Journal of
APPLIED
BIOMEDICINE

J Appl Biomed. **10**: 9–17, 2012
DOI 10.2478/v10136-011-0005-4
ISSN 1214-0287

ORIGINAL ARTICLE

Influence of circulating fibrocytes on the growth, proliferation and migration of keratinocytes and fibroblasts

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Received 3rd March 2011.
Revised 12th October 2011.
Published online 12th October 2011.

Summary
Circulating fibrocytes (CFs) exhibit an extraordinary degree of plasticity and growth factor repertoire, and because of this they have been investigated for their role in the repair and regeneration of damaged tissues, but yet not adequately for their role in wound healing. In the present study, CFs were co-cultured with keratinocytes (KCs) or fibroblasts (Fbs) and the influences of CFs on the growth, proliferation and migration of KCs and Fbs were investigated. Our results showed that the CFs in the co-culture system could inhibit the growth, proliferation and migration of KCs, while CFs promoted the growth and proliferation of Fbs. Our study demonstrates that CFs can regulate the functions of Fbs, which may be a possible cause of fibrosis.

Key words: circulating fibrocytes; cell interaction; keratinocytes; fibroblasts; wound healing

* *These authors contribute equally to the manuscript.*

Abbreviations: CFs: circulating fibrocytes; Fbs: fibroblasts; KCs: keratinocytes

INTRODUCTION

Circulating fibrocytes (CFs) are bone marrow-derived mesenchymal progenitors (Mori et al. 2005, Kisseleva et al. 2006) that can co-express haematopoietic stem cell antigens and markers of the monocyte lineage and fibroblasts (Bucala et al. 1994, Yang et al. 2002, Schmidt et al. 2003, Mori et al. 2005). Studies have shown that CFs are involved in several aspects of wound healing, including inflammation, the production of an extracellular matrix, and angiogenesis (Bucala et al. 1994, Chesney et al. 1997, 1998, Hartlapp et al. 2001). Previous investigations have also provided evidence that CFs participate in fibrotic disorders (Chesney and Bucala 2000, Cowper 2003, Moore et al. 2005, Kisseleva and Brenner 2008). It has been confirmed that the capacity of fibrocytes from healthy subjects or from burn patients to produce collagens is inferior to that of dermal fibroblasts, and that CFs can regulate the activities of local fibroblasts through secreting the transforming

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☎ 86-29-8767-9969
86-29-8740-9503

growth factor beta 1 (TGF-β1) or the connective tissue growth factor (CTGF) in the healing of burn wounds (Wang et al. 2007). Therefore, CFs may play an indirect, regulatory role in wound healing by affecting the functions of dermal fibroblasts.

Nevertheless, the increase of CFs in the hypertrophic scar (Yang et al. 2002, 2005) indicates that CFs contribute to wound repair by regulating not only the fibroblasts' activities, but their differentiation and proliferation. In the proliferative phase, the migration and proliferation of keratinocytes (KCs), fibroblasts (Fbs) and endothelial cells can lead to re-epithelialization and tissue granulation. At the same time, CFs can interact with local KCs and Fbs. We therefore postulate that CFs can impact the proliferation and migration of local cells through direct cell-cell interaction except for the paracrine.

In the present study, the CFs were isolated and co-cultured with KCs or dermal Fbs, and the influences of CFs on the growth, proliferation, migration and cell cycle of KCs and Fbs were investigated. Our results showed that CFs in the co-culture system compromised the functions of KCs but promoted those of Fbs. Therefore, we conclude that CFs play an important role in fibrosis during wound healing.

MATERIALS AND METHODS

Cell culture

This study was approved by the Ethical Committee of the Fourth Military Medical University. CFs were isolated by the leukopheresis of healthy human donors. Briefly, the sample was diluted with phosphate-buffered saline (PBS) at the volume ratio of 1:1 in heparinized tubes. Then, 7 ml of this solution was transferred into a tube containing 7 ml of Percoll-Paque (1.073 g/ml; GE Healthcare, UK), and the nucleated cells were isolated by density gradient centrifugation at 2,100 rpm for 20 min. The nucleated cells were seeded in T25 plastic dishes at a density of 1×10^5 cells/cm^3 and followed by incubation in a low-glucose Dulbecco's modified Eagle's medium (DMEM) containing 7% foetal calf serum (FCS; Gibco, USA), 2 mM-glutamine, 100 U/ml penicillin, 100 mg/ml of streptomycin at 37 °C in a humidified atmosphere with 5% CO_2. Three days later, non-adherent cells were removed by refreshing the medium every 3 days. After 10–14 days of culture, the proportion of Fbs with spindle-shaped morphology was analysed (greater than 75%). When the cells reached 100% confluence, the adherent cells were harvested by incubation in 0.05% (w/v) EDTA/PBS for 5 min at room temperature, and then were gently aspirated and collected for the following experiments.

Proliferation of CFs

At same condition of cell culture, CFs were inoculated into 24 well plates at 4×10^3/cm^3, and every eight wells were regarded as a individual induction group. One of these groups was treated with 50 ng/ml macrophage colony-stimulating growth factor (M-CSF, Sigma, USA) and 50 ng/ml recombinant human thrombopoietin (TPO, Sigma, USA) for another. An individual group was leaved untreated. Cell counting for each well of different groups was performed by inspection under a light microscopy every 24 h, lasted for 8 days, and cell growth curves were drawn. The results are the mean of cell counts from individuals.

Phenotype of CFs

Because of the difficulties in passaging, CFs in the early and late phase (3 and 7 days of primary culture) were stained with the following mouse anti-human antibodies (mAbs) conjugated with fluorescein isothiocyanate (FITC), phycoerythrin (PE) or peridinin-chlorophyll-protein complex (PerCP): CD11b, CD14, CD34, CD44, CD45 and CD90. Cells in the negative controls were incubated with isotype-matched mouse anti-human mAb to an irrelevant antigen. The cells were analysed in an FACS Calibur flow cytometer (BD Biosciences, USA) with CellQuest software.

Osteogenic and adipogenic induction

CFs were seeded in a 6-well plate at a density of 5×10^5 cells/well in basic medium for 24 h to allow adherence. Then, the medium was replaced with osteogenic induction medium containing DMEM, 10% FCS, 0.1 μM dexamethasone, 10 mM β-glycerolphosphate and 50 mg/l ascorbate-2-phosphate, and adipogenic induction medium consisting DMEM, 10% FCS, 0.25 μM dexamethason, 100 μM indomethacin, 0.5 mM 3-isobutyl methylxanthine and 10 mg/l insulin. Cells were maintained for 2 weeks and media were replaced twice weekly. The mineralized nodules were stained with Alizarin Red S and neutral lipid vacuoles with Oil-red O (Sigma, USA).

Analysis of proliferation, migration and cell cycle

A CFs feeder layer was obtained after 10 to 14 days of culture in a 24-well plate and then irradiated at 1400 cGy. The prepared feeder layer was maintained in a humidified atmosphere with 5% CO_2 at 37 °C and used for the subsequent experiments within 5 days after irradiation. The irradiation of CFs layers at 1400

cGy prevents the overgrowth of the feeder while maintaining optimal viability and growth factor production. Four groups were included: 1) KCs alone; 2) KCs+CFs; 3) Fbs alone; 4) Fbs+CFs.

To determine the effects of CFs on the proliferation of KCs and Fbs, KCs and Fbs were seeded on the CFs feeder layer (2×10^5 cells/well) for 24 h in a conditioned medium (DMEM containing 10% fetal bovine serum, Fbs or serum-free medium for the culture of human keratinocytes, K-SFM). When the cell confluence reached 80%, cells were trypsinized for detection of proliferation by MTT assay which was performed from day 1 to day 7. The culture medium was then removed, cells were washed with PBS and a serum-free DMEM was added. About 100 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) solution (5 μg/ml) were added to each well followed by incubation for 2 h in a CO_2 incubator at 37 °C. Then, 1 ml of 20% SDS/DMF solution was added to each well followed by shaking for 15 min on an orbital shaker. These cells were transferred to cuvettes and the optical density was measured at 570 nm. For the BrdU assay, 50 μM 5-bromo-2'-deoxyuridine, BrdU (Sigma, USA) was added.

The migration of co-cultured KCs and Fbs was determined by scratching assay. A representative area of KCs and Fbs at 12 h after scratching was presented. KCs, Fbs and co-cultured KCs+CFs and Fbs+CFs were trypsinized and then fixed in 70% alcohol followed by analysis of cell cycle by flow cytometry (Beckman Coulter, USA).

Statistical analysis

Statistical analysis was carried out with SPSS version 13.0 statistic software package (Cary, USA). We used two sided t-test at the significance level $2\alpha=0.05$.

RESULTS

CFs has a spindle-shaped morphology, grows in the presence of anti-macrophage colony stimulating factor (M-CSF) and can transform into macrophage-like cells

The adherent cells exhibited a spindle-shaped morphology, and some transformed into macrophage-like cells during the culture (Fig. 1a, b). In the presence of M-CSF and TPO (50 ng/ml), CFs could maintain the spindle-shaped morphology and proliferate for a long time (Fig. 1c). CFs were difficult to proliferate and passage under normal conditions, and macrophage-like cells could spread in the disk for more than one month supporting the absence of M-CSF (Fig. 1d). The cell count was significantly increased after 6 days of incubation with M-CSF and TPO at a concentration of 50 ng/ml (Fig. 1e).

Phenotypes of CFs

The CFs displayed CD11b/CD14low/CD34/CD45high/CD44high/CD90 (Fig. 2a), and showed enhanced expressions of CD34 (from 2.99% to 21.06%) and CD45 (from 96.8% to 98.92%) in the early and late phase of primary culture (Fig. 2b). The expression of isotype-control was about 1%. These results indicate the maturation of CFs, and the high expression of CD45 confirms its haematopoietic origin.

Multipotency of CFs

Maintained in the specific induction medium, the CFs possessed osteogenic and adipogenic potentials and were positive for Alizarin Red S and Oil red-O (Fig. 3a, b).

CFs inhibit the proliferation and migration of KCs in vitro

In the scratching assay, cells migrated toward the center of the scratch and only a small amount of cell were confluent after 12 h of co-culture of CFs and KCs, which was much less than that after culture of the KCs alone (Fig. 4a). By MTT assay, the growth and proliferation of KCs were significantly inhibited in the co-culture system of CFs and KCs when compared with those after culture of KCs alone (statistically significant) (Fig. 4b). A smilar result was achieved by BrdU assay (statistically significant) (Fig. 4c). Moreover, the proportion of cells in the G2+S phase and S phase was 13.4 and 13.4 respectively in the co-culture system of CFs and KCs, and 20.6 and 20 respectively in the culture of KCs alone (Fig. 4d), which indicates the decrease of DNA synthesis. These results suggest that CFs co-cultured with KCs can suppress the growth, proliferation and migration of KCs.

CFs promote the proliferation and migration of Fbs in vitro

In contrast to the effects of CFs on KCs in the scratching assay, cells migrated toward the center of the scratch and a large number of cells were confluent after 12 h of co-culture of the CFs and Fbs, which was much more than that in the culture of Fbs alone (Fig. 5a). The MTT assay showed that the growth and proliferation of KCs were significantly promoted after co-culture with CFs when compared with those in the culture of Fbs alone (statistically significant) (Fig. 5b). A similar result was achieved by BrdU

assay (Fig. 5c). Moreover, the proportion of cells in the G2+S phase and S phase was 51 and 39, respectively in the co-culture system of CFs and Fbs, and 31 and 20.5 in culture of Fbs alone (Fig. 5d). These findings suggest that CFs co-cultured with Fbs can promote the growth, proliferation and migration of Fbs.

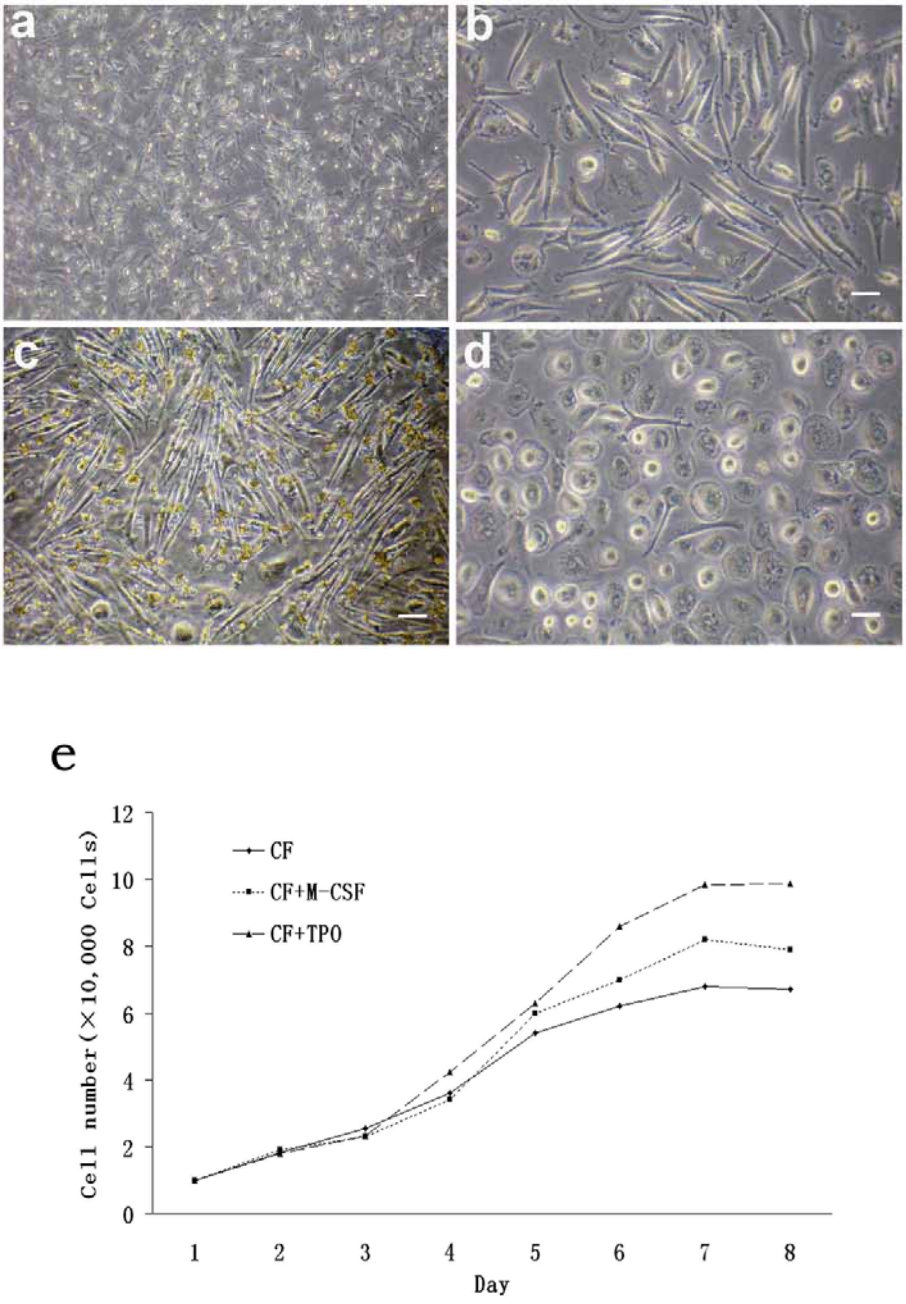

Fig. 1. **CFs exhibits a spindle-shaped morphology, grows in the presence of M-CSF and transforms into macrophage-like cells.** Adherent cells exhibit a spindle-shaped morphology, and some transformed to macrophage-like cells during the culture (*a*, *b*). In the presence of M-CSF (50 ng/ml), CFs could maintain the spindle-shaped morphology and proliferate for a long time (*c*). CFs were difficult to proliferate and passage under normal condition, and macrophage-like cells could spread in the disk for more than one month supporting the absence of M-CSF (*d*). The cell count was significantly increased after 6 days of incubation with M-CSF and TPO at a concentration of 50 ng/ml (*e*).

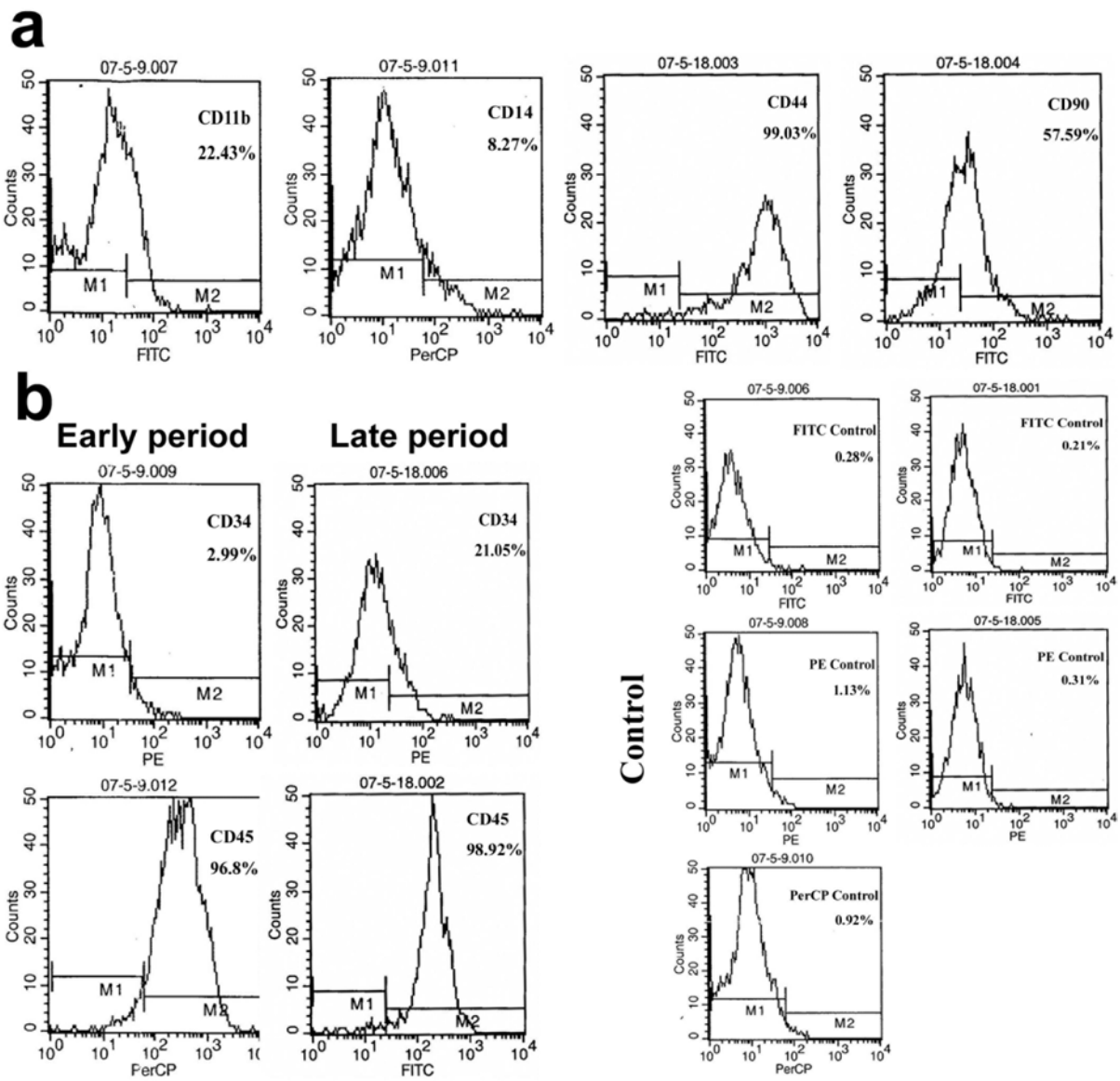

Fig. 2. **CFs displayed CD11b/CD14low/CD34/CD45high/CD44high/CD90** (*a*), and showed an enhanced expression of CD34 (from 2.99% to 21.06%) and CD45 (from 96.8% to 98.92%) in the early and late phase of primary culture (*b*). The expression of isotype-control was about 1%.

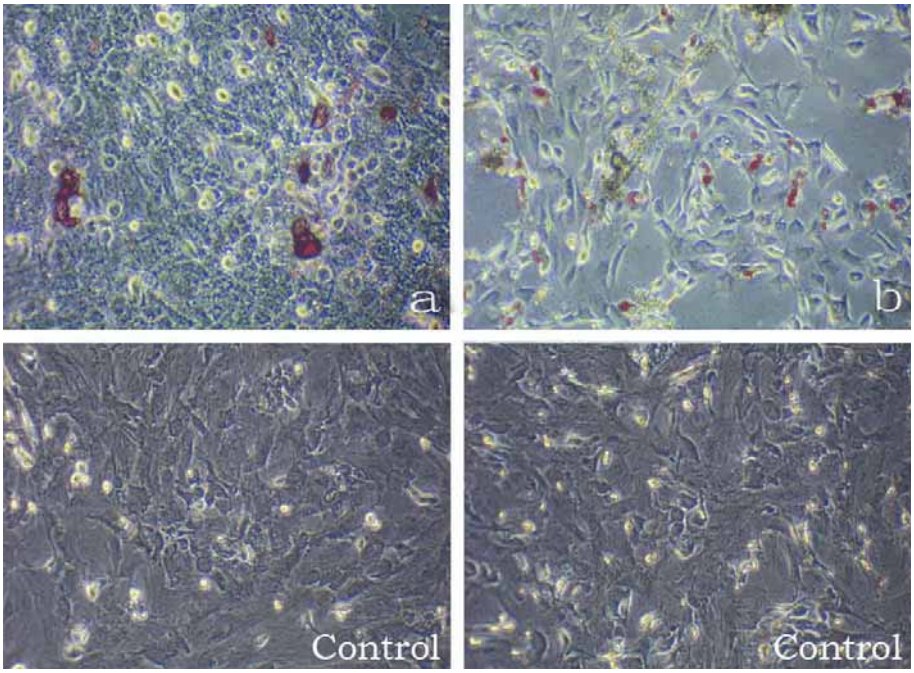

Fig. 3. **CFs maintained in the specific induction media**, CFs possessed osteogenic and adipogenic potentials and were positive for Alizarin Red S and Oil red-O stainings, respectively (*a, b*).

DISCUSSION

The present study aimed to determine the influences of CFs on the cell functions of KCs and Fbs in the co-culture system. Our results demonstrated that CFs could promote the growth, proliferation and migration of Fbs *in vitro* in the co-culture system. In humans, CFs can be found in tumours, skin wounds, hypertrophic scars, bronchial asthma, pulmonary fibrosis, and nephrogenic fibrosing dermopathy (Schmidt et al. 2003, Quan et al. 2004, Mori et al. 2005, Yang et al. 2005, Mehrad et al. 2007). In animal models, CFs are associated with experimental fibrosis of lung, kidney and liver, carotid artery intimal hyperplasia, chronic granulomatous disease, and skin wounding (Direkze et al. 2003, Epperly et al. 2003, Hashimoto et al. 2004, Phillips et al. 2004, Quan et al. 2004, Kisseleva et al. 2006, Moore et al. 2006, Sakai et al. 2006, Varcoe et al. 2006). Therefore, CFs may play an important role in fibrosis in numerous forms.

Although the specific impact of CFs on healing is unclear, there are at least two ways in which CFs exert their effects. One is that CFs can differentiate into fibroblasts secreting an essential extracellular matrix at the injury site. Fibrocytes cultured *ex vivo* can express numerous extracellular matrix molecules, including vimentin, fibronectin, collagen I and collagen III, and fibrocytes in the wounds has the ability to express collagen, suggesting their roles in wound repair (Bucala et al. 1994, Chesney et al. 1998, Yang et al. 2005). The other is that CFs can regulate the biological behavior of fibroblasts via paracrine. Wang et al. (2007) demonstrated that CFs could enhance the activities of local fibroblasts through producing growth factors. In both ways, CFs may result in severe fibrosis, which has been demonstrated by the findings that CFs have been found in the areas with connective tissue matrix deposition in fibrotic liver, lung and kidney (Chesney et al. 1998, Chesney and Bucala 2000, Hashimoto et al. 2004, Phillips et al. 2004, Kisseleva et al. 2006, Sakai et al. 2006, Bellini and Mattoli 2007, Mehrad et al. 2007).

In addition, our results also revealed that CFs could inhibit the growth, proliferation and migration of KCs. Few studies report the influence of CFs on KCs and *in vivo* transplantation of CFs or KCs showed enhanced re-epithelialization, which implies the mechanism of epithelial regeneration is more complex than that of fibrosis. It is well known that the migration and proliferation of epithelial cells can be

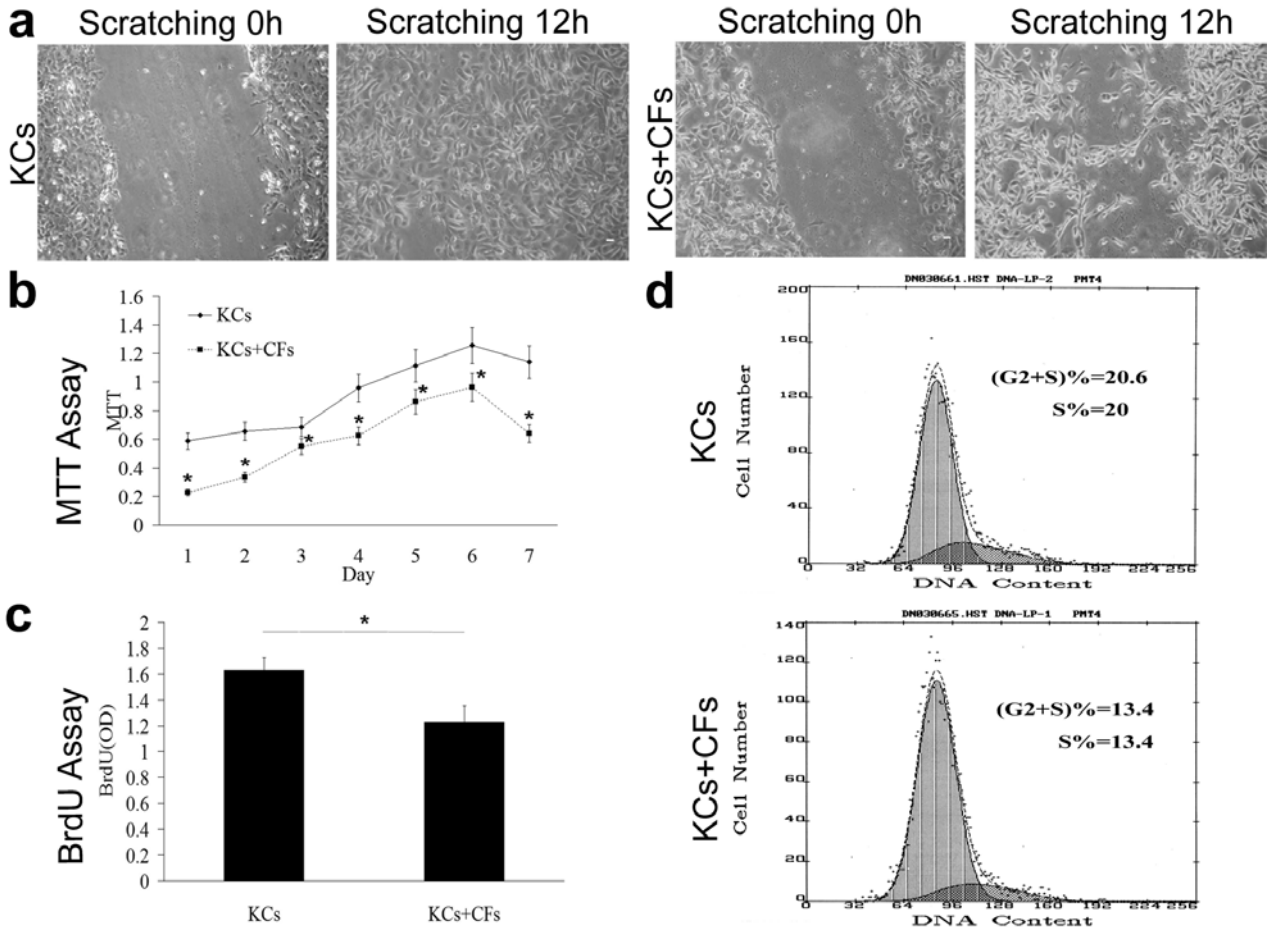

Fig. 4. **CFs inhibit the proliferation and migration of KCs *in vitro*.** In the scratching assay, cells migrated toward the center of the scratch and only a small amount of cells were confluent after 12 h of co-culture of CFs and KCs, which was much less than that in the culture of KCs alone (*a*). In the MTT assay, the growth and proliferation of KCs were markedly inhibited in the co-culture system of CFs and KCs when compared with those in the culture of KCs alone (statistically significant) (*b*). A similar result was obtained by BrdU assay (statistically significant) (*c*). Moreover, the proportion of cells in the G2+S phase and S phase was 13.4 and 13.4 respectively in the co-culture system of CFs and KCs, and 20.6 and 20 respectivel in the culture of KCs alone (*d*), which indicates the decrease of DNA synthesis.

promoted by the surrounding cells at the wound margin and/or by the increase of secreted growth factors and their receptors on the epithelial cells. Future studies are required to explain this discrepancy.

In conclusion, CFs can improve the cell functions of Fbs, which may be an important cause of fibrosis.

ACKNOWLEDGEMENTS

The study was supported by the National High Technology Research and Development Program of China (863 Project) (No: 2006AA02A119) and Fundamental Research Funds for the Central.

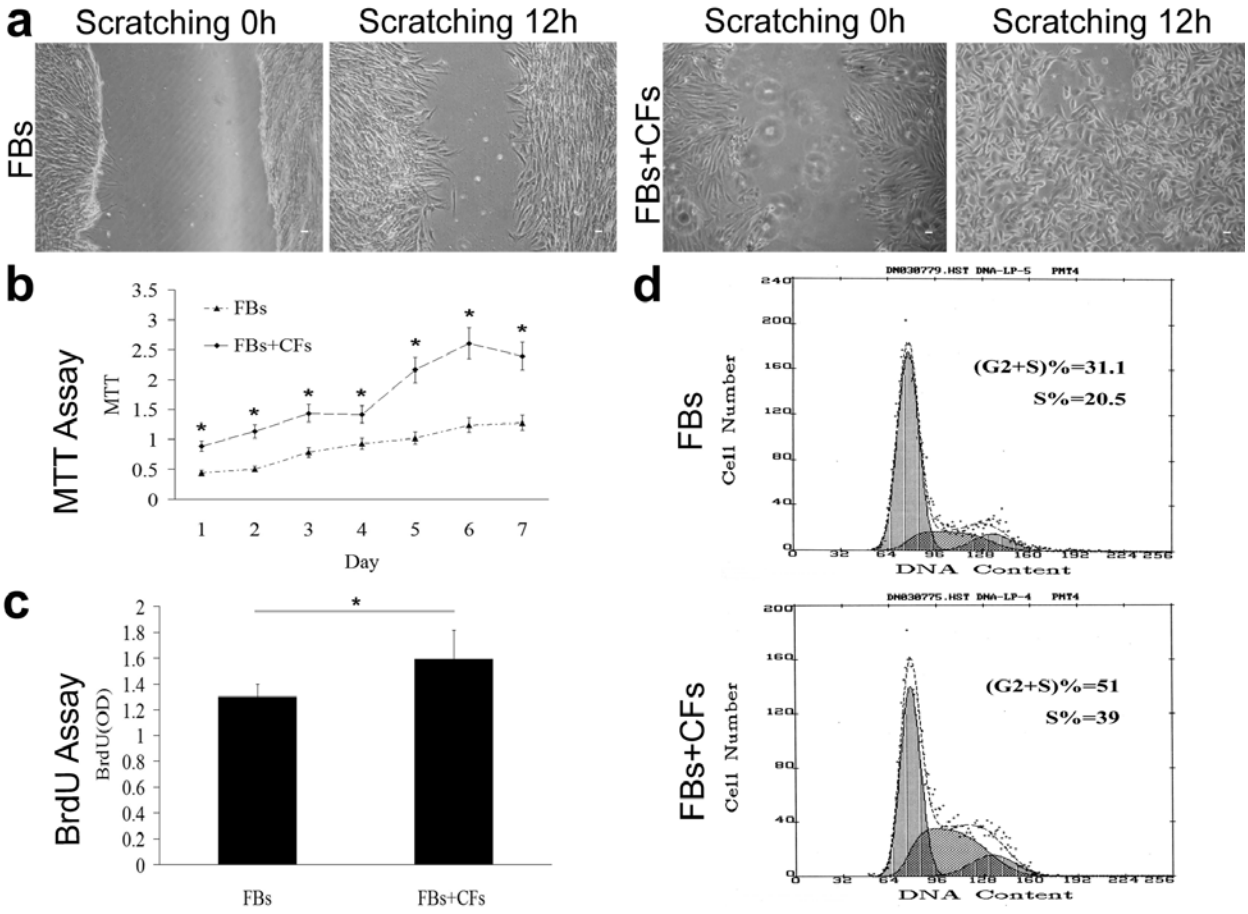

Fig. 5. **CFs promotes the proliferation and migration of Fbs *in vitro*.** In contrast to the effects of CFs on KCs in the scratching assay, cells migrated toward the center of the scratch and a large amount of cells were confluent after 12 h of co-culture of CFs and Fbs, which was much more than that in the culture of Fbs alone (*a*). An MTT assay showed the growth and proliferation of Fbs were significantly increased in the co-culture system of CFs and KCs when compared with those in the culture of Fbs alone (statistically significant) (*b*). A similar result was obtained by a BrdU assay (statistically significant) (*c*). Moreover, the proportion of cells in the G2+S phase and S phase was 51 and 39 respectively in the co-culture system of CFs and Fbs, and 31 and 20.5 in culture of Fbs alone (*d*).

CONFLICT OF INTERESTS

The authors declare no conflict of interests in this manuscript.

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