行政院國家科學委員會專題研究計畫　成果報告

骨母細胞受靜磁場刺激後進行增生與分化現象之機轉研究

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The effects of static magnetic field on the early differentiation stage of MG63 osteoblast-like cells

Abstract

MG63 osteoblast-like cells were exposed to static magnetic field (SMF). The effects of SMF on factors of osteoblastic differentiation at early stages were assessed by observed the change in cell number, productive levels of local regulatory factors and morphologic images. Our results showed that SMF exposure reduce the proliferative rate of MG63 cells with a cell density dependent manner. However, cell exposed to SMF tend to increase the level of various regulatory factors released in conditioned media when compared to the untreated cells. These findings provide an evidence for the hypothesis that SMF affect osteoblastic maturation at early stage.

1. Introduction

Osteoblast has three major roles: to synthesize and secret a calcifiable matrix, to calcification of the matrix, and to participate in the regulation of bone remodeling. The sequential expression of osteoblastic cell growth has been profiled by Stein & Lain (1993). During progressive development of bone cell phenotype, the sequence of gene expression has defined three distinct periods: down-regulation of proliferation, development and maturation of extracellular matrix (ECM), and ECM mineralization. During the period of proliferation down-regulation, type I collagen and transforming growth factor-β (TGF-β) are actively expressed and then gradually down-regulated. After the down-regulation of proliferation, alkaline phosphatase activity increased immediately, and extracellular matrices formed. When mineralization occurred, gene of osteopontin and osteocalcin are induced. On the other hand, matrix vesicles produced by cells have been observed in mineralizating tissues. They are small extracellular organelles enriched in alkaline phosphatase, proteoplipids, and calcium-phospholipid-phosphate complexes. Because hydroxyapatite crystals can be found to form on the matrix vesicle membrane surface, these organelles may serve as sites for initial crystal formation in calcification.

Several investigations indicated that SMF tend to influence the osteoblasts differentiation at early stage. However, the effects of SMF on factors of osteoblastic differentiation at early stages such as collagen type I, TGF-β, matrix vesicles, and

osteopontin are still unclear. In this study, we hypothesized that if SMF stimulates osteoblast differentiation at early stage, and it may drive the pre-osteoblast to a mature phenotype with a limited cultured density. Therefore, we examined the effects of SMF on early local factors of osteoblastic differentiation. On the other hand, morphologic changes of the cell after SMF exposure were assessed by analyzing the ultrastructural images.

2. Materials and methods

MG63 cells were maintained in Dulbecco's modified Eagle's medium, supplemented with L-glutamine, 10% fetal bovine serum (FBS), and 1% penicillin-streptomycin. Cells were seeding onto petri disheswith a cultivation surface of 78.5 cm^2 containing 10 ml culture medium. Cultures were incubated in a 5% CO_2 atmosphere at 37℃ and 100% humidity.

The cultured MG63 cells were divided into two groups, i.e., sham exposed group and exposed group, and were separately incubated inside of two identical incubators. Neodymium ($Nd_2Fe_{14}B$) magnets with a diameter of 10 cm and a thickness of 1 cm were used for providing static magnetic field. The average of the magnetic flux density on the surfacewas 0.4 T. The exposed group cells were transferred and placed on the magnets. The observation times were set at 12, 24, 48, and 72 hrs after SMF exposure. Triplicate tested were used for each observation time.

The effects of the SMF on the proliferative activity of the MG63s were evaluated by cell number assay. To assess the influences of cell density on the SMF effects, growth curves of the exposed cells were recorded with various plated densities of 5×10^3, 1×10^4, 5×10^4, and 1×10^5 cells/ml.

TGF-β1, osteopontin, type Ⅰ collagen production of MG63 cells exposed to SMF was measured in the conditioned media via an enzyme-linked immunoassay (ELISA) kit specific for human TGF-β1. Alkaline phosphatase activity in the MG63 cell layer was determined as the rate of conversion of *p*-nitrophenyl phosphate to *p*-nitrophenol at a pH of 10.2. SEM, and TEM were used for morphology and ultrastructure assessment. All the results including cell number, level of local regulatory factors, and enzyme activity measured in this study were presented as mean±SD and were consistent between triplicate experiments. In each experiment, the differences between controls and exposed cell were tested using Student's *t*-test. P values less

than 0.05 were considered significant.

3. Results

Figure 1 demonstrates growth curves for MG63 cells in incubator 1 (solid line) and 2 (dashed line) during simultaneous cell proliferation assays. The cells in the both incubators reveal a typical growth over the 72-hr period. At each observation time, no statistical difference in cell number was found between the two identical incubators. Figure 2 demonstrates the SMF effects on growth curves of MG63 cells plated with various densities. When the cells were plated on the dishes with a density of 1×10^4 cells/ml, SMF exhibits 10%-15% inhibition in cell proliferation during the periods between 24 to 72 hrs. When the plated density larger than 1×10^4 cells/ml, growth curve between SMF exposure and control groups reveal no significant differences throughout the 3 days tests. Because significant SMF effects on MG63 cells proliferation can be found only when the cells were plated with a initial density of 1×10^4 cells/ml, the plating density was used for the following experiments.

When assessed as a function of cell number, cells exposed to SMF exhibited higher TGF-β1 production than did the cells with sham exposure(Fig. 3a). The collagen level produced by the untreated cells was time-dependent decrease during the experimental period (Fig.3b). The PIP amount of exposure group maintained at a high level within 24 hrs, and then slightly decreased by 78% and 73% at 48 and 72 hrs compared with 0 hr, respectively. As showed in Fig. 3c, static magnetic field stimulation also significantly affected intracellular osteopontin production by MG63 cells. The maximal difference was observed at 12 hr, that osteopontin producrion was 1.6-fold greater in the exposed cultures compared with untreated control cells. At 48 and 72 hrs, the alkaline phosphatase activity of the SMF exposed cells was significantly greater than controls with increases of 1.34 and 1.18 fold, respectively (Fig. 3d).

As a result of SMF treatment, the MG63 cells changed from polygonal into more stellar shapes with extensive processes (Fig. 4b). In addition, they appeared to form multiple layers on the surface. In addition, Thick fiber and extracellular matrix accumulation can be found around the cells (Fig. 4d, e). The ultrastructure of the MG63 cells was observed by transmission electron microscopy. The most distinct different between untreated control and SMF exposed cells were found at the

extracellular area. Matrix vesicles released as a cluster from plasma membrane were first observed around stimulated cells at 24 hr (Fig. 5b). At 48 hr, a substantial amount of matrix vesicles released was observed (Fig. 5d). At the same time, matrix vesicles can also be observed close to the plasma membrane (Fig. 5e).

Conclusion

Taken together, we found SMF stimulation enhances the cells to exhibit a more differentiated phenotype by preventing the decrease of various factors production associated with early stage osteogenic differentiation. Sequentially, the SMF treated cells revealed an increase in alkaline phosphatase activity and displayed a more differentiated morphology, compared with untreated cells. Thus, base on these observations, we suggest that the effects of SMF on the MG63 osteoblast-like cells is to enhance the early phases of osteoblastic differentiation. In addition, our results show for the first time that osteoblast-like cells exposed to SMF exhibited a decrease in cell number was reveals as a manner of cell density dependence.

Figure legend

Fig. 1 Comparison of MG63 cell growth in the two identical incubators. Cell were seeded at 1×10^4 cells/ml. Cell numbers between SMF exposure and control groups reveal no significant differences at each observation time. Data are from one of the three replicate experiments, each yielding similar results (n= 3 cultures. * denotes $p<0.05$).

Fig. 2 The SMF effect on MG63 cells plating at four different initial densities were performed in different experiments. Only when the cells were plated on the dishes with a density of 1×10^4 cells/ml, SMF exhibits significant inhibition in cell proliferation. Data are from one of the three replicate experiments, each yielding similar results (n= 3 cultures. * denotes $p<0.05$).

Fig. 3 MG63 cell exposed to SMF increase the level of various local regulatory factors released in conditioned media when compared to the untreated cells. (a) and (b)

demonstrated that SMF exposure prevent the reduction rates of the TGF-β and type I collagen release from the cell, respectively. Further, SMF stimulation increased the expression of osteopontin at early maturation stage (c). After 48 hrs exposure, the alkaline phosphatase activity of the SMF exposed cells was significantly greater than controls (d). Data are from one of the three replicate experiments, each yielding similar results (n= 3 cultures. * denotes $p<0.05$).

Fig. 4 SEM of MG63 cells treated (b and d) and untreated (a and c) with SMF. After 24-hrs SMF treatment, cells appeared to form multiple layers with extensive processes (b). Abundant fiber and extracellular matrix accumulation (arrow) can be found around the cells after 48 hrs SMF exposure (d), and shown in greater magnificent in e. Bar equals 100 μm (a, b, c, and d), and 20 μm (e).

Fihg.5 TEM of MG63 cells treated (b and d) and untreated (a and c) with SMF. After SMF treatment, cells appeared to release matrix vesicle around the cells (b and d). Abundant matrix vesicles accumulated close to the plasma membrane ion (arrow) can be found after 48 hr SMF treatment. Bar equals 12 μm (a, b, c, and d), and 2 μm (e).

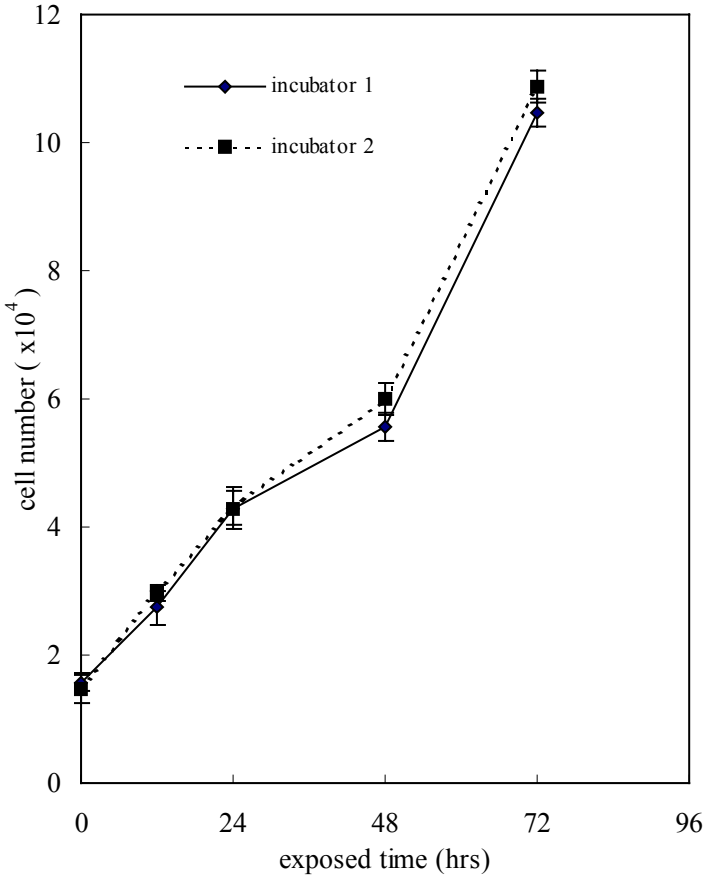
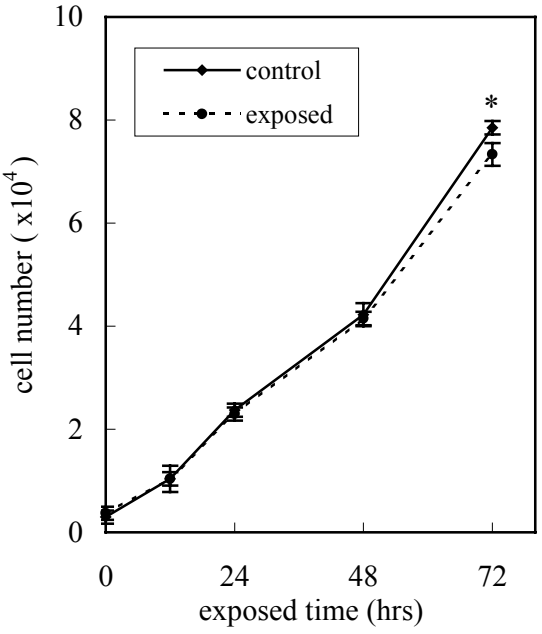
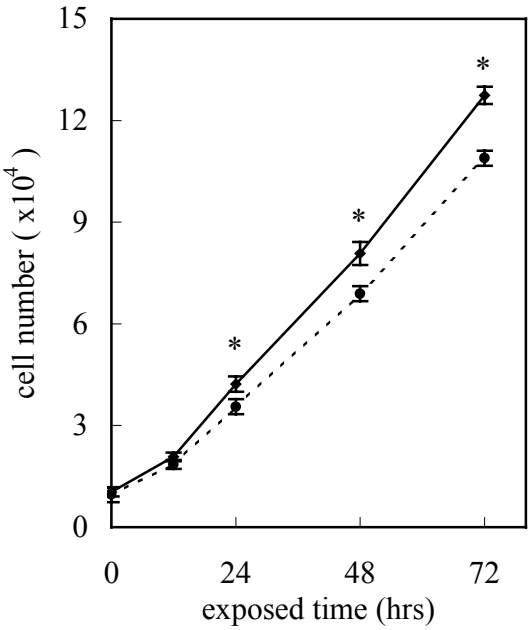

12
10
8
6
4
2
0
cell number (x10^4)
incubator 1
incubator 2
0
24
48
72
96
exposed time (hrs)

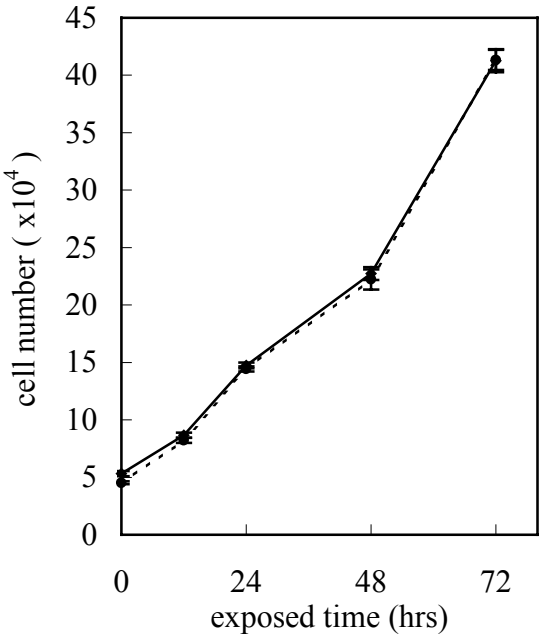
Fig. 1


control
exposed
cell number (x10^4)
exposed time (hrs)
0
2
4
6
8
10
0
24
48
72
*

(a)


cell number (x10^4)
exposed time (hrs)
0
3
6
9
12
15
0
24
48
72
*
*
*

(b)


cell number (x10^4)
exposed time (hrs)
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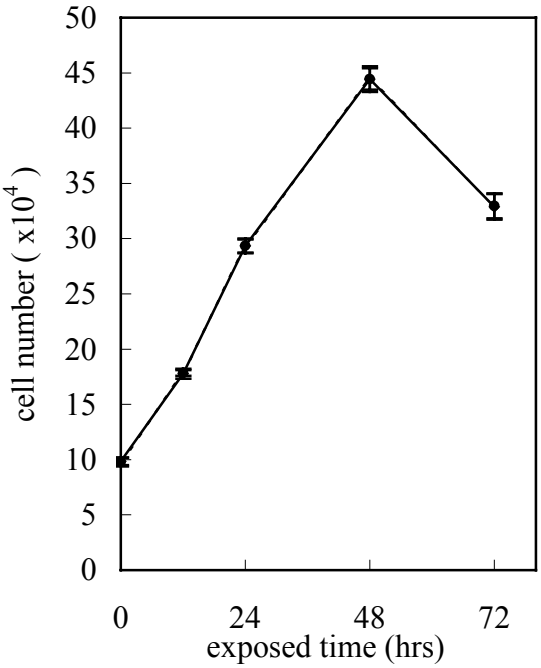
5
10
15
20
25
30
35
40
45
0
24
48
72

(c)


cell number (x10^4)
exposed time (hrs)
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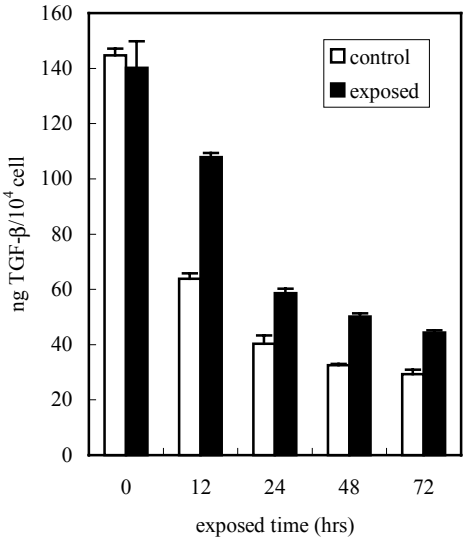
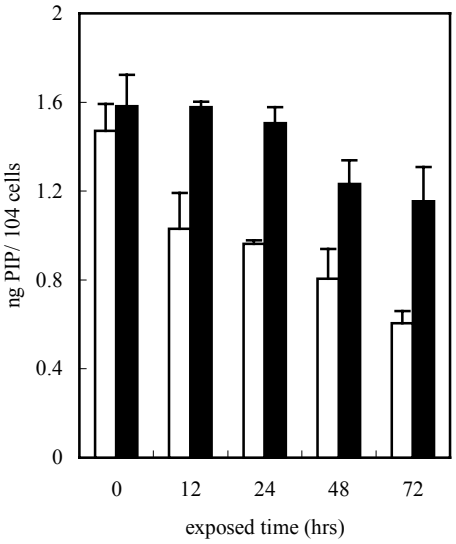
5
10
15
20
25
30
35
40
45
50
0
24
48
72

(d)

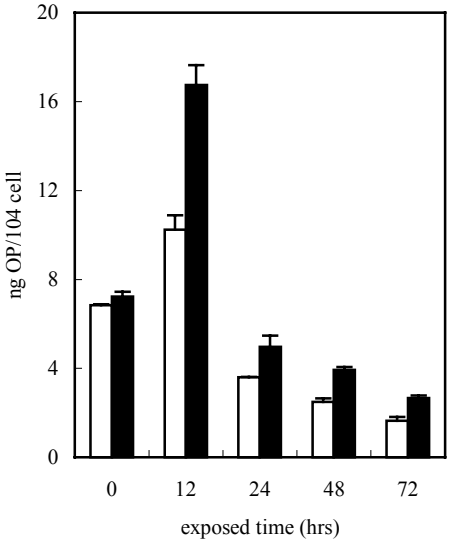
Fig. 2


160
140
120
100
80
60
40
20
0
control
exposed
ng TGF-β/10^4 cell
0
12
24
48
72
exposed time (hrs)

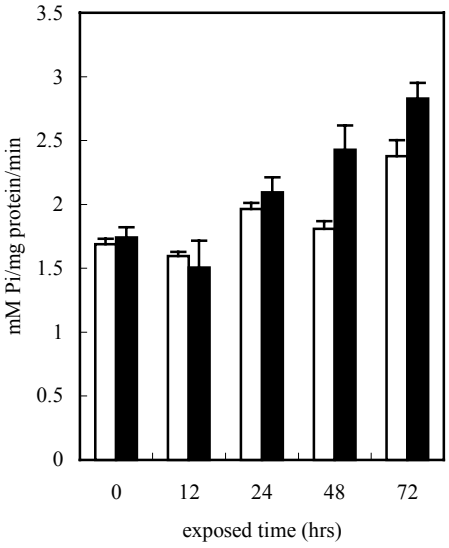
(a)


2
1.6
1.2
0.8
0.4
0
ng PIP/ 104 cells
0
12
24
48
72
exposed time (hrs)

(b)


20
16
12
8
4
0
ng OP/104 cell
0
12
24
48
72
exposed time (hrs)

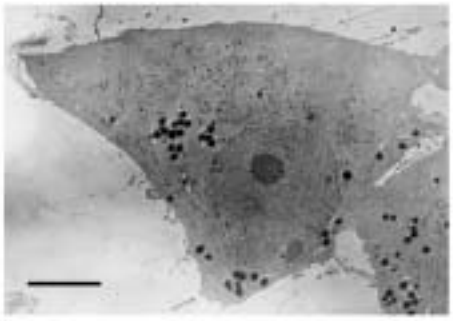
(c)


3.5
3
2.5
2
1.5
1
0.5
0
mM Pi/mg protein/min
0
12
24
48
72
exposed time (hrs)

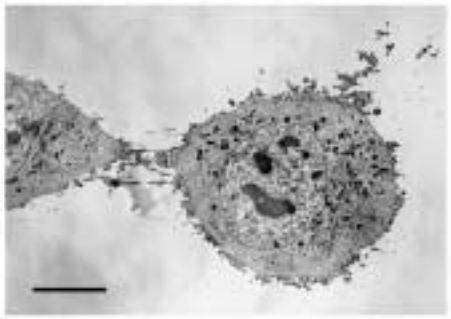
(d)

Fig. 3

24 hrs

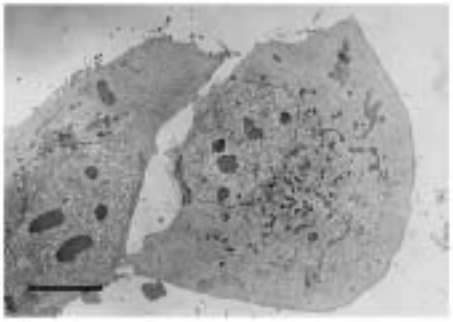


(a)

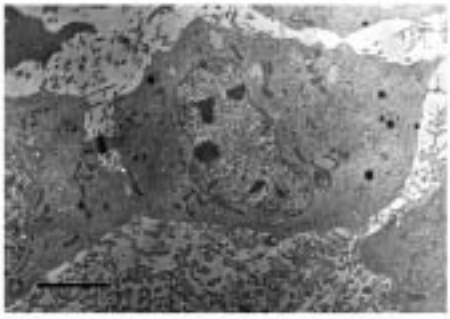


(b)

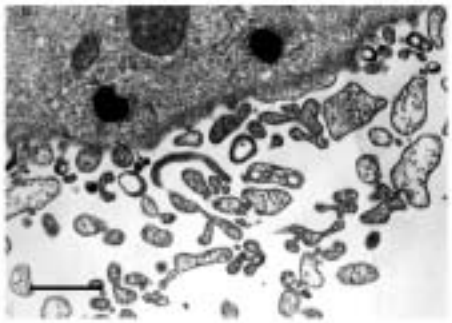
48 hrs



(c)



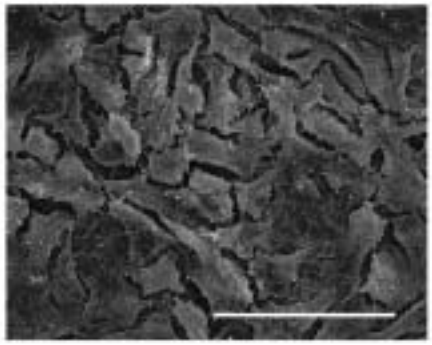
(d)



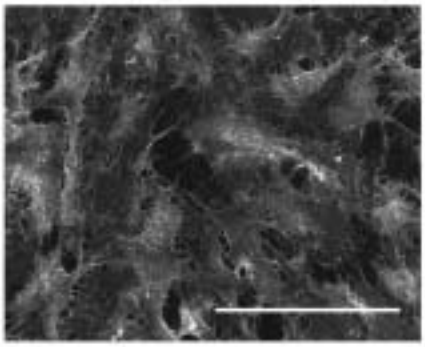
(e)

Fig. 4

24 hrs

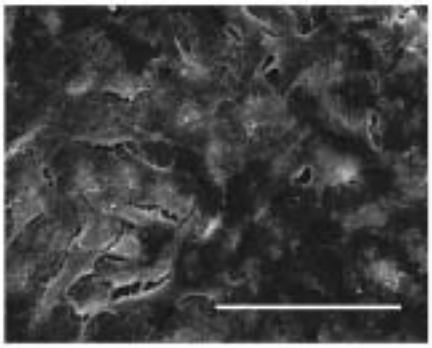


(a)



(b)

48 hrs



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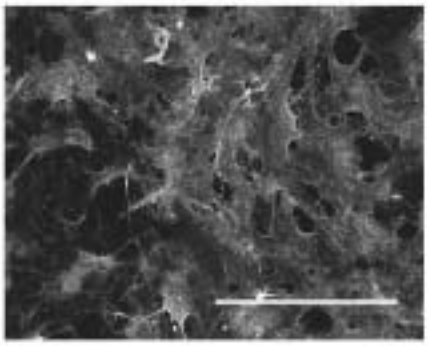

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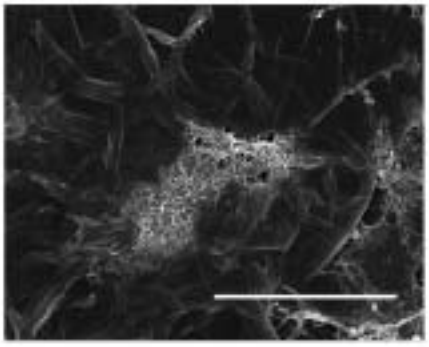

(e)

Fig. 5