31st Annual International Conference of the IEEE EMBS
Minneapolis, Minnesota, USA, September 2-6, 2009

Differentiation of Pluripotent Stem Cells on Multiwalled Carbon Nanotubes

Jon Holy, Edward Perkins, and Xun Yu

Abstract—**This paper studies the adhesion, growth, and differentiation of stem cells on carbon nanotube matrices. Glass coverslips were coated with multiwalled carbon nanotube (MWNT) thin films using layer-by-layer self-assembling techniques. Pluripotent P19 mouse embryonal carcinoma stem cells were seeded onto uncoated or MWNT-coated coverslips, and either maintained in an undifferentiated state or induced to differentiate by the addition of retinoic acid. We found that cell adhesion was increased on the MWNT-coated surfaces, and that the expression patterns of some differentiation markers were altered in cells grown on MWNTs. The results suggest that MWNTs will be useful in directing pluripotent stem cell differentiation for tissue engineering purposes.**

I. Introduction

Combining nanotechnology approaches with tissue engineering efforts promises to remarkably extend the biomedical applications of these disciplines. A particularly intriguing and newly emerging field involves testing how nanomaterials can be used to influence stem cell differentiation. Because of their abilities to self-renew, differentiate, and give rise to various types of adult somatic cells, stem cells have been recognized as potentially revolutionary medical tools. The purpose of this study is to test whether multiwalled carbon nanotubes (MWNTs) are able to support the growth and differentiation of stem cells *in vitro*, and if so, how these processes compare to those occurring under standard culture conditions.

Since their discovery by S. Iijima in 1991 [1], carbon nanotubes have been widely used for a variety of applications due to their mechanical, electrical, and electrochemical properties. Their extremely high aspect ratio (>200) promotes network formation and a high surface-area-to-volume ratio, which facilitates their use as biosensors, biological transporters, tumoricidal agents, DNA assembly scaffolds, and substrates for cell adhesion and growth in tissue engineering efforts [2-7]. Recently, the interactions between CNTs and various types of stem cells have begun to be studied. To date, most stem cell studies have employed cells of restricted developmental potential, such as neuronal stem cells and mesenchymal stem cells. Sridharan et al. [8] studied the ability of a combination collagen/single-walled carbon nanotube substrate to direct the differentiation of human embryonic stem cells, but little else is known about the interaction of carbon nanotubes with pluripotent cells. In this paper, we studied alterations in morphology, adhesion, and expression patterns of differentiation-associated genes in the pluripotent P19 stem cell line.

The P19 cell line was derived from a teratocarcinoma resulting from the introduction of seven-day-old mouse embryos into adult mouse testes [9]. They are a popular developmental model because they exhibit pluripotency, having the ability to differentiate in vitro into cell lineages representing all three germ layers [10]. Retinoic acid is commonly used to induce differentiation of P19 cells into both endoderm cells and neurons. In this study, we examined the ability of MWNTs to influence the retinoic acid-stimulated developmental program of P19 cells in vitro.

II. Experiments

A. Preparation of MWNT substrates for stem cell culture

The high purity MWNTs (>95%) for this study were supplied by Timesnanoweb (Chengdu, China), and were synthesized using chemical vapor deposition (CVD) techniques. Cell culture glass coverslips were coated with MWNT thin films using layer-by-layer self-assembly techniques [11]. MWNTs were treated with a mixture of sulfuric acid and nitric acid to facilitate dispersal in aqueous solution. Glass coverslips were immersed in 1.5 wt % poly(diallyldimethylammonium chloride) PDDA solution (with 0.5 M NaCl), which introduces positively a charged PDDA molecular layer on the surfaces. Glass coverslips were then alternately dipped in MWNT and PDDA solutions for three cycles, and then dried at 50°C. Fig. 1 shows a scanning electron microscope (SEM) picture of a MWNT thin film produced by this method.

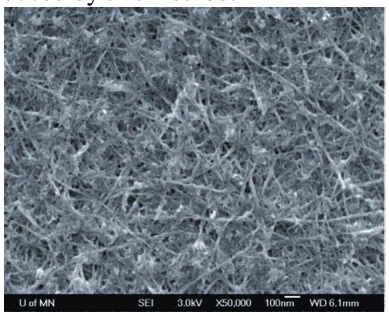

Fig. 1. SEM picture of MWNT thin film

Manuscript received June 18, 2009.

J. Holy is with the Department of Anatomy, Microbiology and Pathology, University of Minnesota School of Medicine-Duluth, Duluth, MN 55812, USA (e-mail:jholy@d.umn.edu).

E. Perkins is with the Department of Biomedical Sciences, Mercer University School of Medicine, Savannah Campus, Savannah, GA 31404, USA (e-mail: PERKINS_EL@mercer.edu).

X. Yu is with the Department of Mechanical and Industrial Engineering, University of Minnesota, Duluth, MN 55812, USA (tel: 218-726-8415, fax: 218-726-8596, e-mail: xunyu@d.umn.edu).

B. Stem cell culture and differentiation

P19 mouse embryonal carcinoma cells (ATCC, Manassas, VA) were maintained in Dulbecco's modified Eagle's medium, containing 8% Fetal Clone III (HyClone, Logan, UT). Cells were seeded onto uncoated glass coverslips or glass coverslips coated with MWNTs and grown in the absence (for undifferentiated cells) or presence of 1 micromolar all-*trans* retinoic acid to induce differentiation for four days.

C. Cell adhesion experiments

Cell adhesion was measured by seeding 100,000 cells/ml on multiple uncoated or coated coverslips, and after 24 hours, exposing coverslips to a diluted trypsin-EDTA solution (1:3 dilution of a liquid solution of 0.25% trypsin and 1 mM EDTA, Invitrogen, Carlsbad, CA) for six minutes. Coverslips were then gently rinsed in PBS and fixed with cold (-10C) absolute methanol-1% acetic acid for at least 20 minutes. Coverslips were air-dried and stained with sulforhodamine B (SRB) as described by Skehan et al. [1990]. Eluted SRB was measured spectrophotometrically at 540 nm.

D. Fluorescence labeling

Cells were fixed either with 4% paraformaldehyde in PBS for rhodamine-phalloidin labeling, or cold (-10C) absolute methanol for antibody labeling. Fixed samples were subsequently labeled with either rhodamine-conjugated phalloidin (Invitrogen) for filamentous actin, or antibodies to cytokeratin 8 (TROMA-1 from Developmental Studies Hybridoma Bank, Iowa City, IA), or beta-3-tubulin (TUJ-1 from Covance, Denver, PA).

E. Western blotting

For Western blotting, coverslips were rinsed in PBS, and Laemmli sample buffer added to solubilize proteins. Sample buffer was collected, sonicated, and heated to 95C for 5 minutes before storage at -75C. Equal protein loads were separated on polyacrylamide gels and transferred to nitrocellulose membranes, which were probed with antibodies to Oct3/4 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), cytokeratin 8 (TROMA-1), beta-3-tubulin (TUJ-1), and lamin B (the kind gift of Dr. H. Worman, Columbia University, N.Y.).

III. Results and Discussion

A. Cell adhesion experiments

Adhesion experiments demonstrate that P19 cells adhere more strongly to MWNTs than to glass (Fig. 2). About 42% of the P19 cells remained attached to MWNT-coated coverslips after six minutes in trypsin-EDTA, whereas only about 12% remained attached to uncoated glass coverslips at the same time point. Laser scanning confocal microscopy of P19 cells fixed 24 hours after seeding does not reveal any marked differences in the degree of flattening of the cells on uncoated and MWNT-coated coverslips, however (Fig. 3).

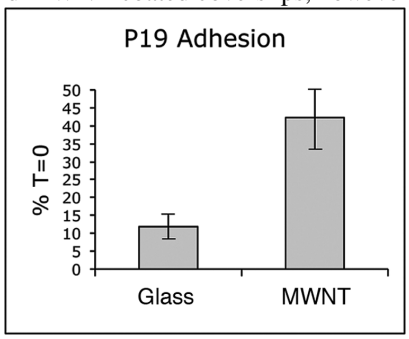

Fig. 2. Graph showing percent of cells remaining on uncoated or MWNT-coated coverslips after six minutes of trypsin-EDTA treatment, expressed as percent of a control coverslip fixed just before addition of trypsin-EDTA (t=0). Average of four experiments; error bars show standard error of the mean.

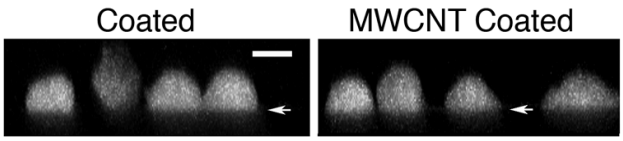

Fig. 3. Side view of undifferentiated P19 cells fixed with glutaraldehyde to preserve morphology and impart autofluorescence. Side views were obtained by rotating a projected z-stack of optical sections 90 degrees. The white arrows identify the surface of the coverslips. Scale bar equals 10 micrometers.

To test whether the increased adhesion of P19 cells on MWNT-coated coverslips was accompanied by alterations in the actin cytoskeleton, filamentous actin (f-actin) was labeled with rhodamine phalloidin. By phase contrast microscopy, P19 cells on uncoated coverslips display their typical stem cell morphology as small cells with relatively smooth outlines and a high nucleus-to-cytoplasm ratio that tend to grow in clusters (Fig. 4). In contrast, a few differences become noticeable in cultures of cells grown on MWNTs. The cells appear to become somewhat more flattened after four days, and many cells exhibit more irregular outlines, reflecting the presence of filopodial cell extensions. Time-lapse movies show that these extensions are highly dynamic structures, continually extending and retracting (not shown).

B. Effects of MWNTs on differentiation

To determine whether growth on MWNTs affected the P19 program of RA-induced differentiation, cells on uncoated and MWNT-coated coverslips were cultured in the presence of 1 micromolar RA for four days, and then labeled with antibodies to cytokeratin 8, an endodermal marker, and beta-3-tubulin, a neuronal marker. After RA treatment, cells can be found both in clusters, and as more dispersed individual cells that are usually more flattened. Differentiation into both neurons and endoderm cells occurs, with the former residing predominantly in cell clusters, and the latter representing many of the individual, flattened cells. Whereas neuronal differentiation was similar on both uncoated and MWNT-coated coverslips, there appeared to be fewer endodermal

cells displaying strong labeling for cytokeratin 8 intermediate filaments on MWNT-coated coverslips (Fig. 5).

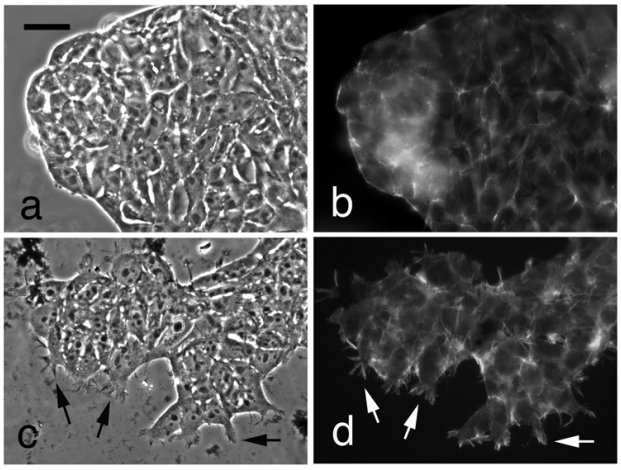

Fig. 4. P19 cells labeled with rhodamine-phalloidin to show the filamentous actin cytoskeleton. (a) and (c) show clusters of cells on uncoated and MWNT-coated coverslips, respectively, by phase-contrast microscopy. (b) and (d) show f-actin organization in the same cells by epifluorescence microscopy. Note the filopodial cell extensions in the MWNT culture (arrows, c and d). Scale bar in (a) equals 25 micrometers.

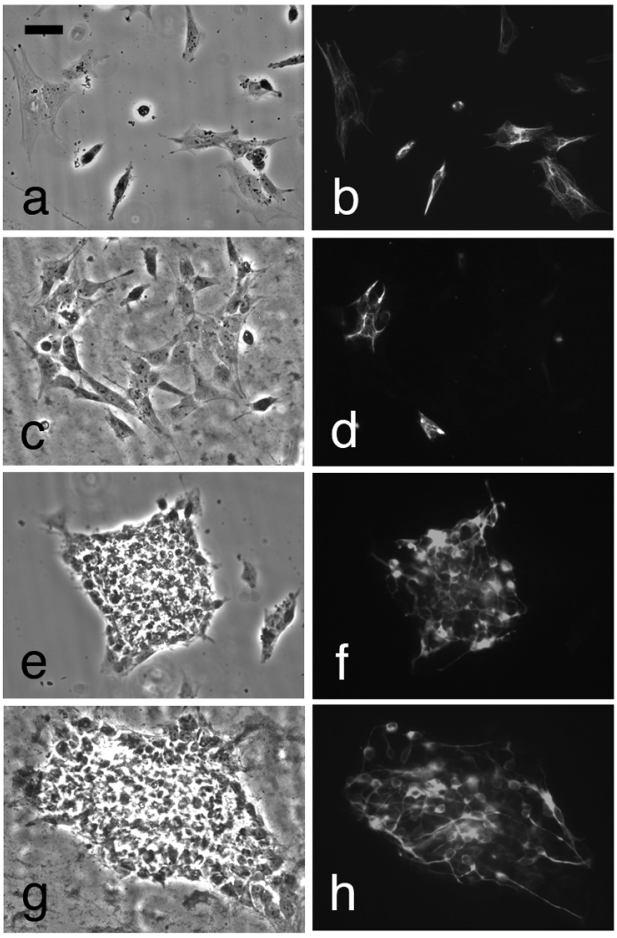

Fig. 5. P19 cells induced to differentiate for four days with RA, and then fixed and labeled with antibodies to cytokeratin 8 (a-d), or beta-3-tubulin (e-h). a,b,e, and f show cells on uncoated glass coverslips; c,d,g and h show cells on MWNT-coated coverslips. MWNT-coated coverslips appeared to contain slightly higher numbers of cells expressing neuronal tubulin, but fewer cells containing well-developed arrays of cytokeratin 8 intermediate filaments. Scale bar in (a) equals 25 micrometers.

Western blots indicate that neuronal tubulin was strongly upregulated to a similar extent in differentiating cells on both uncoated and MWNT-coated coverslips (Fig. 6). In addition, P19 cells grown on uncoated coverslips displayed the typical pattern of a low level of cytokeratin 8 expression in undifferentiated cultures, with increased expression in RA-treated cultures due to endodermal differentiation. By contrast, cells grown on MWNT-coated coverslips exhibit an increased level of cytokeratin 8 expression in the undifferentiated state, which then appears to be suppressed after the addition of RA.

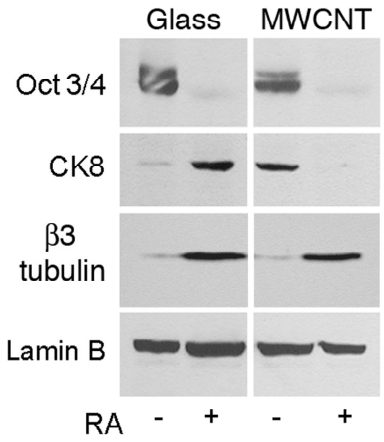

Fig. 6. Western blot showing expression of the differentiation-related markers Oct3/4, cytokeratin 8 (CK8), and beta-3-tubulin. The normal developmental patterns of downregulated Oct3/4 and upreregulated beta-3-tubulin occur in both uncoated and MWNT-coated coverslips after RA treatment. However, the expression patterns of CK8 are altered in cells grown on MWCNT-coated coverslips. Lamin B is a nuclear structural protein that generally does not change during differentiation and is included as a protein loading control.

Expression patterns of the differentiation marker Oct3/4 was similar on both uncoated and MWNT-coated coverslips, with a high level of expression in undifferentiated cells, and a loss of expression in differentiating cells.

C. Relationship between substrate interactions and differentiation

The physical dimensions of carbon nanotubes (CNTs) make them interesting candidates as artificial substrates for cell growth, mimicking some of the structural features of the extracellular matrix (ECM). The ECM provides a number of crucial signals that cells integrate into decisions impacting cell function, including the process and direction of differentiation. Thus, manipulation of the ECM is a critically important factor in the development of tissue engineering and regeneration techniques [13]. Supporting the concept that CNT substrates could have significant influences on

stem cell function, studies have shown these types of substrates to affect stem cell morphology, adhesion, proliferation, and metabolic activity [14-16].

A number of investigators have examined the influence of CNTs on mesenchymal stem cells, and have tested their ability to modulate the differentiation and survival of bone, cartilage, and adipose cells [5], [17], [18]. Interestingly, emerging data suggests that CNTs may constitute a particularly favorable environment for neuronal differentiation. Neurons from explanted central and peripheral nervous tissues survive and develop growth cones and neuronal processes on carbon nanotube sheets to a comparable extent as established, optimized approaches [14]. Neural stem cells have been shown to differentiate on CNT substrates [19], [20], and neuronal differentiation of human embryonic stem cells is enhanced on a hybrid collagen-CNT substrate [8].

In this study, MWNTs constituted a suitable substrate for neuronal differentiation, but appeared to be less permissive for endodermal differentiation. Actually, western blots of cytokeratin 8 levels indicate that the situation may be more complex; it is possible that growth on MWNTs triggers an initial upregulation of cytokeratin 8, but responses to RA are then significantly altered such that endodermal differentiation is aborted, or at least progresses in a direction that exhibits changes in typical reporter gene expression patterns.

D. Toxicity concerns of carbon nanotubes

The biocompatibility and cytotoxicity of CNTs is a fundamental issue that needs to be thoroughly investigated. Currently, our understanding of CNT biocompatibility and toxicity is fragmentary. Zanello *et. al.* [5] found CNTs can sustain osteoblast growth and bone formation, and Meng *et. al.* [7] described enhanced cell adhesion and proliferation on CNTs. However, Cui *et. al.* [6] found that single-walled CNTs inhibit human HEK293 cell proliferation through decreased substrate adhesion. In this study, we did not observe any reductions in cell proliferation, or increases in cell death, in the MWNT cultures. There also appears to be a lack of long-term effects, as preliminary experiments indicate that the rate of cell proliferation of P19 cells initially grown on MWNTs but subsequently transferred to uncoated coverslips is the same as cells grown continuously on uncoated coverslips (not shown).

IV. CONCLUSION

Altogether, our results indicate that MWNTs are at least permissive, and may facilitate, neuronal differentiation of P19 stem cells. Notably, the reduction in the numbers of cells with well-developed cytokeratin 8 cytoskeletons suggests that MWNTs may be useful to help direct stem cell differentiation which, in this case, involves a reduction in endodermal differentiation and a possible facilitation of neuronal differentiation.

REFERENCES

[1] S. Iijima, "Helical microtubes of graphitic carbon," *Nature*, vol.354, pp. 56-58, 1991.

[2] K. Balasubramanian, and M. Burghard, "Biosensors based on carbon nanotubes," *Anal. Bioanal. Chem.*, vol. 385. pp. 452-468, 2006

[3] N. W.S. Kam, M. O'Connell, J. A. Wisdom, and H. Dai, "Carbon nanotubes as multifunctional biological transporters and near-infrared agents for selective cancer cell destruction," *PNAS*, vol. 102, pp. 11600-11605, 2005.

[4] K. Rege, G. Viswanathan, G. Zhu, A. Vijayaraghavan, P. Ajayan, and J. S. Dordick, "In vitro transcription and protein translation from carbon nanotube-DNA assembles," *Small*, vol. 2, pp. 718-722, 2006

[5] L. P. Zanello, B. Zhao, H. Hu, and R. C. Haddon, "Bone cell proliferation on carbon nanotubes," *Nano Letters*, vol. 6, pp.562-567, 2006.

[6] D. Cui, F. Tian, C. S. Ozkan, M. Wang, and H. Gao, "Effect of single wall carbon nanotubes on human HEK293 cells," *Toxicology Letters*, vol. 155, pp.73-85, 2005.

[7] J. Meng, L. Song, J. Meng, H. Kong, G. Zhu, C. Wang, L. Xu, S. Xie, and H. Xu, "Using single-walled carbon nanotubes nonwoven films as scaffolds to enhance long-term cell proliferation in vitro," *Journal of Biomedical Materials Research, Part A*, vol.79, pp. 298-306, 2006.

[8] I. Sridharan, T. Kim, and R. Wang, "Adapting collagen/CNT matrix in directing hESC differentiation," *Biochem. Biophys. Res. Commun*., vol. 381, pp. 508-512, 2009.

[9] M.W. McBurney, E.M. Jones-Villeneuve, M.K. Edwards, and P.J. Anderson, "Control of muscle and neuronal differentiation in a cultured embryonal carcinoma cell line," *Nature*, vol. 299, pp. 165-167, 1982.

[10] D.R. Soprano, B.W. Teets, and K.J. Soprano, "Role of retinoic acid in the differentiation of embryonal carcinoma and embryonic stem cells," *Vitam. Horm.,* vol. 75, pp. 69-95, 2007.

[11] X. Yu, R. Rajamani, K. A. Stelson, and T. Cui, "Fabrication of carbon nanotube based transparent conductive thin films using layer-by-layer technology", *Surface & Coating Technology*, vol.202, pp. 2002-2007, 2008.

[12] P. Skehan, R. Storeng, D. Scudiero, A. Monks, J. McMahon, D. Vistica, J.T. Warren, H. Bokesch, S. Kenney, and M.R. Boyd, "New colorimetric cytotoxicity assay for anticancer-drug screening," *J. Natl. Cancer Inst.,* vol. 82, pp. 1107-1112, 1990.

[13] J.A. Burdick, and G. Vunjak-Novakovic, "Engineered microenvironments for controlled stem cell differentiation," *Tissue Eng., Part A*, vol. 15, pp. 205-219, 2009.

[14] P. Galvan-Garcia, E. W. Keefer, F. Yang, M. Zhang, S. Fang, A. Zakhidov, R. H. Baughaman, and M. I. Romero, "Robust cell migration and neuronal growth on pristine carbon nanotube sheets and yarns," *J. Biomater. Sci. Polymer Edn,* vol. 18, pp. 1245-1261, 2007.

[15] M. Kalbacova, M. Kalbac, L. Dusch, H. Kataura, and U. Hempel, "The study of the interaction of human mesenchymal stem cells and monocytes/macrophages with single-walled carbon nanotube films," *Phys. Stat. Sol. (b)*, vol. 243, pp.3514-3518, 2006.

[16] S. Y. Park, S. Y. Park, S. Namgung, B. Kim, J. Im, J. Y. Kim, K. Sun, K. B. Lee, J.-M. Nam, Y. Park, and S. Hong, "Carbon nanotube monolayer patterns for directed growth of mesenchymal stem cells," *Advanced Materials*, vol. 19, pp. 2530-2534, 2007.

[17] E. Mooney, P. Dockery, U. Greiser, M. Murphy, and V. Barron, "Carbon nanotubes and mesenchymal stem cells: biocompatibility, proliferation and differentiation," *Nano Letters*, vol. 8, pp. 2137-2143, 2008.

[18] S. Mwenifumbo, M.S. Shaffer, and M.M. Stevens, "Exploring cellular behaviour with multi-walled carbon nanotube constucts," *J. Materials Chem*., vol. 17, pp. 1894-1902, 2007.

[19] E. Jan, and N. A. Kotov, "Successful differentiation of mouse neural stem cells on layer-by-layer assembled single-walled carbon nanotube composite," *Nano Letters*, vol. 7, pp. 1123-1128, 2007.

[20] N.W.S Kam, E. Jan, and N.A. Kotov, "Electrical stimulation of neural stem cells mediated by humanized carbon nanotube composite made with extracellular matrix protein," *Nano Letters*, vol. 9, pp. 273-278, 2009.