
26. Rodd, C.; Jourdain, N.; Alini, M. Action of estradiol on epiphyseal growth plate chondrocytes. Calcif. Tissue Int. 75:214–224; 2004.
27. Shukunami, C.; Ohta, Y.; Sakuda, M.; Hiraki, Y. Sequen-

tial progression of the differentiation program by bone morphogenetic protein-2 in chondrogenic cell line ATDC5. Exp. Cell Res. 241:1–11; 1998.
28. Tsutsumi, S.; Shimazu, A.; Miyazaki, K.; Pan, H.; Koike, C.; Yoshida, E.; Takagishi, K.; Kato, Y. Retention of multilineage differentiation potential of mesenchymal cells during proliferation in response to FGF. Biochem. Biophys. Res. Commun. 288:413–419; 2001.
29. von der Mark, K.; Gauss, V.; von der Mark, H.; Muller, P. Relationship between cell shape and type of collagen synthesised as chondrocytes lose their cartilage phenotype in culture. Nature 267:531–532; 1977.
30. Wroblewski, J.; Edwall-Arvidsson, C. Inhibitory effects of basic fibroblast growth factor on chondrocyte differentiation. J. Bone Miner. Res. 10:735–742; 1995.
31. Yasuda, T.; Poole, A. R. A fibronectin fragment induces type II collagen degradation by collagenase through an interleukin-1-mediated pathway. Arthritis Rheum. 46:138–148; 2002.
32. Zerega, B.; Cermelli, S.; Bianco, P.; Cancedda, R.; Cancedda, F. D. Parathyroid hormone [PTH(1-34)] and parathyroid hormone-related protein [PTHRP(1-34)] promote reversion of hypertrophic chondrocytes to a prehypertrophic proliferating phenotype and prevent terminal differentiation of osteoblast-like cells. J. Bone Miner. Res. 14:1281–1289; 1999.

STEM CELLS®
Original Article

Characterization and Localization of Side Population Cells in Mouse Skin

SHOICHIRO YANO,[a,b] YURIKO ITO,[a] MANABU FUJIMOTO,[b] TATSUO S. HAMAZAKI,[a]
KUNIHIKO TAMAKI,[b] HITOSHI OKOCHI[a]

[a]Department of Regenerative Medicine, Research Institute, International Medical Center of Japan, Tokyo;
[b]Department of Dermatology, Faculty of Medicine, University of Tokyo, Japan

Key Words. Side population • Stem cell • Breast cancer resistance protein 1 • Skin

ABSTRACT

Recently, the detection of side population (SP) cells, which have the ability to strongly efflux Hoechst 33342 fluorescence dye, has attracted attention as a method of stem cell isolation. We identified SP cells from mouse skin using the same method as from bone marrow. This population almost completely disappeared after treatment with the calcium channel blocker verapamil. SP cells were mainly localized in the epidermis, with a few in the dermis. The ratio of SP cells decreased as the mouse became older. Surface marker analysis revealed that the sorted SP cells expressed α6-integrin, β1-integrin, Sca-1, keratin 14, and keratin 19, which are proliferating and progenitor cell markers, at levels higher than in non-SP cells, while they expressed E-cadherin, CD34, and CD71 at lower levels. The expression of breast cancer resistance protein 1 (BCRP1), which participates in dye efflux, was expressed at high levels at both the protein and mRNA level in sorted SP cells. Immunohistochemical analysis showed that BCRP1 was expressed in the basal layers and hair bulge regions of mouse skin. BCRP1 mRNA was found in basal layers and hair follicles of newborn skin by in situ hybridization. These results indicate that the localization of BCRP1-positive cells is compatible with that of keratinocyte stem cells. Based on the close relationship between BCRP1 and the SP cell phenotype, we conclude that keratinocyte stem cells are closely related to the SP- or BCRP1-positive cells. STEM CELLS *2005;23:834–841*

INTRODUCTION

Many studies have investigated the localization, character, and function of stem cells in the skin. Multipotent skin stem cells have been thought to be localized in the basal layers or the arrector pili muscle–attaching area in the follicle, called the bulge area, where they have been detected by labeling skin with titrated thymidine or bromodeoxyurindine [1, 2] or colony-forming culture [3]. Several specific molecular markers can distinguish stem cells from other skin cells; α6-integrin, β1-integrin, CD34, and keratin 19–positive and CD71-negative skin cells are thought to be keratinocyte stem cells [4–8]. Although many methods have been developed for the isolation and analysis of specific cell types, these cannot be used for medical experimentation on living material because of the damaging techniques, such as isotope radiation and cell fixation.

Recently, it was discovered that hematopoietic stem cells with the characteristics of immature stem cells could be isolated as a specific cell population that had the capability to strongly efflux Hoechst 33342 DNA-binding fluorescent dye [9]. The method relies on incubating the target cells with Hoechst 33342 and performing subsequent fluorescence-activated cell sorter analysis of dual-wavelength Hoechst fluorescence with gating on a specific side population (SP) displaying low red and low blue fluorescence. This low-staining population is called the SP.

These interesting phenomena are explained by the mechanism of a novel stem cell half-transporter. Breast cancer resis-

Correspondence: Hitoshi Okochi, M.D., Ph.D., Department of Regenerative Medicine, Research Institute, International Medical Center of Japan, 1-21-1 Toyama, Shinjuku-ku, Tokyo 162-8655, Japan. Telephone: 81-3-3202-7181, ext. 2825; Fax: 81-3-3202-7192; e-mail: hokochi@ri.imcj.go.jp Received September 4, 2004; accepted for publication February 21, 2005. 1066-5099/2005/$12.00/0 doi: 10.1634/stemcells.2004-0226

tance protein (BCRP1), which is one of the multidrug resistance proteins (MDRPs) on the cell membrane and an ATP-binding cassette transporter, predominantly effluxes Hoechst 33342 [10]. MDRPs are associated with resistance to some carcinostatics and are overexpressed in several cancer cell lines [11, 12]. In hematopoietic stem cells, SP cells express relatively high levels of this BCRP1 [13]. Zhou et al. [10, 14] determined that bone marrow SP cells require surface expression of BCRP1 for the efflux of Hoechst dye. Therefore, BCRP1 expression may serve as a new marker for stem cells, not only in hematopoietic cells but also in other types of cells.

As for skin SP cells, only a few studies have been previously performed [15–18]. It has not been clarified where SP cells are localized and whether skin SP cells possess stem cell characteristics. Accordingly, in the present study, we analyzed skin cells from newborn and adult mice for the presence of SP cells. We further examined the characteristics of the isolated skin SP cells and localized the SPs that were BCRP1-positive in mouse skin.

Materials and Methods

Isolation of SP Cells from Mouse Skin

C57BL/6J mice (Clea Japan, Tokyo, http://www.clea-japan.com) were used. Animal studies were conducted according to protocols approved by the International Medical Center of Japan. For the isolation of skin cells, mice were shaved and the dorsal and ventral skin was removed. The skin was minced in cold phosphate-buffered saline (PBS) and incubated for 60 minutes at 37°C in 0.25% trypsin-EDTA (Gibco, Grand Island, NY, http://www.invitrogen.com). Newborn skin was first incubated in 2000 U/ml dispase (Godo Shusei, Tokyo, http://www.godo.jp/english) overnight at 4°C and then separated into epidermis and dermis. Each tissue was incubated for 30 minutes at 37°C in 0.25% trypsin-EDTA. Cells were washed, resuspended at 1×10^6 cells/ml in PBS with 3% fetal bovine serum, and incubated in 5 μg/ml Hoechst 33342 dye (Sigma, St. Louis, http://www.sigmaaldrich.com) for 60 minutes at 37°C. An aliquot was stained with Hoechst in the presence of 100 μM verapamil (Wako Chemical, Osaka, Japan, http://www.wako-chem.co.jp/english), which blocks the action of the transporter responsible for Hoechst exclusion. After terminating the staining, the cells were washed and then analyzed and sorted by EPICS ALTRA flow cytometer (Beckman Coulter, Fullerton, CA, http://www.beckman.com). They were first excited with 50 mW of UV (351–364 nm), and then the emission was detected through a 450/20-nm (Hoechst blue) band-pass filter and a 675-nm (Hoechst red) long-pass filter. All of the parameters were collected using linear amplification in list mode and displayed in a Hoechst blue versus Hoechst red dotplot to visualize SP.

Antibody Staining

After sorting, aliquots of the cells were incubated for 30 minutes at 4°C with anti-mouse α6-integrin antibody (fluorescein isothiocyanate [FITC]–conjugated, 1:100), anti-mouse β1-integrin antibody (FITC-conjugated, 1:100), anti-mouse stem cell antigen-1 (Sca-1) antibody (FITC-conjugated, 1:100), anti-mouse CD34 antibody (FITC-conjugated, 1:100), anti-mouse CD71 antibody (phycoerythrin [PE]-conjugated, 1:100), anti-mouse E-cadherin antibody (FITC-conjugated, 1:100), anti-mouse CD3 antibody (FITC-conjugated, 1:100), anti-mouse B220 antibody (FITC-conjugated, 1:1000,), or anti-mouse CD45RO antibody (PE-conjugated, 1:100) (all from BD Biosciences, San Jose, CA, http://www.bdbiosciences.com). For keratin 14, keratin 19, and BCRP1 staining, we used an IntraPrep Permeabilization Kit (Immunotech, Marseille, France, http://www.bc-cytometry.com). Anti-mouse keratin 14 and 19 monoclonal antibodies (1:100) were kind gifts from Professor E.B. Lane, University of Dundee School of Life Sciences, Dundee, Scotland, U.K. Anti-mouse BCRP1 antibody (BXP-9, 1:50) came from Kamiya Biomedical (Seattle). FITC-conjugated anti-mouse immunoglobulin G (IgG) antibody (1:1000, Molecular Probes, Eugene, OR, http://probes.invitrogen.com) and PE-conjugated anti-rat IgG antibody (1:200, BD Biosciences) were used as the secondary antibodies.

Western Blotting for BCRP1 Expression on SP and Non-SP Cells

Each fraction of sorted and unsorted cells from epidermal and dermal tissues was collected by centrifugation at 4°C. The pellets were homogenized in RIPA buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, and 1 μg/ml leupeptin) at 4°C. The lysates were analyzed by SDS-PAGE. Equal amounts of protein were electrophoresed in a 7.5% SDS-polyacrylamide gel and then transferred onto a nitrocellulose membrane. The membrane was blocked with 2% bovine serum albumin in tris-buffered saline (TBS) for 2 hours. Primary antibody staining with anti-mouse BCRP1 monoclonal antibody (BXP-53, 1:100, Kamiya Biomedical) was performed in TBS with 0.1% Tween 20 (TBST) overnight at 4°C. Secondary antibody staining with anti-rat IgG (HRP-conjugated, 1:1000, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, http://www.scbt.com) was performed for 1 hour at room temperature. Last, chemiluminescence was performed with the SuperSignal Chemiluminescent Substrate (Pierce, Rockford, IL, http://www.piercenet.com).

Reverse Transcription–Polymerase Chain Reaction for *BCRP1* Gene

Total RNA was extracted from the pellets of sorted cells using ISOGEN (Nippon Gene, Tokyo, http://www.nippongene.com) according to the manufacturer's protocol. Reverse transcription–polymerase chain reaction (RT-PCR) was then performed using

an OneStep RT-PCR Kit (Qiagen, Hiden, Germany, http://www.qiagen.com). The primers used for PCR amplification were as follows: BCRP1: 5'-CCA TAG CCA CAG GCC AAA GT-3' and 5'-GGG CCA CAT GAT TCT TCC AC-3', ACTIN: 5'-GCT CGT TGC CAA TAG TGA TG-3' and 5'-AAG AGA GGT ATC CTG ACC CT-3'. Conditions for BCRP1 were 94°C for 1 minute, 56°C for 1 minute, 72°C for 1 minute, 38 cycles. Conditions for ACTIN were 94°C for 1 minute, 55°C for 1 minute, 72°C for 1 minute, 33 cycles. Acquired DNA samples were loaded onto a 2% agarose gel and analyzed.

Immunohistochemical Staining of Mouse Skin with Anti-BCRP1 Antibody

Five micrometers of frozen mouse skin fragments were dried and fixed in 100% ethanol for 10 minutes at 4°C. After washing and blocking, they were incubated with primary anti-mouse BCRP1 monoclonal antibody (BXP-9, 1:100, Kamiya Biomedical), anti–Ki-67 monoclonal antibody (SP6, Lab Vision, Fremont, CA, http://www.labvision.com), or anti-CD31 antibody PE-conjugated (1:100, BD Biosciences) for 1 hour at room temperature. Anti-rat IgG (1:100, HRP-conjugated, Amersham Biosciences, Piscataway, NJ, http://www.amersham.com) enhanced with TSA system Alexa 488–conjugated (Molecular Probes) and anti-rabbit IgG (1:300, Alexa 594–conjugated, Molecular Probes) were used as secondary antibodies. The samples were mounted and observed under a confocal microscope LSM510 (Carl Zeiss, Thornwood, NY, http://www.zeiss.com).

In Situ Hybridization

These methods have been previously described [19]. Briefly, for the synthesis of the probe, a 386-bp BCRP1 cDNA sequence was subcloned into pGEM-T (Promega, Madison, WI, http://www.promega.com). The linearized plasmid was incubated with T7 or SP6 RNA polymerase, transcription buffer, digoxigenein-labeled UTP, and nucleotides at 37°C for 2 hours. For in situ hybridization, rehydrated paraffin-embedded tissue sections were fixed in 4% paraformaldehyde in PBS for 15 minutes and then treated with 7.5 μg/ml proteinase K in PBS at 37°C for 1 hour. They were refixed with 4% paraformaldehyde in PBS and placed in 0.2 M HCl for 10 minutes. After washing, they were acetylated by incubation in 0.1 M triethanolamine-HCl, pH 8.0, for 1 minute and further in 0.1 M triethanolamine-HCl, 0.25% acetic anhydride for 10 minutes. After washing, they were dehydrated through an ethanol series. Hybridization was performed with probes at concentrations of 200–500 ng/ml in a hybridization solution (50% formamide, 5 × standard saline citrate [SSC], 1% SDS, 50 μg/ml tRNA, and 50 μg/ml heparin) at 55°C for 16 hours. After hybridization, they were washed in 5 × SSC at 55°C for 15 minutes and then in 50% formamide, 2 × SSC at 55°C for 15 minutes, followed by RNase treatment in 50 μg/ml RNase A in 10 mM Tris-HCl, pH 8.0, 1 M NaCl, and 1 mM EDTA. Then they were washed twice with 2 × SSC at 50°C for 15 minutes, twice with 0.2 × SSC at 50°C for 15 minutes, and once with TBST (0.1% Tween 20 in TBS) for 5 minutes. After treatment with 0.5% blocking reagent (Roche, Mannheim, Germany, http://www.roche.com) in TBST for 1 hour, they were incubated with anti-DIG AP conjugate (Roche) diluted 1:2000 with TBST for 1 hour, washed twice with TBST containing 2 mM levamisole, and then incubated in 100 mM NaCl, 50 mM $MgCl_2$, 0.1% Tween 20, 100 mM Tris-HCl, pH 9.5, and 2 mM levamisole. Coloring reactions were performed with BM purple substrate (Roche) overnight. The samples were dehydrated and mounted on a slide glass.

Statistics

Aging analyses were performed in at least three independent experiments that yielded highly comparable results. Data are presented as mean values ± SD, as indicated in the Figure 1. Differences between mean values were analyzed with Student's *t*-test, and $p < .05$ was considered statistically significant.

Results

SP Cells Were Detected in Mouse Skin

Flow cytometric analysis of skin cell suspensions revealed that approximately 1% of total cells were found distinctly and reproducibly in the tail of the curve (Fig. 2A), representing a side population analogous to bone marrow SP cells (Fig. 2C). This SP was eliminated by verapamil treatment (Figs. 2B, 2D). Interestingly, the frequency of SP cells was relatively higher in mouse skin (approximately 1.4%) than in bone marrow (approximately 0.1%). Cells isolated from the ear skin suspen-

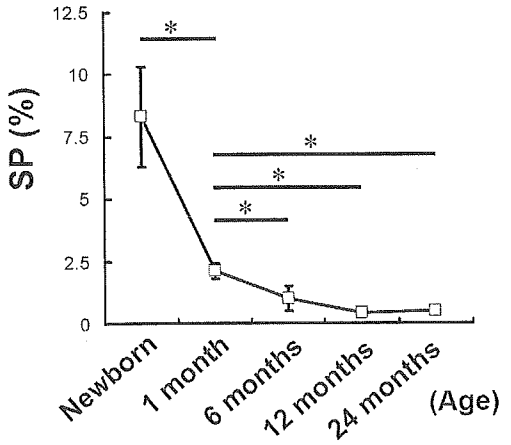

Figure 1. Side population (SP) cells with aging. We examined the correlation with age by examining newborn mice and those that were 1 day, 1 month, 6 months, 12 months, and 24 months old. The frequency of SP cells was found to be age-dependent. Statistical analysis was performed from three sets of independent experiments. Representative results from three sets of independent experiments are shown. *$p < .05$ versus 1-month-old mice.

sion gave similar results (data not shown). Next, we also measured the SP cells from the epidermis and the dermis separately. SP cells were present at high levels in the epidermis (approximately 5.1%, Fig.2E); however, they were rarely found in the dermis (approximately 0.1%, Fig. 2F), suggesting that SP cells are located mainly in the epidermis in vivo. We then compared the ratios of SP cells among various ages of mice. Epidermal cells from newborn mice had a very high ratio of SP cells. The ratio of SP cells significantly tended to decrease in proportion with aging (Fig. 1).

Characterization of the Sorted SP Cells by Several Markers

To investigate whether the SP cells expressed different surface markers than non-SP cells did, epidermal cells were separately sorted as shown in Figure 3A. We then examined putative stem cell markers. We found that SP cells were more strongly stained with α6-integrin, β1-integrin, Sca-1, and keratin 14 than non-SP cells (Figs. 3B–3E). Keratin 19 was weakly stained in SP cells but not in non-SP cells (Fig. 3F). On the other hand, we found that SP cells were very weakly stained by CD34, CD71, and E-cadherin compared with non-SP cells (Figs. 3G–3I). Both SP cells and non-SP cells other than epidermal Langerhans cells were negative for CD3, B220 (Figs. 3J, 3K), and CD45RO (Fig. 3L), suggesting that the preparations were not contaminated by hematopoietic cells.

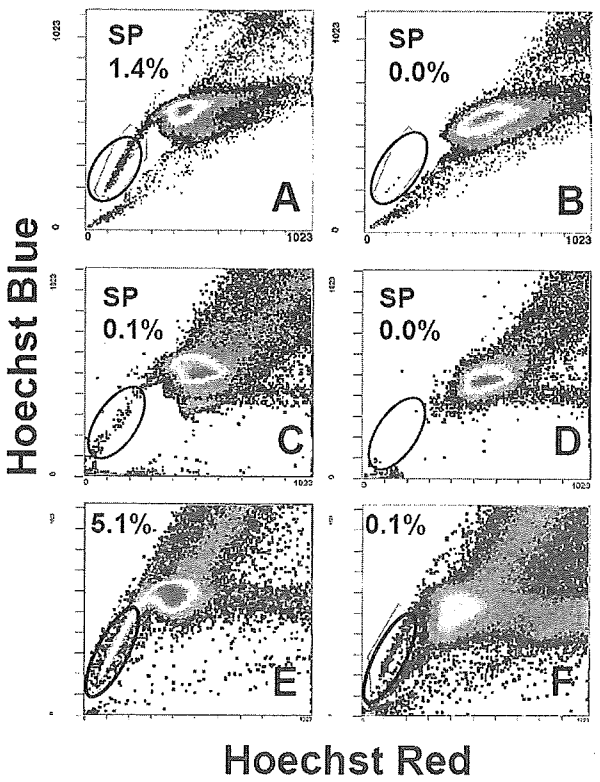

Figure 2. Detection of side population (SP) cells in mouse skin. Hoechst 33342 staining of a mouse skin cell suspension revealed that **(A)** approximately 1% of total skin cells showed the SP pattern of staining behavior, which disappeared with **(B)** verapamil treatment. The gated region suggests SP. The pattern of SP behavior was similar to that of **(C, D)** bone marrow SP cells. The ratio of SP cells in **(E)** newborn epidermis was high; however, the ratio of SP cells in **(F)** newborn dermis was very low.

BCRP1 Was Strongly Expressed in SP Cells at Both the Protein and mRNA Levels

Next we examined BCRP1 expression on skin SP cells, because BCRP1 is a key molecule for the SP cell phenotype [10, 13, 14]. After the sorting of both SP and non-SP cells, we analyzed the cell-surface BCRP1 protein. Flow cytometric analysis revealed that BCRP1 was expressed on the cell membrane of skin SP cells more strongly than on non-SP cells (Fig. 4A). Using an anti-

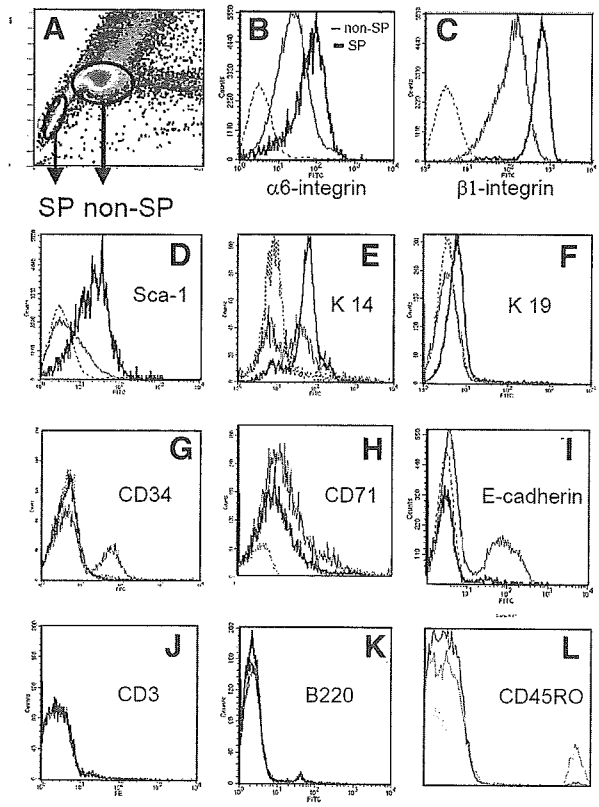

Figure 3. Strong or significant expression of stem cell markers in side population (SP) cells revealed by flow cytometry. SP and non-SP cells were separately sorted (each gate in **A**). We found that the SP cells (thick line) were more strongly stained by **(B)** α6-integrin, **(C)** β1-integrin, **(D)** Sca-1, and **(E)** keratin 14 than non-SP cells (thin line). **(F):** Keratin 19 weakly stained only SP cells; it did not react with non-SP cells. We also found that SP cells were rarely stained by **(G)** CD34, **(H)** CD71, and **(I)** E-cadherin, but non-SP cells were positively stained. Both SP cells and non-SP cells other than epidermal Langerhans cells were negative for **(J)** CD3, **(K)** B220, and **(L)** CD45RO. Isotype controls are shown as broken lines. Representative results from three sets of independent experiments are shown.

BCRP1 monoclonal antibody as a probe during Western blot analysis of sorted cells and tissue lysates, we detected a single protein band with a molecular weight of approximately 72 kDa, the expected size of BCRP1 (Fig. 4B). We found that the SP cells and the epidermis expressed BCRP1; however, non-SP cells and those in the dermis expressed little if any BCRP1. These results suggest that skin SP cells express BCRP1 as do other types of SP cells and that skin SP cells are located mainly in the epidermis, which is consistent with our flow cytometric analysis of epidermal and dermal cell suspensions.

To further analyze *BCRP1* mRNA, RT-PCR was performed. We found that SP cells expressed *BCRP1* strongly but non-SP cells in either adult mouse (Fig. 4C) or newborn mouse (Fig. 4D) showed a weak expression. These results suggest that SP cells strongly express both BCRP1 protein on the cell membrane and *BCRP1* mRNA.

Localization of BCRP1 in Mouse Skin

Based on the correlation between SP cell and BCRP1 expression, we determined where the BCRP1-positive cells existed. Because BCRP1 is also expressed in blood vessels, we performed CD31 double staining to clarify the location of BCRP1 in the skin. The immunohistochemical staining of newborn mouse skin with anti-mouse BCRP1 and CD31 antibodies combined with the usual staining with hematoxylin and eosin revealed that positive staining was found on the cell membrane in almost all of the basal layers and bulge regions of hair follicles in both newborn (Figs. 5A, 5B) and 1-week-old (Figs. 5C, 5D) mice, consistent with another new report of human skin staining [20]. The skin of adult mouse (Figs. 5E, 5F) stained positively for BCRP1 only in the basal layers and hair bulge regions. The staining intensity of adult skin was therefore relatively weaker than that of newborn skin. The dermal papillae and surrounding lower hair follicle cells did not positively stain in either newborn or adult mice.

We further performed in situ hybridization using *BCRP1* mRNA probes. Scattered BCRP1 mRNA expression was detected along the basal layers of the epidermis and hair follicles in newborn mouse skin (Figs. 6A, 6B). No accumulation of positive cells in the bulge regions or dermal papillae was observed. The lung epithelial cells in the newborn mouse were strongly positive, as

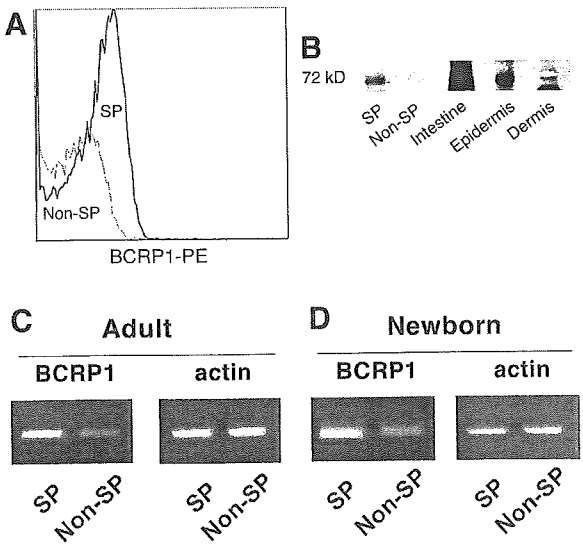

Figure 4. Skin side population (SP) cells strongly express BCRP1 protein and mRNA. **(A):** Flow cytometric analysis revealed that BCRP1 was more strongly expressed on the cell membranes of skin SP cells (thick line) than on non-SP cells (thin line). **(B):** Western blot analysis revealed that SP cells and the epidermis expressed BCRP1 and positive control intestine also expressed BCRP1 strongly; however, non-SP cells and dermis did not. Reverse transcription–polymerase chain reaction analysis of SP cells and non-SP cells for BCRP1 and β-actin (control) was also performed. **(C):** In adult mice skin, SP cells strongly expressed BCRP1 but non-SP cells showed a weak band. **(D):** In newborn mice skin, SP cells expressed BCRP1, but non-SP cells, as in the case of adult skin, showed a weak expression. Representative results from three sets of independent experiments are shown.

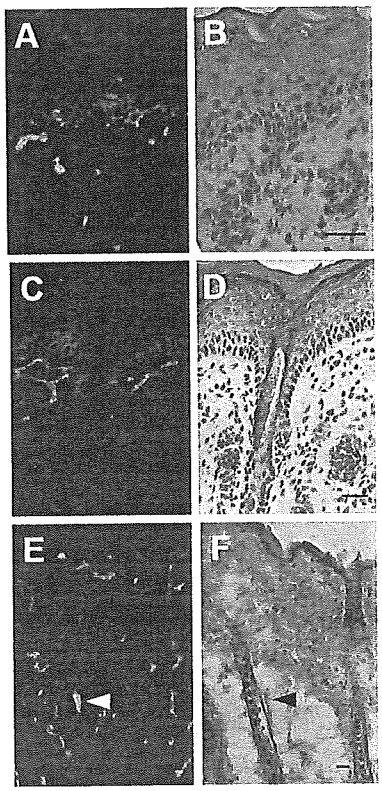

Figure 5. Localization of BCRP1 in mouse skin. Immunohistochemical double-staining with anti-BCRP1 antibody and anti-CD31 antibody revealed that BCRP1-positive staining was found in both the basal layers in **(A, B)** newborn mouse and **(C, D)** 1-week-old mouse skin. **(E, F):** Adult mouse skin also showed BCRP1-positive staining in basal layers and hair bulge regions (arrowheads). Endothelium of blood vessels was also stained by BCRP1 antibody, but it was distinguishable as the reactivity to anti-CD31 antibody (red).

previously reported [21] (Fig. 6C). In adult mouse skin, BCRP1 mRNA expression was scarcely detected (not shown).

Next, we compared the Ki-67 and BCRP1 expression in mouse skin to elucidate the proliferation or self-renewal capacity of the skin SP cells. We found that over half of the BCRP1-positive cells in the basal layer were also positive for Ki-67 (Figs. 7A–7C).

Discussion

SP cells were first discovered in the hematopoietic system by Goodell et al. [9]; they are now considered primitive stem cells. Since then, SP cells have been found in several tissues and experimental cell lines, including bone marrow, muscle, lung, mammary epithelium, embryonic stem cells, neurosphere cells, and the A549 cancer cell line [9, 10, 21–25]. SP cells are also present in epidermal skin suspensions [15–18]. The SP phenotype identifies a subset of stem cells. SP cells from bone marrow have been shown to be able to rescue lethally irradiated mice [9] and to differentiate into both lymphoid and myeloid lineages [9]. SP cells from bone marrow and skeletal muscles express Sca-1 and c-kit [22, 26], and SP cells from mammary epithelium express α6-integrin [23].

We found that the percentage of skin SP cells was relatively higher than that from other organs such as bone marrow, which was reported to be approximately 0.05%–0.1% (Figs. 2A–2D). The probable reason for the high ratio is the fact that the epidermis is a functional barrier organ that can strongly efflux foreign bodies, toxic substances, and dyes, including Hoechst dye. Previous studies have reported that the ratio of skin SP cells is approximately 1.1% in the mouse (varying by the concentration of Hoechst) [17, 18] and 0.01%–5.4% in the human [15]. We are the first to show that newborn skin has a very high ratio of SP cells and that the percentage of skin SP cells decreases in proportion with aging (Fig. 1). Other reports have found no relationship between SP and aging [15, 17]. These discrepancies can be explained by the differences of observation periods; ours extended from newborn to 24-month-old mice, which is much longer than others. Considering the fact that embryo and newborn skin cells can reconstitute hair on nude mice but adult cells cannot [27], it is not surprising that newborn epidermal cells are highly plastic. Our data also clarified that the dermis is composed of only 0.1% SP cells, which is similar to bone marrow (Figs. 2C, 2F). Therefore, epidermal SP cells have a stronger dye-effluxing potential than dermal and bone marrow cells.

Many markers for epidermal stem cells have been investigated. We demonstrated that α6-integrin [6], β1-integrin [4, 5], Sca-1 [28], keratin 14 [29], and keratin 19 [7] were highly expressed in SP cells and that CD34 [8], CD71 [6], and E-cadherin [30] were more weakly expressed in SP cells than in non-SP cells (Fig. 3). These results indicate that SP cells have several stem cell markers and are not differentiated cells. Our data for Sca-1 were compatible with previous reports [15, 17, 18]. However, CD34 expression on epidermal stem cells is controversial. CD34 is reported by several authors to be highly expressed in the bulge region [8, 31, 32], but another study reported that epidermal label-retaining cells do not express CD34 [33]. Our results showed that SP cells rarely expressed CD34 but that non-SP cells did express CD34, in agreement with the report that BCRP1 did not appear to be enriched in CD34-high bulge stem cells [32]. However, Montanaro et al. [17] claimed that CD34 is expressed in SP cells (91%) more frequently than in non-SP cells (73%). It has been suggested that these discrepancies could be due to the fact that SP- or BCRP1-positive cells are located not only in the bulge region but also in the basal layer, but it is certain that further examinations are needed.

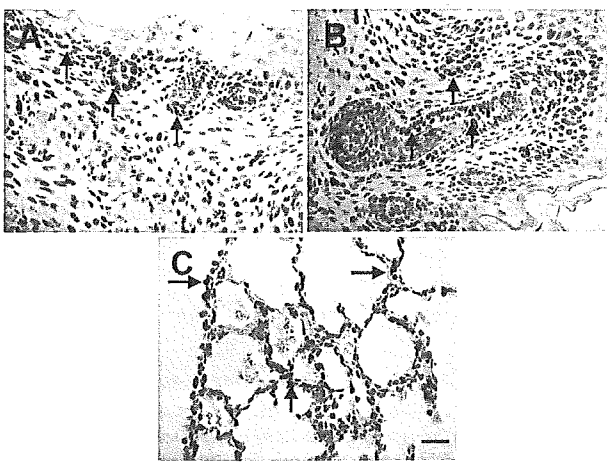

Figure 6. In situ analysis of *BCRP1* mRNA. **(A, B):** In situ hybridization revealed that *BCRP1* mRNA expression (arrows) was detected in parts of the basal layers and hair follicles in newborn mouse skin. **(C):** Lung epithelial cells were used as positive controls (arrows). Scale bar = 100 μm.

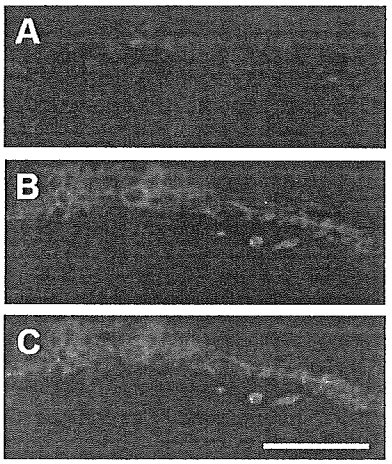

Figure 7. BCRP1-positive cells express Ki-67. Furthermore, over half of the BCRP1-positive cells in the basal layer were positive for Ki-67, which presented in proliferated cells. **(A):** Anti-Ki-67 antibody-Alexa 594; **(B):** anti-BCRP1 antibody-Alexa 488; and **(C)** a merge image of A and B. Scale bar = 100 μm.

BCRP1 is a novel stem cell transporter. *BCRP1* mRNA is expressed at high levels in primitive hematopoietic stem cells and is sharply downregulated with differentiation [10]. Our flow cytometric analysis, Western blotting, and RT-PCR analysis revealed that there is strong expression of BCRP1 in SP cells and epidermis but not in non-SP cells and only weakly in the dermis (Fig. 4B). We initially pinpointed the location of the BCRP1-positive cells in the mouse skin. Immunohistochemical staining and in situ hybridization of BCRP1 revealed that BCRP1-positive cells are located mainly in the epidermal basal layers and hair bulge regions of both newborn and adult mice (Fig. 5). However, BCRP1 expression in the dermal papilla and lower hair follicle was not detected in either newborn skin or in adult skin. The frequency of BCRP1-positive cells in the skin was similar to that of SP cells in skin suspension. These results confirm that the presence of BCRP1 is closely correlated with that of SP cells and that BCRP1 can therefore be used as a marker for primitive quiescent stem cells.

Although it has been suggested that SP cells protect the integrity of stem cells and can contribute to all cell lineages, not all SP cells function as primitive stem cells. To use hematopoietic stem cells, both SP and c-kit$^+$, Sca-1$^+$, CD34$^-$ cells are used for the regeneration of ischemic cardiac muscle [34]. SP cells that have a subset of c-kit$^+$, Thy-1$^+$, Sca-1$^+$, and linage$^-$ represent long-term repopulating cells [24]. There are controversies about the stem cell properties of skin SP cells. Although it has been claimed that adult skin SP cells can engraft in dystrophic skeletal muscle [17], another report showed that overexpression of BCRP1 compromises the growth potential of the cells [15] and that SP cells are not label-retaining cells [16]. It has also been reported that SP cells express Sca-1; however, the bulge region does not express Sca-1 [15], and BCRP1 does not seem to be enriched in CD34-high bulge stem cells [32]. Considering these facts, being SP- or BCRP1-positive is not considered completely indispensable criteria for stem cells, but they are among the characteristics of the stem cells. Therefore, it is reasonable to combine SP cell screening with that for other surface markers to isolate pure epidermal stem cells. We demonstrated the proliferative character and self-renewal capacity of SP cells using Ki-67 and BCRP1 costaining (Fig. 7). To prove their abilities, we need to develop a suitable in vitro culture system for epidermal stem cells in the near future.

In conclusion, we have demonstrated that SP cells can be isolated from mouse skin and that they display several epidermal stem cell markers. These SP cells are abundant in newborn skin, have strong dye-effluxing potential, and significantly decrease in proportion with aging. The location of these SP cells in the skin was elucidated in both newborn and adult mice. Our observations open doors for future research in stem cell biology, skin carcinogenesis, wound healing, and hair and skin regeneration. Using the plasticity of skin SP cells as a source of stem cells for other organ regeneration is both promising and attractive. We are next going to transplant skin SP cells into lethally irradiated mice and determine whether the mice are rescued and the SP cells can differentiate into other cell types.

Acknowledgments

This work was supported by a Health Science Research Grant from the Japanese Ministry of Health, Labor, and Welfare. The authors thank Dr. Miho Mizukami, Yasuhiko Nagasaka, and Eri Watanabe for technical advice.

References

1 Cotsarelis G, Sun TT, Lavker RM. Label retaining cells reside in the bulge area of pilosebaceous unit: implications for follicular stem cells, hair cycle and skin carcinogenesis. Cell 1990;61:1329–1337.

2 Bickenbach JR, Chism E. Selection and extended growth of murine epidermal stem cells in culture. Exp Cell Res 1998;244:184–195.

3 Rochat A, Kobayashi K, Barrandon Y. Location of stem cells of human hair follicles by clonal analysis. Cell 1994;76:1063–1073.

4 Jones PH, Watt FM. Separation of human epidermal stem cells from transit amplifying cells on the basis of differences in integrin function and expression. Cell 1993;73:713–724.

5 Jones PH, Harper S, Watt FM. Stem cell patterning and fate in human epidermis. Cell 1995;80:83–93.

6 Tani H, Morris RJ, Kaur P. Enrichment for murine keratinocyte stem cells based on cell surface phenotype. Proc Natl Acad Sci U S A 2000;97:10960–10965.

7 Michel M, Torok N, Godbout MJ et al. Keratin 19 as a biochemical marker of skin stem cells in vivo and in vitro: keratin 19 expressing cells are differentially localized in function of anatomic sites, and their number varies with donor age and culture stage. J Cell Sci 1996;109:1017–1028.

8 Trempus CS, Morris RJ, Bortner CD et al. Enrichment for living murine keratinocytes from the hair follicle bulge with the cell surface marker CD34. J Invest Dermatol 2003;120:501–511.

9 Goodell MA, Brose K, Paradis G et al. Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo. J Exp Med 1996;183:1797–1806.

10 Zhou S, Schuetz JD, Bunting KD et al. The ABC transporter Bcrp1/ABCG2 is expressed in a wide variety of stem cells and is a molecular determinant of the side-population phenotype. Nat Med 2001;7:1028–1034.

11 Kohno K, Sato S, Takano H et al. The direct activation of human multidrug resistance gene (MDR1) by anticancer agents. Biochem Biophys Res Commun 1989;165:1415–1421.

12 Ferguson PJ, Cheng YC. Phenotypic instability of drug sensitivity in a human colon carcinoma cell line. Cancer Res 1989;49:1148–1153.

13 Kim M, Turnquist H, Jackson J et al. The multidrug resistance transporter ABCG2 (breast cancer resistance protein 1) effluxes Hoechst 33342 and is overexpressed in hematopoietic stem cells. Clin Cancer Res 2002;8:22–28.

14 Zhou S, Morris JJ, Barnes Y et al. Bcrp1 gene expression is required for

normal numbers of side population stem cells in mice, and confers relative protection to mitoxantrone in hematopoietic cells in vivo. Proc Natl Acad Sci U S A 2002;99:12339–12344.

15 Triel C, Vestergaard ME, Bolund L et al. Side population cells in human and mouse epidermis lack stem cell characteristics. Exp Cell Res 2004;295:79–90.

16 Terunuma A, Jackson KL, Kapoor V et al. Side population keratinocytes resembling bone marrow side population stem cells are distinct from label-retaining keratinocyte stem cells. J Invest Dermatol 2003;121:1095–1103.

17 Montanaro F, Liadaki K, Volinski J et al. Skeletal muscle engraftment potential of adult mouse skin side population cells. Proc Natl Acad Sci U S A 2003;100:9336–9341.

18 Montanaro F, Liadaki K, Schienda J et al. Demystifying SP cell purification: viability, yield, and phenotype are defined by isolation parameters. Exp Cell Res 2004;298:144–154.

19 Hoshino M, Sone M, Fukata M et al. Identification of the stef gene that encodes a novel guanine nucleotide exchange factor specific for Racl. J Biol Chem 1999;274:17837–17844.

20 Paiva CS, Chen Z, Corrales RM et al. ABCG2 transporter identifies a population of clonogenic human limbal epithelial cells. STEM CELLS 2005;23:63–73.

21 Summer R, Kotton DN, Sun X et al. Side population cells and Bcrp1 expression in lung. Am J Physiol Lung Cell Mol Physiol 2003;285:L97–104.

22 Jackson KA, Mi T, Goodell MA. Hematopoietic potential of stem cells isolated from murine skeletal muscle. Proc Natl Acad Sci U S A 1999;96:14482–14486.

23 Alvi AJ, Clayton H, Joshi C et al. Functional and molecular characterisation of mammary side population cells. Breast Cancer Res 2003;5:R1–R8.

24 Hulspas R, Quesenberry PJ. Characterization of neurosphere cell phenotypes by flow cytometry. Cytometry 2000;40:245–250.

25 Scharenberg CW, Harkey MA, Torok-Storb B. The ABCG2 transporter is an efficient Hoechst 33342 efflux pump and is preferentially expressed by immature human hematopoietic progenitors. Blood 2002;99:507–512.

26 Pearce DJ, Ridler CM, Simpson C et al. Multiparameter analysis of murine bone marrow side population cells. Blood 2004;103:2541–2546.

27 Itoh M, Hiraoka Y, Kataoka K et al. Novel collagen sponge reinforced with polyglycolic acid fiber produces robust, normal hair in murine hair reconstitution model. Tissue Eng 2004;10:818–824.

28 Welm BE, Tepera SB, Venezia T et al. Sca-1(pos) cells in the mouse mammary gland represent an enriched progenitor cell population. Dev Biol 2002;245:42–56.

29 Purkis PE, Steel JB, Mackenzie IC et al. Antibody markers of basal cells in complex epithelia. J Cell Sci 1990;97:39–50.

30 Behrens J, Vakaet L, Friis R et al. Loss of epithelial differentiation and gain of invasiveness correlates with tyrosine phosphorylation of the E-cadherin/beta-catenin complex in cells transformed with a temperature-sensitive v-SRC gene. J Cell Biol 1993;120:757–766.

31 Tumbar T, Guasch G, Greco V et al. Defining the epithelial stem cell niche in skin. Science 2004;303:359–363.

32 Blanpain C, Lowry WE, Geoghegan A et al. Self-renewal, multipotency, and the existence of two cell populations within an epithelial stem cell niche. Cell 2004;118:635–648.

33 Albert MR, Foster RA, Vogel JC. Murine epidermal label-retaining cells isolated by flow cytometry do not express the stem cell markers CD34, Sca-1, or Flk-1. J Invest Dermatol 2001;117:943–948.

34 Jackson KA, Majka SM, Wang H et al. Regeneration of ischemic cardiac muscle and vascular endothelium by adult stem cells. J Clin Invest 2001;107:1395–1402.

DOI 10.1111/j.1365-2133.2005.06832.x

Long-term culture of adult murine epidermal keratinocytes

S. Yano and H. Okochi

Department of Regenerative Medicine, Research Institute, International Medical Centre of Japan, 1-21-1 Toyama, Shinjuku-ku, Tokyo 162-8655, Japan

Correspondence
Shoichiro Yano.
E-mail: yano-der@h.u-tokyo.ac.jp

Accepted for publication
1 April 2005

Key words:
cholera toxin, epidermal growth factor, keratinocytes, mouse

Conflicts of interest:
None declared.

Summary

Background Long-term cultures of epidermal cells from mouse skin have been notoriously difficult to establish.

Objectives To develop a modified serum-free medium and technique for long-term culture of adult mouse epidermal keratinocytes.

Methods Epidermal cells from trypsin-treated adult mouse dorsal and ventral skin were grown on type I collagen-coated dishes without feeder layers in a serum-free medium supplemented with only 10 ng mL^{-1} epidermal growth factor (EGF) and 10^{-10} mol L^{-1} cholera toxin (CT).

Results After removing coexisting fibroblasts several times, we were able to obtain almost pure basal epidermal keratinocytes. Our technique supports sustained multiplication of mouse basal keratinocytes for more than 100 population doublings, and they retained the capacity to undergo terminal differentiation when given the appropriate stimulus. The epithelial nature of these cultivated cells was demonstrated both by phase-contrast microscopy and by immunostaining with anti-keratin antibodies. EGF and CT, which have been reported to accelerate the cellular growth rate, were essential for successful long-term cultivation during multiple passages.

Conclusions Our technique is very simple. It provides a useful and suitable model for investigations of growth, differentiation and skin remodelling in vitro.

Long-term cultures of epidermal cells from mouse skin have been notoriously difficult to establish.[1] Hager *et al.*[2] reported the establishment of long-term newborn C57/BL6 mouse epidermal keratinocyte cultures using a highly supplemented, fibroblast-conditioned medium (FCM) and fetal bovine serum (FBS). Caldelari *et al.*[3] reported the establishment of long-term neonatal mouse epidermal keratinocyte cultures isolated from the skin of 17·5-day-old embryos of C57/BL6 mice.

Primary cultures and subculturing from the skin of adult mice was not mentioned in either paper. Long-term primary cultures of epidermal cells from normal, untreated adult mice have been difficult to establish and to maintain except under conditions in which the concentration of calcium in the medium has been reduced.[4,5] As for subcultures of adult mouse keratinocytes, only Bickenbach and Chism[6] have reported that mouse ear keratinocytes could be expanded and subcultured three times using FCM and FBS. However, we have not been able to find any reports of successful establishment of long-term adult mouse keratinocyte subcultures from dorsal or ventral skin without using FBS, feeder layers, and FCM up to the present.

In this paper, we report that we have succeeded in newly establishing long-term adult mouse keratinocyte subcultures without using FBS, feeder layers, FCM or bovine pituitary extract.

Materials and methods

Primary cell culture

We attempted to culture and subculture keratinocytes from dorsal skin and ventral skin of one mouse. First, both dorsal skin and ventral skin of an 8-week-old C57/BL6 mouse (Clea Japan, Tokyo, Japan) were removed with a scalpel and washed with ice-cold phosphate-buffered saline (PBS) three times after carefully shaving off all the body hair. Then the tissues were incubated for 2 h in 0·25% trypsin/0·1% ethylenediamine tetraacetic acid (EDTA) (Gibco, Grand Island, NY, U.S.A.) at 37 °C. The superficial skin area involving the full epidermis and part of the dermis was scraped carefully with a cell scraper. The cell suspension was neutralized by PBS with 10% FBS and filtered through a 40-μm cell strainer twice to take off the remaining body hairs and tissue debris. Cells were then centrifuged at 60 *g* for 10 min at 4 °C, washed, and resuspended in defined keratinocyte serum-free medium (Gibco) supplemented with 10 ng mL^{-1} epidermal growth factor (EGF) (R&D Systems, Minneapolis, MN, U.S.A.), 10^{-10} mol L^{-1} cholera toxin (CT) (Calbiochem, San Diego, CA, U.S.A.) and 1 × antibiotics/antimycotics (Gibco), in an incubator at 5% CO_2 and 37 °C. Cells were seeded at a density of 1×10^5 cells cm^{-2} on to a type I collagen-coated flask (Iwaki, Tokyo, Japan)

