European Journal of Orthodontics 29 (2007) 198–203
doi:10.1093/ejo/cjl083
Advance Access publication 20 February 2007

The cytotoxicity of orthodontic metal bracket immersion media

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SUMMARY The purpose of the present study was to evaluate the cytotoxic effects of four different orthodontic metal bracket immersion media on primary human oral gingival fibroblasts (HGFs) and one permanent human osteogenic sarcoma cell line (U2OS). Four different metal brackets (Unitek, Tomy, Ormco, and Dentaurum) were immersed in buffer solutions of $NaHNO_3$ (1 mM) with a pH of 4 or 7, as well as artificial saliva. The concentrations for the experiments were 0.01, 0.1, and 1.0 µl/ml. At the end of the period of bracket immersion, morphological observations were conducted using light microscopy. The tetrazolium reduction assay was used to detect the survival rate of the target cells. Statistical analysis was conducted using one-way analysis of variance.

The results showed microscopically no morphological changes of the HGF or U2OS cells exposed to the metal bracket immersion media. At pH 4, the survival rates of the U2OS cells and the HGFs differed statistically for the Unitek ($P = 0.003$) and Ormco ($P = 0.000$) groups. At pH 7, the survival rate for the HGFs and the U2OS cells differed statistically for the Dentaurum ($P = 0.021$) and Unitek ($P = 0.03$) groups. The results demonstrate that differing cells exhibit various cellular reactions on exposure to metal bracket immersion media, although the four types of brackets appear to be biocompatible with HGF and U2OS cells.

Introduction

Metals and alloys have a wide range of applications in orthopaedics and dentistry. In the field of orthodontics, the types of stainless steel most commonly used in bracket manufacture are American Iron and Steel Institute (AISI) types 303, 304, 304L, 316, 316L, and 317 (Matasa, 1995). Stainless steel alloys containing 8 to 12 per cent nickel and 17 to 22 per cent chromium are generally used for the metallic components of orthodontic appliances (Kerosuo *et al.*, 1997).

In the oral environment, orthodontic attachments are exposed to a number of potentially damaging physical and chemical agents, such conditions possibly contributing to corrosion of the metal components of the appliance (Maijer and Smith, 1986). A previous investigation (Huang *et al.*, 2001) demonstrated that metal ions are released from an orthodontic metal bracket at a pH of 4. A number of studies have also shown that metal ions are released by all dental alloys *in vitro* and *in vivo* (Stenberg, 1982; Covington *et al.*, 1985; Messer and Lucas, 1996).

At present, the incidence of nickel hypersensitivity in the general population is reported to be between 20 and 30 per cent, with case reports of nickel hypersensitivity becoming commonplace (Dalmau *et al.*, 1984; Lamster *et al.*, 1987; Temesvari and Racz, 1988; Guyuron and Lasa, 1992). Adverse corrosion effects related to nickel-containing orthodontic devices such as archwires, brackets, and buckles on headgear devices have also been reported (Peltonen, 1979; Greig, 1983).

Biocompatibility occurs when the tissues of patients that come into contact with the materials do not experience any toxic, irritating, inflammatory, allergic, mutagenic, or carcinogenetic effects (Arvidson *et al.*, 1986; Jacobsen and Hensten-Pettersen, 1989). Orthodontic appliance biocompatibility is more prevalent in young patients who are more susceptible to developing inflammatory reactions to the use of alloys with possible toxic effects (Grimsdottir *et al.*, 1994; Wataha *et al.*, 1994b).

The sensitivity of cells to chemical cytotoxicity is reported to differ depending on the cell lines used (Sato and Ozawa, 1980). Tanaka (1994) reported that BALB3T3 cells, which are fibroblasts derived from murine embryos, have a higher sensitivity to the cytotoxicity of nickel ions released into the medium from a nickel specimen than V79 cells, which are fibroblasts derived from the lung of the Chinese hamster.

In vitro cytotoxicity screening, as a primary index of biocompatibility, is typically determined by means of cell culture. Although research into the biocompatibility of dental materials appears to be increasing, the findings are frequently controversial and non-harmonized experimental approaches often lead to contradictory results. In addition, data pertaining to the cytotoxic response of different cell lines to the influence of orthodontic metal brackets would appear to be scarce. Normal diploid cells differ from established or transformed cells in many ways (Holley, 1975; Reuveny *et al.*, 1982). Since metal brackets can be corroded in the oral environment, the corroded material can directly contact with the periodontal tissues. The effects of metal bracket immersion media on the alveolar bone and gingival tissue need to be identified. The purpose of present study was to compare the cytotoxic effects of four different orthodontic metal bracket immersion media

on primary human oral gingival fibroblasts (HGFs) and one permanent human osteogenic sarcoma cell line (U2OS).

Materials and methods

Sample preparation

Four different brands of metal bracket were analysed (Table 1). The method of sample preparation followed that of a previous study (Huang *et al.*, 2004). For each brand, a total of 160 brackets were tested. The brackets were immersed in the relevant solutions and incubated at a temperature of 37°C for a period of 48 weeks. The buffer solutions included $NaHNO_3$ (1 mM), with a pH of 4 or 7, as well as artificial saliva (Sinphar, Taipei, Taiwan; Table 2). The concentrations for the experiments were 0.01, 0.1, and 1.0 µl/ml.

Cell cultures

Human primary gingival fibroblast culture. The research was approved by the ethical board of Chung Shan Medical University Hospital. Following informed consent, gingival tissues were obtained by excision of premolar gingiva from a 12-year-old female patient undergoing orthodontic treatment. The resultant tissue was cut into 1- to 2-mm^3 sized pieces, washed twice with phosphate-buffered saline supplemented with penicillin (100 U/ml; Sigma Chemical Co., St Louis, Missouri, USA), streptomycin (100 µg/ml, Sigma Chemical Co.), and placed into 25 cm^3 tissue-culture flasks. The explants were incubated with culture medium consisting of alpha minimum essential medium (Sigma Chemical Co.), 30 per cent foetal bovine serum (FBS; Sigma Chemical Co.), penicillin (100 U/ml), and streptomycin (100 µg/ml), at 37°C in a humidified atmosphere of 5 per cent CO_2 in air. When outgrowth cells were observed in the cultures, the medium was replaced twice, sequentially, and the cells were then reincubated until the proliferating cells had reached confluence. The cells were detached from the monolayer by brief treatment with 0.02 per cent trypsin/0.04 M ethylenediaminetetraacetic acid (EDTA) and recultured in 100 cm^2 tissue-culture flasks until confluent monolayers were again obtained. Cells between the fifth and the seventh passages were used in the subsequent experiments.

Human osteogenic sarcoma cell culture. The U2OS cell line (BCRC no. 60187, Food Industry Research and Development Institute, Taiwan) was used. Briefly, the cells were cultured in McCoy's medium (Sigma Chemical Co.) containing 10 per cent FBS and penicillin, streptomycin, and fungizone, and L-glutamine (1 per cent; Sigma Chemical Co.). The cultures were maintained at 37°C in a humidified atmosphere of 5 per cent CO_2 in air. Confluent cells were detached with 0.025 per cent trypsin and 0.05 per cent EDTA for a period of 5 minutes, following which, aliquots of separated cells were subcultured. Cells were cultivated as monolayers in plastic culture flasks.

Cell viability test—tetrazolium reduction assay

The tetrazolium reduction (MTT) [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] colorimetric assay, a measure of succinic dehydrogenase activity, was performed following the method of Mossman (1983). HGF and U2OS cells were inoculated into 96-well plates (Falcon, Teterboro, New Jersey, USA) at a density of 4×10^3 cells per well, and incubated at 37°C, in 5 per cent CO_2-in-air for a period of 2 days. The cells were then incubated under identical conditions to the above for a further 3 days. Subsequent to incubation, the cells were treated with various concentrations of metal bracket extracts, following which MTT dye (50 µg/l) was added to each well. The plates were incubated at 37°C, and 5 per cent CO_2 in air for a period of 4 hours. For each well, the degree of light absorbance at 570 nm was then measured using an enzyme-linked immunosorbent assay reader (U2000, Hitachi, Tokyo, Japan). The cell viability results were presented as the ratio (in per cent) of the absorbance at 570 nm in the experimental wells to that detected in the control wells. Five replicates of each concentration were used for each test. All assays were repeated three times to ensure reproducibility. Statistical analysis was conducted using the SAS program for Unix 6.09 (SAS Institute, Cary, North Carolina, USA) using one-way analysis of variance, with a value of $P < 0.05$ showing statistical difference.

Results

Morphological observation

The morphology of the cells appeared to have been maintained subsequent to metal bracket immersion media exposure. Cell membranes appeared to be intact with no obvious damage or apoptosis (Figures 1 and 2).

Table 1 Details of the metal brackets.

Company	Bracket type	Position	Size (inch)	Order no.
Tomy Co., Tokyo, Japan	Micro-LOC bracket, standard edgewise	Upper premolar	0.018	920-45
Ormco Co., Glendora, California, USA	Diamond bracket, standard edgewise	Upper premolar	0.018	340-0604
Unitek 3M Co., Monrovia, California, USA	Twin torque bracket Andrews prescription	Upper premolar	0.018	018-203
Dentaurum Co., Pforzheim, Germany	Discovery direct bond bracket. System Ricketts universal	Upper premolar	0.018	790-136-00

Table 2 The content of the artificial saliva.

Composition	Content (mg)
Sali lube (saliva substitute)	
Sodium chloride	0.844
Potassium chloride	1.2
Calcium chloride anhydrous	0.146
Magnesium chloride 6 H_2O	0.052
Potassium phosphate dibasic	0.34
Sorbitol solution 70%	60
Methyl paraben	2
Hydroxyethyl cellulose	3.5

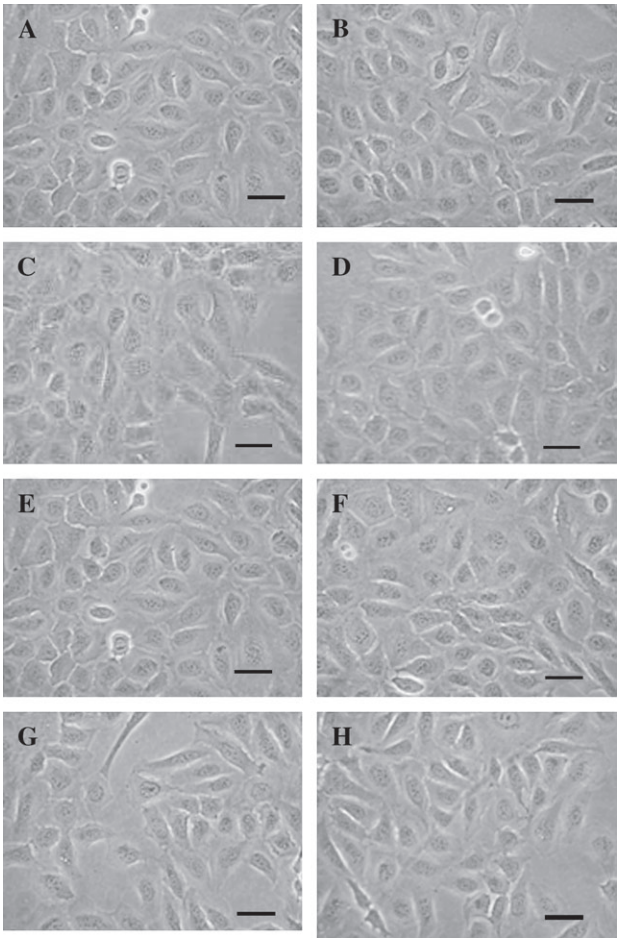

Figure 1 Light electron photomicrograph of U2OS cells treated with various concentrations of immersed metal brackets solutions. (A) Dentaurum group, pH 4, 1 µl/ml; (B) Dentaurum group, pH 7, 1 µl/ml; (C) Unitek group, pH 4, 1 µl/ml; (D) Unitek group, pH 7, 1 µl/ml; (E) Tomy group, pH 4, 1 µl/ml; (F) Tomy group, pH 7, 1 µl/ml; (G) Ormco group, pH 4, 1 µl/ml; (H) the Ormco group, pH 7, 1 µl/ml. Cell membranes appeared to be intact with no obvious cell damage or apoptosis (×200 magnification, bar = 50 µm).

Cytotoxicity of pH 4 metal bracket immersion media evaluated by MTT assay

The dose-response curve showed a dose-dependent increase in toxicity for the Unitek (P = 0.03) and Ormco (P = 0) groups, but not for the Dentaurum (P = 0.667) or Tomy (P = 0.138) groups (Figure 3). The greatest cell survival rate (in per cent) for the Unitek group was noted at a concentration of 0.01 µl/ml for the HGF cell group (89.48 ± 5.37 per cent) while the lowest survival rate was observed at a concentration of 1 µl/ml for the U2OS cell line (74.76 ± 4.89 per cent). The greatest cell survival rate (in per cent) for the Ormco group was noted at a concentration of 0.01 µl/ml for the U2OS cell line (90.32 ± 8.99 per cent) while the lowest survival rate for the HGF cell group was observed at a concentration of 1 µl/ml (69.42 ± 4.77 per cent).

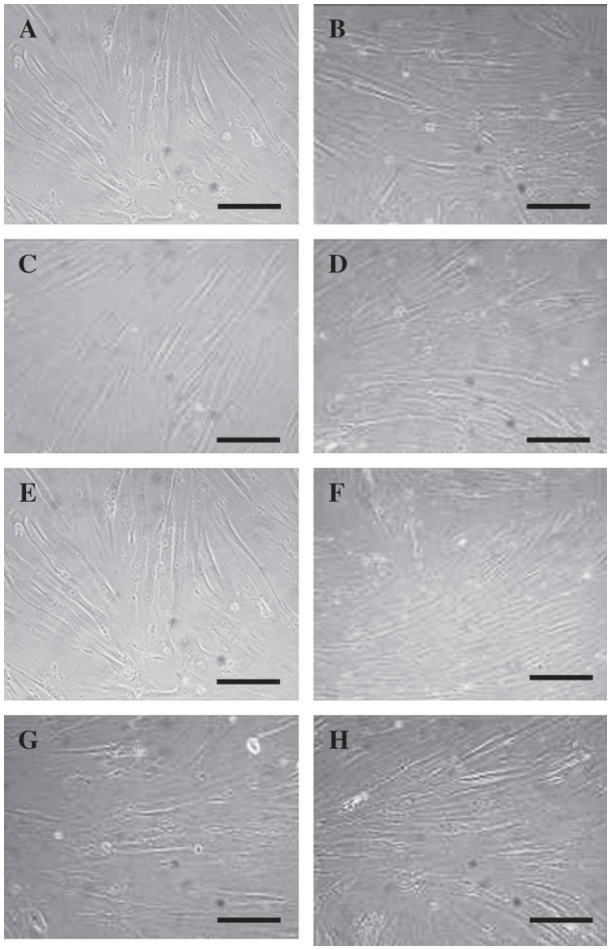

Figure 2 Light electron photomicrographs of human gingival fibroblasts treated with various concentrations of immersed metal brackets solutions. (A) Dentaurum group, pH 4, 1 µl/ml; (B) Dentaurum group, pH 7, 1 µl/ml; (C) Unitek group, pH 4, 1 µl/ml; (D) Unitek group, pH 7, 1 µl/ml; (E) Tomy group, pH 4, 1 µl/ml; (F) Tomy group, pH 7, 1 µl/ml; (G) Ormco group, pH 4, 1 µl/ml; (H) Ormco group, pH 7, 1 µl/ml. Cell membranes appeared to be intact with no obvious cell damage or apoptosis (×200 magnification, bar = 50 µm).

Cytotoxicity of pH 7 metal bracket extracts evaluated by the MTT assay

The dose-response curve reflecting the level of relative cytotoxicity of the metal bracket immersion media appeared

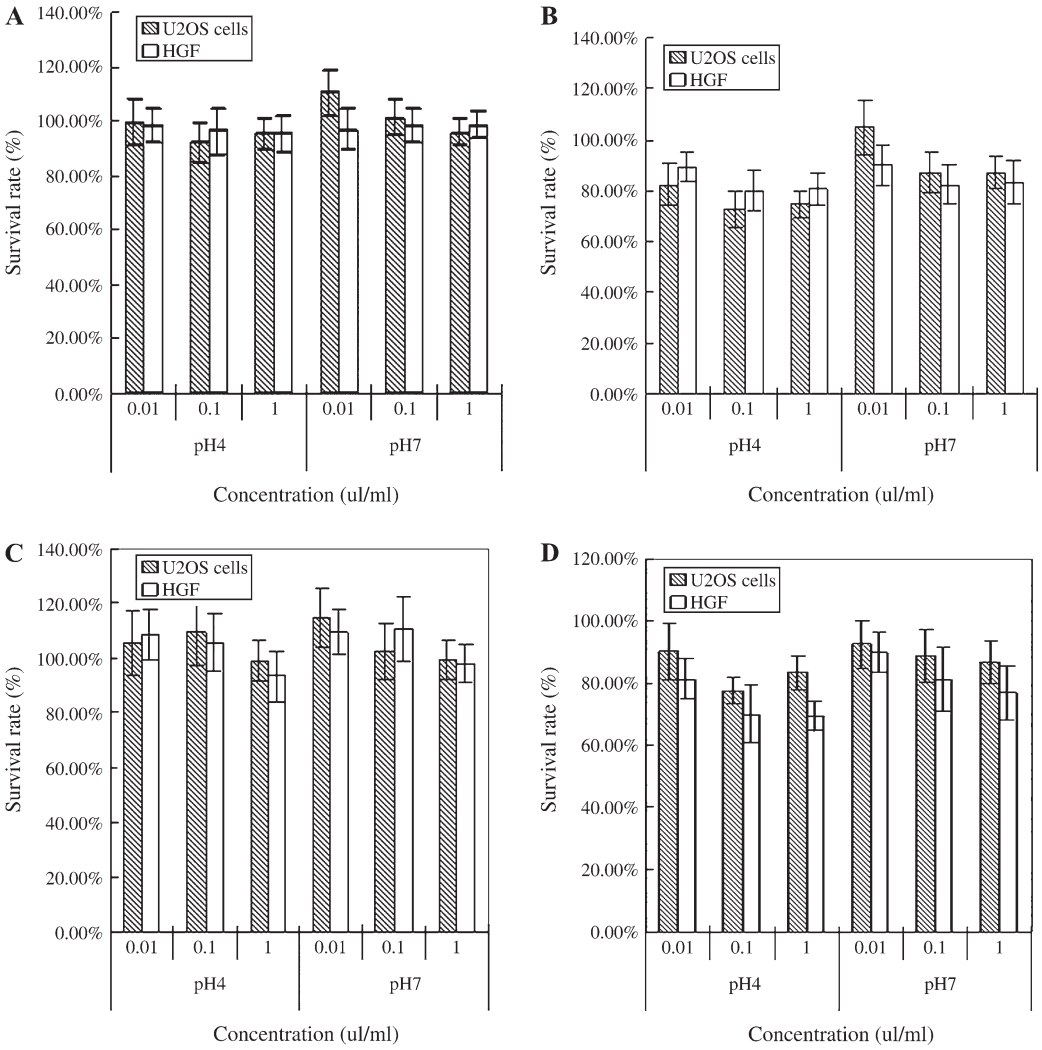

Figure 3 The survival rate of the U2OS and human gingival fibroblast cells in the metal bracket immersion media (a) Dentaurum, (b) Unitek, (c) Tomy, and (d) Ormco metal bracket immersion media. Survival rate (in per cent) = (absorbance of experiment/absorbance of control) × 100 per cent.

to reveal a dose-dependent increase in toxicity for the Dentaurum ($P = 0.021$) and Unitek ($P = 0.03$) groups but not for the Tomy ($P = 0.054$) or Ormco ($P = 0.06$) groups (Figure 3). The greatest cell survival rate for the Dentaurum group (110.43 ± 8.38 per cent) was observed at a concentration of 0.01 µl/ml for the U2OS cell group, while the test cell survival rate appeared to be similar for the other remaining metal bracket groups. The greatest cell survival rate (in per cent) for the Unitek group was observed at a concentration of 0.01 µl/ml for the U2OS cell group (104.83 ± 10.74 per cent).

Discussion

Dental materials used in the oral environment are subject to electrochemical and chemical reactions, mechanical forces of mastication, and wear. Since orthodontic metal brackets are located in proximity to periodontal tissues, it is essential to determine the relative levels of biocompatibility of the various metal brackets in such an environment. A previous study has shown that metal brackets may corrode in the oral environment and metal ion leaching may occur (Huang *et al.*, 2001). The extraction assay described above would appear to be one of the most frequently used methods to investigate the mechanism of intraoral cytotoxicity with regard to the study of dental materials and their oral environmental interaction (Leyhausen *et al.*, 1998). The MTT assay, although not as accurate, is often used to evaluate the activity of mitochondrial succinic dehydrogenase by measuring the amount of formazan produced by this enzyme (Mossman, 1983; Lehmann *et al.*, 1993). In the present study, this method was chosen to evaluate the relative toxicity to tested cells of orthodontic metal bracket extracts.

It has been shown that human primary gingival cells can provide a more sensitive and discriminating cultured-cell

model for the cytotoxic assessment of dental materials than various permanent cell lines originally derived from animal tissue (Caughman *et al.*, 1990; Andreotti *et al.*, 1994). Andreotti *et al.* (1994) noted that the resistance to dental material toxicity of normal cells was likely to be greater than that for cell lines, it being suggested that this was due to the high growth-rate conditions in which cell lines are cultured. Thus, in the present study, primary cultured HGF and U2OS cell lines, which were considered as a representation of the alveolar bone and periodontal tissue, were chosen.

Comparison of the results for the Ormco HGF group with those for the Ormco U2OS group, showed that the latter's survival rate (83.44 ± 5.38 per cent) was statistically greater than the corresponding result for the HGF group ($P < 0.05$). The findings demonstrated that cells of different origins have a different cellular reaction to foreign bodies. From this result, HGF would appear to be more sensitive to Ormco metal bracket extracts, similar to the findings of Andreotti *et al.* (1994).

Previous studies have revealed that a greater number of metal ions are released into the solution from metal brackets immersed in pH 4 than in pH 7 extract (Huang *et al.*, 2001, 2004). The present results demonstrated that HGFs and Ormco in metal bracket immersion media at a concentration of 1 µl/ml and at pH 4 had the lowest overall cell survival rate (69.42 ± 4.77 per cent). Interestingly, Huang *et al.* (2004) showed that the immersed Ormco metal bracket group elicited the most substantial nickel concentration of all tested metal bracket groups (260.5 ± 17.9 µg/ml). It has been demonstrated that nickel ions present in bracket immersion media are able to enter test cells in a number of different ways and induce cellular reactions (Costa and Mollenhauer, 1980; Brommundt and Kavaler, 1987; Kavaler and Brommundt, 1987; Raffa *et al.*, 1987). Essentially, under such circumstances, nickel ions bind with several biological compounds, and thus decrease the extent of a number of cellular functions (Sunderman and Selin, 1968; Coogan *et al.*, 1989), including succinic dehydrogenase activity and protein synthesis (Wataha *et al.*, 1994a). The higher nickel content of the Ormco metal bracket may be the cause of the observed lower survival rate for HGF cells exposed to metal bracket immersion media.

Metal bracket fabrication may utilize welding or brazing, with the brazing alloys generally consisting of silver and copper and sometimes palladium. Locci *et al.* (2000) showed that brazing alloy is more cytotoxic than stainless steel on HGF. The present results revealed that for the low concentrations of Tomy metal bracket immersion media (0.01 and 0.1 µl/ml), under either pH 4 or pH 7 conditions, the survival rate for HGF and U2OS cells was greater than 100 per cent; such findings suggesting that Tomy metal brackets actually contribute to a minor exposure-related proliferative response to the test cells. Huang *et al.* (2004) showed that the Tomy metal brackets released lower concentrations of metal ions such as nickel, chromium, and copper into the immersion solution than Unitek, Ormco, and Dentaurum metal brackets. These results may explain why the Tomy metal bracket was relatively biocompatible with U2OS cells and HGFs.

The morphological changes in the U2OS cells and by HGFs following treatment with the four different metal bracket immersion media did not appear to include any obvious cellular alterations. From microscopic observation, there appeared to be no evidence of any apoptotic change or necrosis, neither cell membrane demonstrating any evidence of bulb formation or destruction of the treated cells, suggesting that these four types of metal bracket are biocompatible. Further, according to the results obtained from mitochondrial activity and morphology investigations, the four different types of orthodontic metal brackets demonstrated good biocompatibility with the U2OS cells and HGF.

Conclusion

The study showed that cells of different origins exhibit various cellular responses to exposure to metal bracket immersion media, although the four types of brackets appear to be biocompatible with HGF and U2OS cells.

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Acknowledgement

The authors acknowledge grants from the National Science Center of Taiwan (NSC-91-2314-B-040-010, NSC-92-2314-B-040-007) supporting this project.

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