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Different fate of cancer cells on several chemical functional groups

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ABSTRACT

Self-assembled monolayers with different terminal chemical groups including thiol (–SH), methyl ($-CH_3$), carboxyl (–COOH) and hydroxyl (–OH) were employed as substrates for the culture of hepatoma cells (HepG2s). X-ray photoelectron spectroscopy and atomic force microscopy confirmed the similar density of different functional groups occupation. The adhesion and proliferation of cancer cells exhibited significant difference on different surfaces. The HepG2s adhered to $-CH_3$ surfaces but exhibited the smallest contact area with mostly rounded morphology, while those on –SH surfaces exhibited the largest contact area with extensive spreading. The proliferation of HepG2s in prolonged culture was significantly inhibited on $-CH_3$ surface. Cells on other surfaces of various chemical groups proliferated at different levels. After 7 days of culture, the proliferation of HepG2s on the different surfaces followed the trend: $-OH \approx -COOH > -SH \gg -CH_3$. Due to the strong hydrophobic property, the $-CH_3$ group inhibited the cell adhesion, which led to the death of cancer cells. Compared with other chemical functional groups, the $-CH_3$ group exhibited its unique effect on the fate of cancer cells, providing a potential way on prevention and treatment of liver cancer.

1. Introduction

Cell behavior can be significantly influenced by extracellular matrix of cellular microenvironment [1–4]. Several recent works have reported that the fate of cancer cells depends on their surrounding microenvironment modified by various materials [5–9]. However, due to the complexity of materials chemical and physical properties in the cellular microenvironment, the exact function of material factors is still unknown [1,2]. Previous studies have proved that surface chemical functional groups on the cell culture substrate could control cell behavior including adhesion, migration and differentiation, by using human osteoblast-like cells [10], human fibroblasts [11], mesenchymal stem cells [12], and neural stem cells [13]. However, the influence of chemical functional groups on cancer cells has not been reported in the literature, to the best of our knowledge.

Self-assembled monolayers (SAMs) technique has been well developed for the modification of metal surface with chemical functional groups over the past two decades [14]. The SAMs on gold surface can provide chemical functional group surfaces with same surface density for cells to seed on. Our previous study have shown the different effects of the thiol (–SH), methyl ($-CH_3$), carboxyl (–COOH) and hydroxyl (–OH) groups on neural stem cells [10,15,16]. In the present study, hepatoma cells (HepG2s) were selected as an example of cancer cells, because hepatoma is one of the most frequent malignant tumors with uncontrolled growth. To investigate the effect of chemical groups on HepG2s, the surfaces were prepared with –SH, $-CH_3$, –COOH and –OH groups. The adhesion and proliferation of cells after seeding on the different surfaces were examined. We provided the first evidence for the effects of chemical functional groups on cancer cell behavior. Especially we found that methyl surface can inhibit cell adhesion and proliferation. As we know, cancer cells have the characteristics of unlimited proliferation and easy migration. Hence, our findings provide helpful insights for designing biomaterials rich in methyl groups to prevent or treat liver cancer.

2. Materials and methods

2.1. Preparation of SAMs on Au surface

Au {111} surfaces (thickness of ~40 nm) were grown on glass coverslips after Ti layer (thickness of ~10 nm) as a buffer layer by ion beam sputtering (IBS) technique. All the Au substrates were rinsed

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with deionized water and stored in deionized water prior to introduction of different chemical functional groups on the surfaces.

The technology of SAM was applied to form well-ordered chemical surfaces with the $-SH$, $-CH_3$, $-COOH$ and $-OH$ groups [17,18]. The Au substrates were dipped into four types of 1% thiol (Sigma, USA) solution with $-SH$, $-CH_3$, $-COOH$ and $-OH$ groups for the same period of over 2 h. Thus the chemical functional groups seeding on gold surface with same concentration can be obtained. These modified surfaces were then rinsed with deionized water and dried in nitrogen before characterization by contact angle system, X-ray photoelectron spectroscopy (XPS) and atomic force microscope (AFM).

2.2. Contact angle test

Different surfaces with $-SH$, $-CH_3$, $-COOH$ and $-OH$ groups were characterized by contact angle measurements. Ambient air–water substrate contact angle measurements (4 ml ultra-pure H_2O) were applied with a contact angle system OCA20 (Dataphysics, Germany) fitted with a digital camera and analyzed using in-house image analysis software.

2.3. Occupations of chemical functional groups

The modified Au surfaces with $-SH$, $-CH_3$, $-COOH$ and $-OH$ groups were characterized by XPS (Axis Ultra, UK) with carbon (284.8 eV) as a marker. The spot size was 700 μm × 300 μm and penetration depth is 2–3 nm. The source type was Al K Alpha and the energy step was 1 eV.

2.4. Morphology of SAMs modified surfaces

The modified Au surfaces with different functional groups were observed by AFM (MFP-3D-S, Asylum Research, USA) under contact mode in an air atmosphere. The photographs were taken with open-loop condition and Olympus AC240TS probe was applied.

2.5. Cell culture

The HepG2 cells were cultured in medium of DMEM-F12 containing 10% serum medium plus 1% penicillin and streptomycin solution at a density of 80000 cells/cm^2, which were in T75 culture flasks (Corning, USA) at 37 °C under a humidified atmosphere of 95% air/5% CO_2. The number of live cells was counted by trypan blue exclusion assay in a hemocytometer.

After 3 days of culture, the cells were centrifuged, mechanically dissociated into single cells by syringe, and then seeded on the Au surfaces which were modified with $-SH$, $-CH_3$, $-COOH$ and $-OH$ chemical functional groups, respectively. The cells were cultured in the same culture medium as described above. The medium was changed every 2 days.

2.6. Morphological observation of the cells

The morphology of the cells was observed by scanning electron microscopy (SEM). The samples were removed from the medium after seeding on surfaces for 12 h and fixed by 4% formaldehyde for 10 min. Then the samples were dehydrated through gradient of alcohol and allowed to air dry in a fume hood. After sputter-coated with gold, the samples were examined with SEM (LEO-1530).

2.7. Focal adhesion assembly

The HepG2 cells cultured on modified gold surfaces at 1 day were fixed in 4% paraformaldehyde for 15 min and permeabilized with 0.1% Triton X-100 in PBS for 3 min. Non-specific binding was blocked with 1% BSA in PBS for 30 min. Focal adhesion kinase was shown using a commercially available kit (FAK 100 and AP124F, Millipore) according to the manufacturer's protocol. The samples were then washed, mounted and examined by laser scanning confocal microscope (LSM710-3channel, Leica SP5) with 40× objective.

2.8. Fluorescence staining

At 1, 3 and 5 day of culture, the samples were removed from the medium and washed three times in PBS before fixed in 4% formaldehyde for 10 min. Then all the cells were stained by Rhodamine R415 (Invitrogen) (1:2500) and the nuclei were contra-stained by DAPI (1:4000).

2.9. MTT assay

The cell proliferation was tested by the 3-(4,5-dimethylthiazol-2-yl)-diphenyltetrazolium bromide (MTT) (Sigma, USA) assay (n = 3 donors). The cells were cultured on the Au surfaces modified with $-SH$, $-CH_3$, $-COOH$ and $-OH$ groups in 24-well plates. At the indicated time points, 50 ml of MTT solution was added into the 24-well plates (5 mg/ml in PBS) and incubated at 37 °C for 4 h. After removing the culture medium and washing in PBS for three times, the formozan reaction products were dissolved in dimethylsulfoxide (DMSO) for 20 min. The optical density was read on an ELISA plate reader at 490 nm. Statistical analysis was performed with one-way ANOVA method.

3. Results

3.1. Physicochemical characterization

The contact angles of water for chemical functional groups modified Au surfaces are listed in Table 1. The $-OH$ surface appeared to be the most hydrophilic with a contact angle of 13.50 ± 0.5°, while the $-CH_3$ surface was the most hydrophobic with a contact angle of 103.00 ± 5.0°, because of its non-polar nature. The surface of $-COOH$ group had slightly higher contact angle values of 29.38 ± 1° than that of $-OH$ group. The contact angles of $-SH$ surfaces was 68.15 ± 2.1°.

3.2. Surface densities of chemical functional groups determined by XPS and AFM

The surface densities of SAMs with different chemical functional groups were studied by XPS (Fig. 1), where the XPS data of $-COOH$ group was taken as a typical because the XPS spectrums of these chemical functional groups are similar. To estimate the surface densities of specific chemical functional group, the following points were assumed: 1) the surface density of the chemical functional group of $-CH_3$, $-COOH$ and $-OH$ is the same as the surface density of S atoms, because one thiol molecule contains one S atom [14]. The surface density of $-SH$ group is the half of the density of S atoms assuming that each thiol molecule stands on the surface with only one $-SH$ group reacting with Au surface. 2) The surface density of S atoms can be obtained from the ratio of the content of S atoms and Au atoms on the top monolayer of Au substrate, and the surface density of Au {111} substrate was taken as $12 \times 10^{18}/m^2$. 3) The area of S_{2p} in XPS spectrum indicates the content of S atoms on the top monolayer due to the nature of the SAM. Numerically, the content of S atoms is equal to the area of S atoms multiplied by the scattering factor of S [19]. 4) The area of Au_{4f} in XPS spectrum involved multilayer of Au atoms. By adopting a sampling depth of 2 nm, the area of Au_{4f} peak had contribution from about 7 layers of Au

Table 1
Contact angle data with water as solvent. Results were means ± SD of 5 independent experiments.

Chemical groups	$-CH_3$	$-SH$	$-COOH$	$-OH$
Contact angle(°)	103.00 ± 5.0	68.15 ± 2.1	29.38 ± 1	13.5 ± 0.5

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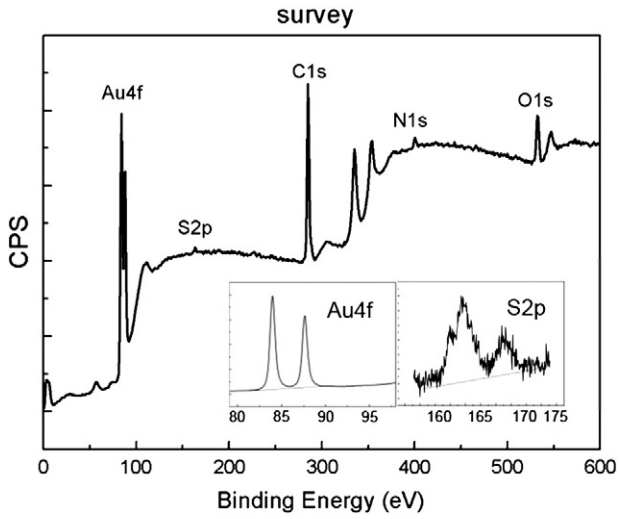

Fig. 1. XPS spectrum of the Au surfaces with –COOH chemical functional group as an example. Inserted is the scan spectrum of Au(4f) and of S(2p), the areas of which are calculated to indicate the content of chemical functional group.

atoms. With these consideration in mind, the surface densities of $-CH_3$, –OH, –SH, and –COOH groups were estimated to be $(3.9\pm0.4)\times10^{18}/m^2$, $(4.0\pm0.3)\times10^{18}/m^2$, $(3.9\pm0.3)\times10^{18}/m^2$ and $(4.0\pm0.6)\times10^{18}/m^2$, respectively. Therefore, we can conclude that surface densities of these groups are almost equal on the micrometer scale.

The AFM photographs of the surfaces with –SH, $-CH_3$, –COOH and –OH were taken to observe the arrangement of chemical functional groups on Au {111} surfaces. The photographs were recorded under contact mode and Olympus AC240TS probe was applied. As shown in Fig. 2a–d, all the samples with different chemical functional groups as –SH, $-CH_3$, –COOH and –OH showed similar molecular arrangement on Au surfaces. Based on the section analysis (Fig. 2e), the average nearest-neighbor spacing was about 0.5–0.6 nm, corresponding to a $\left(\sqrt{3}\times\sqrt{3}\right)R30^\circ$ monolayer absorbed on Au {111} surface [14]. The surface density thus calculated was about $3.2–4.6\times10^{18}/m^2$, which was consistent with the results of XPS. Collectively, the data proved that different surfaces with a similar density of various terminal chemical groups were obtained by SAM technique, as schematically shown in Fig. 2f.

3.3. Morphology of HepG2s

The adhesion and morphology of HepG2 cells on chemical groups modified substrates were investigated. During the initial 12 h of culture, the HepG2 cells attached on different surfaces with chemical functional groups, as shown in Fig. 3. The morphology of HepG2s on the same surface had no significant changes after day 1, 3 and 5.

The different surface chemistry showed different effect on cell adhesion. On $-CH_3$ surface, the cells exhibited spherical cell body in small area, without apparent cellular processes to interact with chemically modified surface. In contrast, for –OH, –SH and –COOH modified surfaces, the cells showed much better adhesion and spread out to a large area than that on $-CH_3$ surface. The cells exhibited spindle or

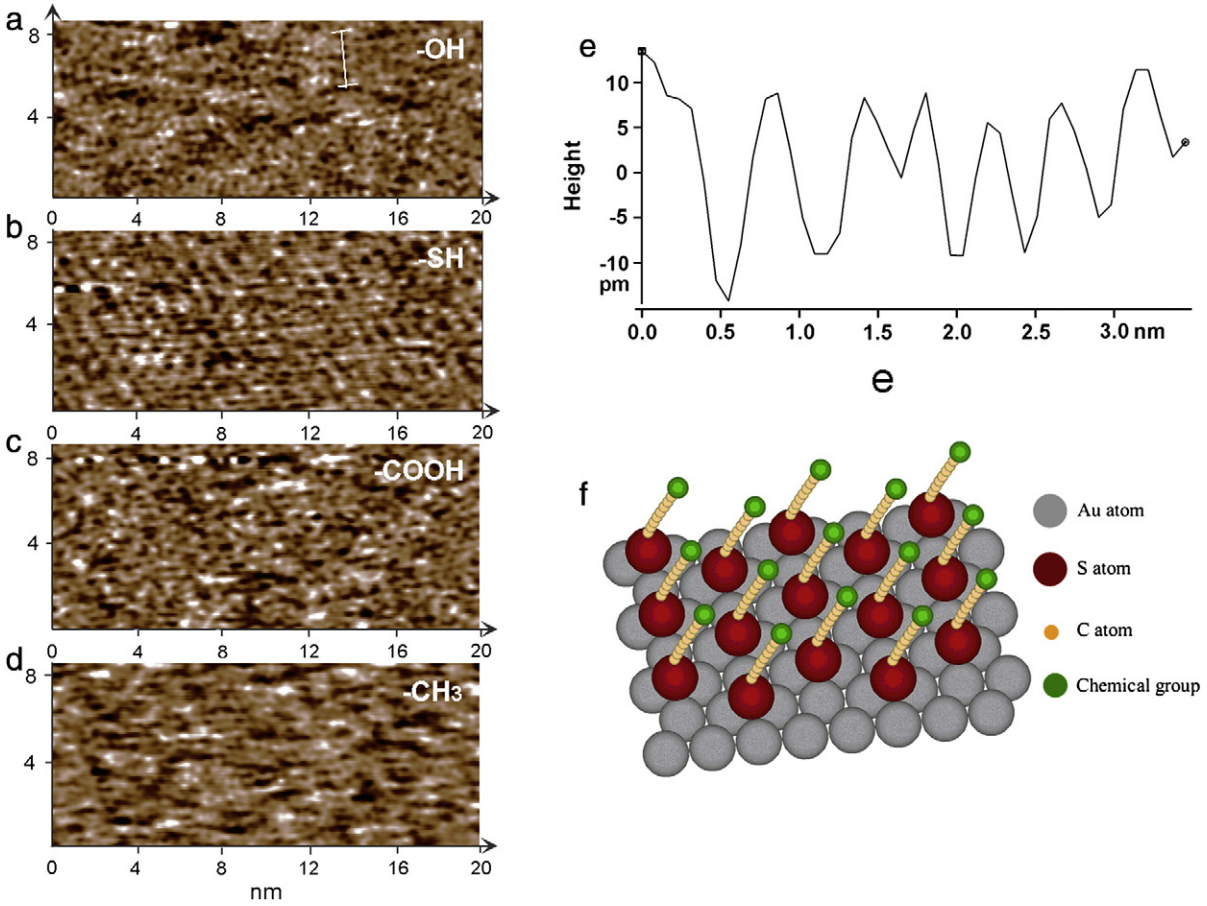

Fig. 2. AFM images of different chemical functional groups modified Au (111) surfaces in 8 nm×20 nm area, which was recorded under contact mode. (a) –OH group, (b) –SH group, (c) –COOH group, (d) $-CH_3$ group. (e) Section analysis of the surface contour along the line in (a). The nearest-neighbor spacing was about 0.5 nm. (f) Schematic of a thiol monolayer self-assembled on an atomically flat gold substrate.

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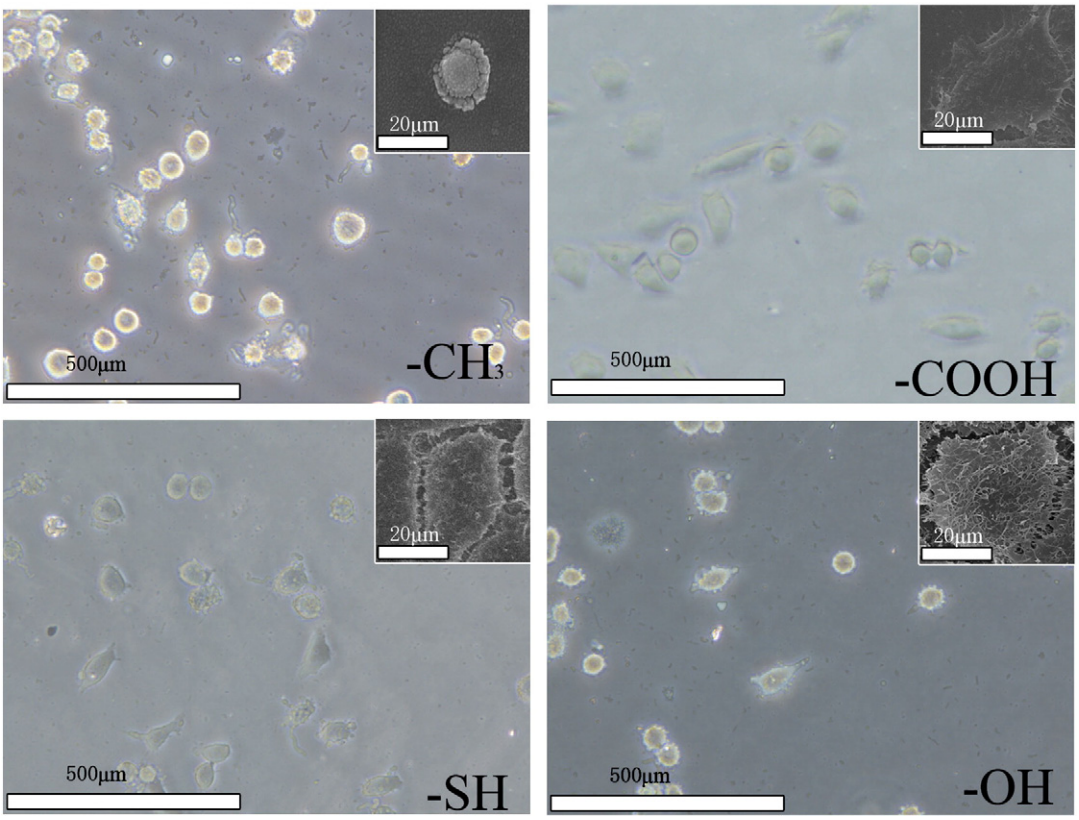

Fig. 3. The optical photographs of HepG2s cultured on surfaces with different chemical functional groups after 12 h of culture. Inserted is the figure of a single cell cultured on the same surface revealed by SEM.

polygonal morphology, with many processes contacting with each other and surfaces. The cells on −SH and −OH surfaces exhibited mainly polygonal shape, those on −COOH surface exhibited both polygonal and spindle cell body.

The focal adhesion analysis further confirmed the differences of initial cell spreading modulated by different chemical functional groups (Fig. 4). Actin was partially colocalized with vinculin in the focal adhesion plaques indicated by the green color. Both focal adhesion formation and the cell size were different for the HepG2s cultured on different chemical functional groups. Cells on $-CH_3$ groups were ~20 μm wide, while those on −COOH group was ~50 μm. The cell size was about 40–55 μm on −SH and −OH groups. Most cells on the surfaces with −OH and $-CH_3$ groups were roundish and on those with −COOH and −SH were polygonal. It was clear that the formation of focal adhesion on the surface of $-CH_3$ groups was significantly inhibited.

3.4. Proliferation of HepG2s

HepG2s cultured on different chemical functional groups were observed by a fluorescence confocal microscope at day 1, 3 and 5. The HepG2s on different surfaces were uniformly seeded at start of experiment. It is seen from Fig. 5 that proliferation of HepG2 cells on the four surfaces exhibited significantly different characters. For cells on $-CH_3$ surface, they migrated towards each other into colony of ~15 cells at day 1. Few colonies can be found and only separated cells were left on the surface at day 3. For cells on −COOH and −OH surfaces, they started to proliferate and form some small colonies of ~4 cells at day 1. They continued to proliferate and form colonies of 5–10 cells at day 3. At day 5, the number of cells obviously increased and large colonies of around 50 cells or more was formed. The large colonies further contacted each other. For cells on −SH surface, they were separated at day 1, proliferated and formed small colonies of 2–7 cells at day 3. Large colonies of ~40 cells could be seen at day 5. Among the four types of chemical groups modified surfaces, the growth of HepG2 cells appeared to be greatly inhibited on $-CH_3$ surface. This phenomenon would be potentially useful in cancer therapy.

The results of MTT assay of HepG2s cultured on different chemical groups modified surfaces at day 3, day 5 and day 7 were shown in Fig. 6. From day 3 to day 5, there was a small but not significant increase in proliferation of HepG2s on the surfaces with the −SH, −COOH and −OH groups. At day 7, the cells on −OH, −SH and −COOH surface increased significantly, but the cells on $-CH_3$ surface remained at a very low level. The cells in the control culture showed a moderate proliferation. It appeared that the −OH, −SH and −COOH groups had a promoting effect on HepG2s proliferation in a longer culture period. However, the $-CH_3$ group had less effect on HepG2s proliferation than other groups, in other words, it inhibited the proliferation of HepG2s. Furthermore, the proliferation capacity of HepG2s on different surfaces followed the trend: $-COOH \sim -OH > -SH \gg -CH_3$.

4. Discussion

The experimental results showed that significantly different adhesion and proliferation of cancer cells can be controlled simply by different chemical functional groups when other factors including surface densities of chemical functional groups remained the same.

The SAMs formed by the adsorption of different thiols on gold surfaces have generally compact and even crystalline structure [14]. The theoretical value of surface density of chemical groups in this process is $5\times10^{18}/m^2$ by calculation from the configuration of one S atom bonding to three Au atoms in the hexagonal packing overlayer structure [14,15]. The experimental measurements for the four different types of surfaces in this study were very close to the theoretical value.

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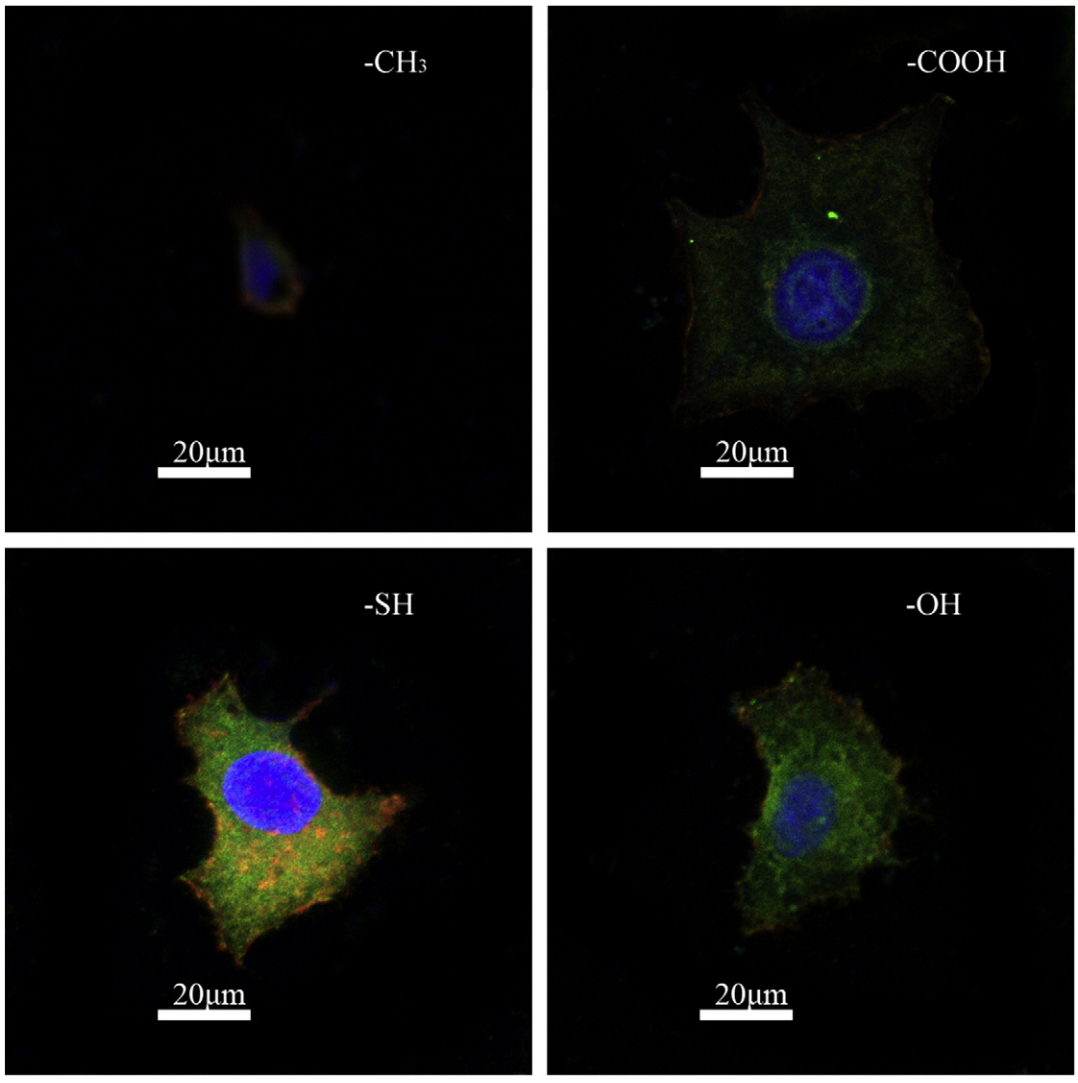

Fig. 4. The focal adhesion of HepG2s cultured on surfaces with different chemical functional groups after 12 h of culture. Scale bar = 20 μm. (For interpretation of the references to color in the text with the citation of this is figure, the reader is referred to the web version of this article.)

The surface densities of the different types of chemical groups were considered to be similar.

Although no previous study has been done in HepG2 from the viewpoint of effect of chemical functional groups on cancer cells, some studies have investigated the interaction of HepG2s with various materials including polymers and biological materials [9,12]. It is well established that cell adhesion and morphology can influence subsequent activity of cells [13]. Cell adhesion belongs to the first stage of cell–material interactions and the quality of this first stage will influence the following proliferation and growth of cells [17]. Surface properties of the polymer materials such as hydrophilicity gave rise to different morphology of HepG2s on different tested surfaces. For example, the cells on hydrophilic –OH surfaces expanded larger area and extended more processes than those on other surfaces [11]. In terms of interaction between cells and chemical functional groups, the –OH, –SH and –COOH groups tend to form ligands bonding with receptors of cells in contrast to the $-CH_3$ groups. Yin et al. [20] reported that the spreading, adhesion contact dynamics and adhesion strength for hepatocytes were influenced by the nature of ligand–receptor interactions and the accessibility of ligands. The ligand–receptor interaction should play an important role in cancer cells behavior controlled by chemical surfaces. Based on the results of adhesion and proliferation, the ligand–receptor interaction between the HepG2 cells and chemical functional groups should be the major cause of the proliferation trend of $-COOH \sim -OH > -SH \gg -CH_3$. This is consistent with the results of water affinities of the surfaces (Table 1). The hydrophilicity of surface may facilitate the adhesion and spreading of tumor cells and then promote their proliferation.

Tumor cells share many characteristics with stem cells such as the regulation mechanism of self-renewal. It is interesting to note that the surface chemistry has different effect on the fate of stem cell and tumor cells. Curran et al. [12] investigated the effect of surface chemistry on the phenotype and function of mesenchymal stem cells (MSC). The MSC cultured in contact with the –COOH surfaces showed the trend of chondrogenic differentiation. In contrast, MSCs cultured in contact with the $-CH_3$, –SH and –OH surfaces exhibited the behavior of cell stasis. The different interaction of surface functional groups with the normal stem cells and the cancer cells should play the vital role in this respect. On the other hand, the interactions of cells cannot be ignored due to the low cell seeding density in the study. In addition, there might be communications between $-CH_3$ group and liver cancer cells [21,22], which caused inhibition of HepG2 proliferation. These possible communications deserves further investigation in the view of interactions between protein and cell in the future.

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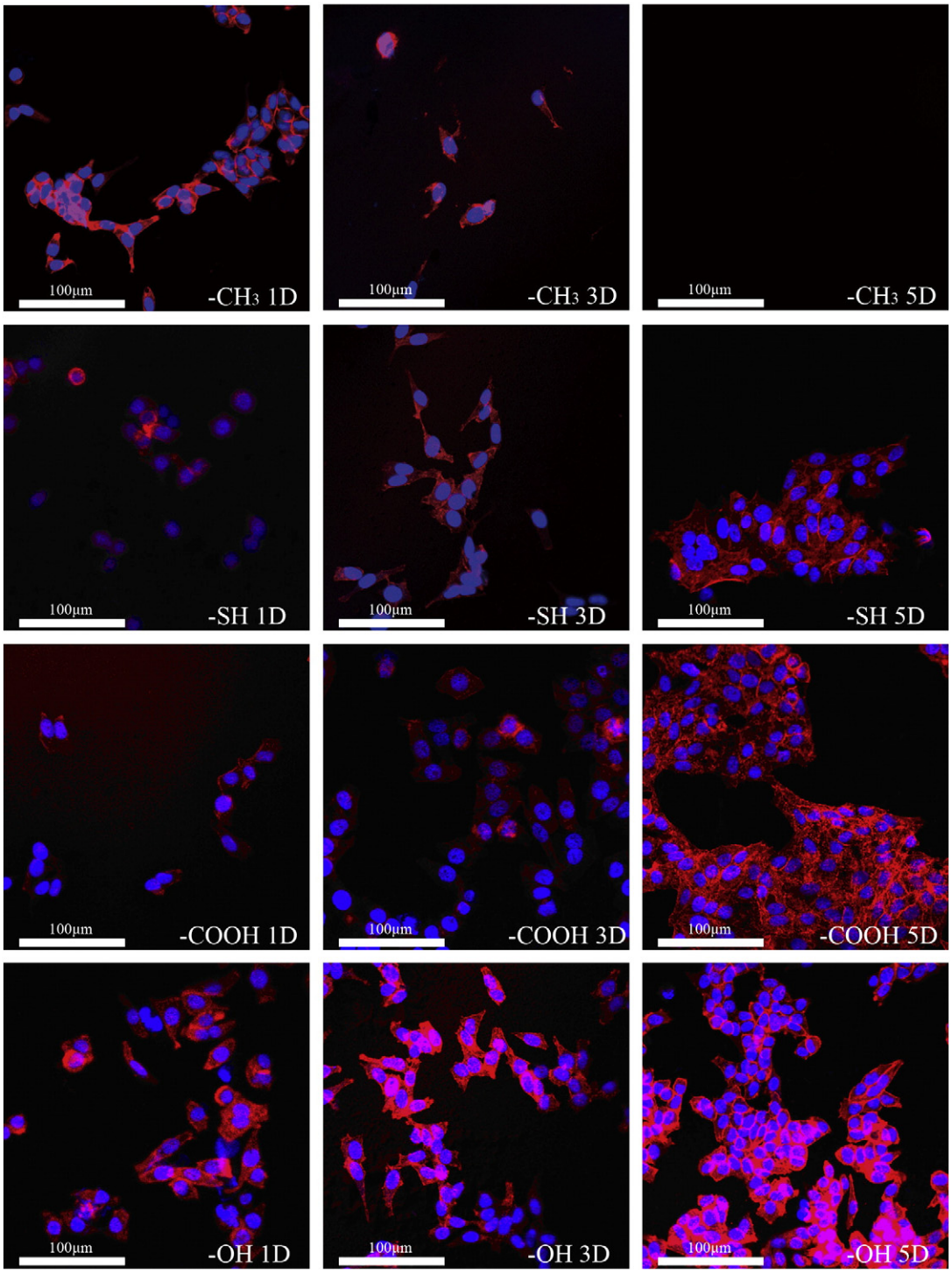

Fig. 5. The morphology of HepG2s cultured on the chemical functional groups modified surfaces at day 1, 3 and 5 with a fluorescence confocal microscope. Scale bar = 500 μm.

5. Conclusions

The adhesion and proliferation of HepG2 cells on the surfaces with terminal functional groups of –SH, $-CH_3$, –COOH and –OH were studied. The surface densities of different chemical functional groups were similar. The cells can attach on the $-CH_3$ surface and exhibit spherical cell body in smaller area than those on the –OH, –SH and –COOH groups. The MTT assay provided evidence that the $-CH_3$ chemical group significantly inhibited the reproduction of HepG2s. The biomaterials modified by $-CH_3$ chemical group may give an application to promote the death of liver cancer.

Acknowledgments

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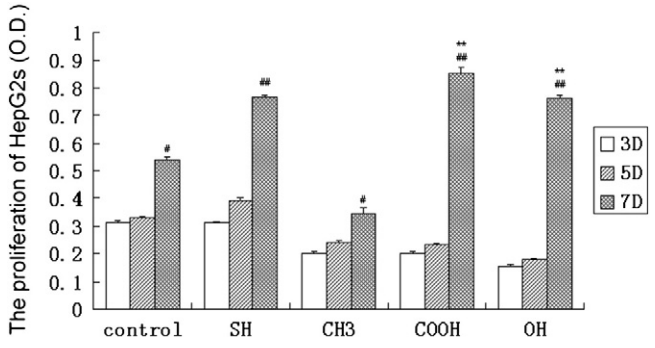

Fig. 6. The proliferation of HepG2s cultured on different chemical functional groups modified substrates at day 3, 5 and 7 by MTT assay. Error bars represent means ± SD for n = 3. # or ##, compared to the same substrates at day 3. **, compared to the substrates modified with $-CH_3$ group. # refers to $p<0.05$; ## and ** refer to $p<0.01$.

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