Neural differentiation regulated by biomimetic surfaces presenting motifs of extracellular matrix proteins

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Abstract: The interaction between cells and the extracellular matrix (ECM) is essential during development. To elucidate the function of ECM proteins on cell differentiation, we developed biomimetic surfaces that display specific ECM peptide motifs in a controlled manner. Presentation of ECM domains for collagen, fibronectin, and laminin influenced the formation of neurites by differentiating PC12 cells. The effect of these peptide sequences was also tested on the development of adult neural stem/progenitor cells. In this system, collagen I and fibronectin induced the formation of beta-III-tubulin positive cells, whereas collagen IV reduced such differentiation. Biomimetic surfaces composed of multiple peptide types enabled the combinatorial effects of various ECM motifs to be studied. Surfaces displaying combined motifs were often predictable as a result of the synergistic effects of ECM peptides studied in isolation. For example, the additive effects of fibronectin and laminin resulted in greater expression of beta-III-tubulin positive cells, whereas the negative effect of the collagen IV domain was canceled out by coexpression of collagen I. However, simultaneous expression of certain ECM domains was less predictable. These data highlight the complexity of the cellular response to combined ECM signals and the need to study the function of ECM domains individually and in combination. © 2009 Wiley Periodicals, Inc. J Biomed Mater Res 93A: 824–832, 2010

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INTRODUCTION

The differentiation of cells in tissues is affected by a complex combination of different factors within their local environment. These factors include soluble molecules, local geometry, and interactions with the extracellular matrix (ECM), which together form a niche and allow for correct cellular development and tissue function.[1] Direct evidence that local microenvironments are ideal for certain cell types has been demonstrated when neural progenitor cells transplanted into the subventricular zone, rostral migratory stream, hippocampus, or striatum undergo neurogenesis in a similar fashion to endogenous cells.[2]

Cell culture is widely used to create model systems to study cell behavior *in vitro*. However, such models are often not representative of the *in vivo* environment resulting in cell behavior that is likely to be abnormal. Consequently, efforts have been made to recreate the complex interactions observed *in vivo* to produce *in vitro* models that more faithfully mimic the environment cells experience *in situ*. Within the tissues, cells are in contact with other cells via the ECM which plays an important role in

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controlling cell behavior. These interactions can be recreated *in vitro* by coating surfaces with ECM molecules.[3] For example, cells in the subventricular zone of the brain grow in contact with ECM proteins, collagen I, and laminin.[4] Combined with the correct culture media, immobilization of the ECM proteins, entactin, collagen, and laminin, onto the culture surface partially recreates *in vivo* growth conditions and allows the differentiation of murine embryonic stem cells into cells characteristic of the subventricular zone.[5]

Previous work has demonstrated that immobilization of whole ECM proteins, such as fibronectin, results in conformational changes in the molecule.[6,7] Alteration of the molecular conformation can result in decreased ECM activity through reducing the availability of functional domains to the cell.[8] In some instances of coating surfaces with whole ECM proteins, multiple layers of molecules are produced instead of a single monolayer.[9] This can result in some molecules being hidden by those above, making it difficult to determine the actual amount of ECM protein available to interact with the cultured cells. The effects produced by ECM proteins can usually be attributed to short peptide sequences or motifs. By immobilizing these motifs on surfaces, it is possible to imitate the behavior elicited by the whole molecule. Peptides can be immobilized to surfaces in a controllable manner with a defined conformation and hence it is sometimes advantageous to present the functional peptide motif(s) as opposed to the whole ECM molecule. Many factors contribute to the effective presentation of peptide motifs within biomimetic surfaces, including density of the molecules,[10] the nature of the substratum supporting the biomimetic surface,[11] the distance from the surface,[12–14] and the conformation of the molecules presented on the surface.[15,16] Accordingly, the method by which ECM peptide motifs are presented by the biomimetic surface is important.

We have previously demonstrated that peptide motifs can be engineered into the extracellular loops of outer membrane protein A (OmpA). We have engineered such Omps to form self assembled monolayers (SAMs) where the protein is correctly oriented on a solid surface, enabling the presentation of the peptide in a highly controlled manner.[17] This technology can be used in cell culture applications and we have recently demonstrated that presenting cell adhesion motifs using this technology enhanced attachment of cultured cells to the growth surface.[18] In this study, we have used this method of creating biomimetic surfaces to investigate the function of certain ECM protein sequences during neural development. Through presenting specific ECM peptides either individually or in combination, we can identify those domains that play a role in the differentiation of cultured neural cells. This approach can be used to assist in the development of defined culture conditions for the generation of specific cell types for use in basic research and tissue engineering.

MATERIALS AND METHODS

Culture and differentiation of PC12 cells

PC12 cultures were purchased from American Type Culture Collection (ATCC; http://www.lgcpromochem-atcc.com) and maintained as previously described.[19] Stock cultures were grown on cell culture plasticware (VWR; http://uk.vwr.com) coated with a 0.1% solution of collagen IV (Sigma-Aldrich; http://www.sigmaaldrich.com) in 0.25% glacial acetic acid (Fisher Scientific, http://www.fishersci.com) in RPMI 1640 medium (Cambrex; http://www.cambrex.com) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen; http://www. invitrogen.com), 2 m*M* L-glutamine (Cambrex), 20 units/mL of penicillin and 20 μg/mL of streptomycin (Invitrogen). Cultures were maintained under standard conditions at 37°C in a humidified 5% CO_2 incubator. The medium was changed three times a week and cells passaged as required using trypsin-EDTA solution as standard.

In preparation for differentiation, a single cell suspension of PC12 cells was achieved by trypsinization and the number of cells determined using a hemocytometer. Cells were seeded at a density of 300,000 cells/well of a 6-well culture plate (VWR) containing cover slips presenting alternative growth surfaces. Cells were maintained at 37°C in a humidified, 5% CO_2 incubator for 24 h. The medium was replaced with 50 ng/mL nerve growth factor (NGF, Sigma-Aldrich) containing media and cells differentiated for 10 days with medium changes every 3 days. Imunocytochemical analysis was carried out as detailed below. Experiments were repeated at least three times. Images were captured of 20 randomly selected single differentiated PC12 cells per condition. Cells were examined and the number of neurites per cell body was recorded. Also the length of the longest neurite was measured and the number of neurite branches was determined. Neurite branching data was normalized to neurite length to take into account that longer neurites are more likely to have an increased number of branches.

Culture and differentiation of neural/stem progenitor cells (NSPCs)

NSPCs were isolated from the subventricular zone (SVZ) of the lateral ventricles of the adult enhanced GFP transgenic male Wistar rat forebrains, as described previously.[20] Briefly, subependymal tissue was harvested from 8–12 week old rats and subjected to papain dissociation (Papain Dissociation System; Worthington Biochemical Corporation; http://www.worthington-biochem.com). The resultant cell suspension was centrifuged and the pelleted cells were subjected to a discontinuous density gradient to remove cell debris. Dissociated cells were resuspended in

complete medium (CM) containing Neurobasal media (Invitrogen), B27 neural supplement (Invitrogen), 2 m*M* L-glutamine (Sigma-Aldrich), 100 μg/mL penicillin-streptomycin (PenStrep; Sigma-Aldrich), 20 ng/mL epidermal growth factor (recombinant human EGF; Gibco-Invitrogen), 20 ng/mL basic fibroblast growth factor (recombinant human bFGF; Invitrogen) and 2 ng/mL heparin (Sigma-Aldrich) and incubated at 37°C in a humidified incubator with 5% CO_2. GFP-positive neurospheres appeared in 2–3 weeks, after which cells were passaged every week.

Neurospheres were mechanically dissociated using a pipette to produce a single cell suspension and the number of cells was determined using a hemocytometer. Cells were subsequently seeded at a density of 400,000 cells/well of a 6-well culture plate containing cover slips presenting alternative growth surfaces. Cells were maintained at 37°C in a humidified, 5% CO_2 incubator for 24 h in CM. The medium was replaced with differentiation medium containing Neurobasal media; B27 neural supplement, 2 m*M* L-glutamine, 100 μg/mL penicillin-streptomycin, and 1% fetal bovine serum (FBS, Invitrogen) and cells were differentiated for 4 days. Immunocytochemical analyses were carried out (see below), cells were visualized using a fluorescence microscope and digital images recorded. Experiments were repeated in triplicate. Images were captured from nine random fields of view per condition per replicate, which equated to a total of 27 fields of view per surface type tested. Neural differentiation was assessed by the number of beta-III-tubulin positive cells observed per field of view. These data were normalized against the number of beta-III-tubulin positive cells observed on the control (OmpA) surface and the results were plotted as the change in number of beta-III-tubulin positive cells observed when compared with the control surface.

Biomimetic surface preparation

Surfaces were prepared as previously described.[17,18,21] We have previously shown by polarized neutron deflection data that the OmpA molecules attach in an oriented manner with the molecules standing perpendicular to the gold substrate and displaying the engineered motifs on the uppermost surface.[22] Furthermore, it has been shown by surface plasmon resonance that the orientation of the protein monolayer was controlled by specific attachment of the protein molecules to the gold layer mediated by the single cysteine residue present in each protein molecule. The SAM assembly conditions resulted in a reproducible and homogeneous, high density protein layer of ~1.5 ng protein/mm^2 as determined by surface plasmon resonance (data not shown). When compared with the theoretical maximum 2D crystal protein density of 5.7 ng/mm^2, this equates to ~26% of the total surface being covered with protein fitting well with the percentage protein coverage we have previously observed by atomic force microscopy.[23]

Peptide sequences for ECM motifs are shown in Table I and were acquired from previously published work as follows: fibronectin (RGDS)[24–26]; collagen I[27,28]; collagen IV[29–31]; fibronectin (PHSRN).[32–34] In brief, oligonucleotides designed to encode the relevant motifs of interest were ligated into a modified *E.coli* OmpA coding sequence so that the motif was within outer loop-1 of OmpA. The resulting constructs are shown in Table I. The parental unmodified OmpA was used as a negative control (OmpA-control). Surfaces displaying multiple peptides were produced by mixing equal amounts of the different OmpA proteins containing the relevant peptides. SAMs of the modified proteins were produced as described previously,[18] except that 11-mercaptoundecanoic acid (MUDA, Sigma-Aldrich) was used to fill the gaps between the protein molecules to create an intact monlayer. Cover slips with fully assembled surfaces were dried under N_2 and stored at 4°C with desiccant until required for cell culture. Before use, the cover slips were sterilized by immersion in 70% ethanol for 15 min followed by two washes with sterile phosphate-buffered saline (PBS, Cambrex).

TABLE I
Types of Extracellular Matrix Protein and their Motifs Used to Create Selective Biomimetic Surfaces

ECM protein	Surface	Motif(s)
Fibronectin	Orla 1	RGDS
Collagen I	Orla 31	GTPGPQGIAGQRGVV
Collagen IV	Orla 32	MNYYSNS
Fibronectin	Orla 34	PHSRN
Laminin	Orla 36	YIGSR
Collagen I Collagen IV	MOS1	GTPGPQGIAGQRGVV MNYYSNS
Fibronectin Laminin	MOS2	RGDS YIGSR
Fibronectin Collagen I Collagen IV	MOS3	RGDS GTPGPQGIAGQRGVV MNYYSNS
Fibronectin Collagen I Laminin	MOS4	RGDS GTPGPQGIAGQRGVV YIGSR
Fibronectin Fibronectin Laminin Collagen I Collagen IV	MOS5	RGDS PHSRN YIGSR GTPGPQGIAGQRGVV MNYYSNS

Table provides information about the different ECM protein motifs used in this study and the sequence of the motifs used.

See text for further details and citation of the appropriate literature concerning specific peptide domains.

Immunocytochemistry

Immunocytochemical analysis was performed using standard methods. Subsequent to removal of the culture medium, cells were washed once with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde (PFA, Sigma-Aldrich) in PBS for 30 min. Blocking was carried out using a solution of 1.5% bovine serum albumin (BSA, Sigma), 0.2% Triton-X 100 (Fisher) in PBS for 1 h. Cells were subsequently incubated with primary antibody beta-III-tubulin (Covance, http://www.covance.com) diluted 1 : 250 in 1.5% BSA, PBS for 1 h. The cells were then washed

in PBS and incubated with anti-mouse cy3 1 : 600 secondary antibody (Covance) for 1 h. Following further washing in PBS, the samples were mounted using 4′,6-diamidino-2-phenylindole (DAPI, Invitrogen) in Vectashield (Vector Laboratories; http://www.vectorlabs.com) containing 4′,6-diamidino-2-phenylindole (DAPI).

Statistical analysis

For normally distributed data, an ANOVA test was preformed with a post-hoc Bonferoni comparison. For data that were not normally distributed Kruskal-Wallis and Mann Whitney-U tests were conducted and subsequent post-hoc analysis was carried out as described elsewhere.[35] $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***) were used to indicate levels of statistical significance.

RESULTS AND DISCUSSION

Cell differentiation is influenced by multiple factors and their reaction to cues within their environment, notably the interaction with adjacent cells, soluble signals, and the ECM. Within a developing tissue, cells differentiate as a combined response to these multiple signalling events. This is especially relevant to the ECM which is often composed of mixtures of different molecules that surround cells and tissues and are important in determining cell growth, state of differentiation, and cell migration.[36] It is difficult to elucidate and investigate the exact role of individual ECM molecules in a standardized and reproducible manner.

In this study, we have developed a series of alternative synthetic surfaces that present the functional domain of several different but well known ECM proteins in an orderly and consistent fashion to cultured cells. The biomimetic surfaces were manufactured under controlled conditions resulting in the formation of surfaces that present the active peptide domain of ECM proteins in an appropriate molecular configuration (Fig. 1). We demonstrate that this technology can be used to investigate the function of such active domains when presented individually or in combination to differentiating cultured cells. Specifically, we show how peptide motifs within a biomimetic surface can be used to regulate the growth of neurites from PC12 cells and influence the formation of beta-III-tubulin positive cells from primary neural stem/progenitor cells.

Although it is feasible that serum proteins may preferentially act with the designer substrates created, experiments with the parental unmodified OmpA surface will have partially controlled for this possibility. Serum protein variability was also controlled given that replicate experiments were set up in batches using a common stock of serum containing media. Furthermore, the interaction of serum proteins with specific motifs is unlikely to account for the observed biological response due to the differences in the observed effects induced by the motifs when presented individually and in combination.

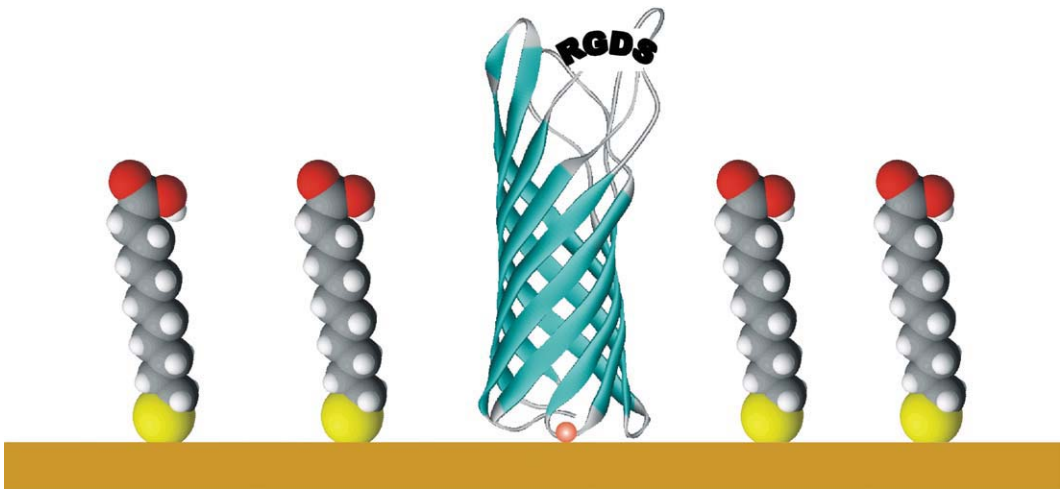

Figure 1. Schematic representation of biomimetic surface presenting a fibronectin RGDS motif. RDGS motifs are presented in a constrained loop by OmpA beta-barrels orientated so that they are located at the extremity of the surface to enable maximal contact with cultured cells. Orientation of the motifs is achieved due to the presence of a cysteine residue (red) that allows self assembly onto the gold surface. Spaces between beta-barrels are filled using 11-mercaptoundecanoic acid by a process of self assembly and orientation which is achieved by the terminal sulphur atom (yellow) bonding to the gold surface, resulting in the formation of an intact, stable monolayer. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

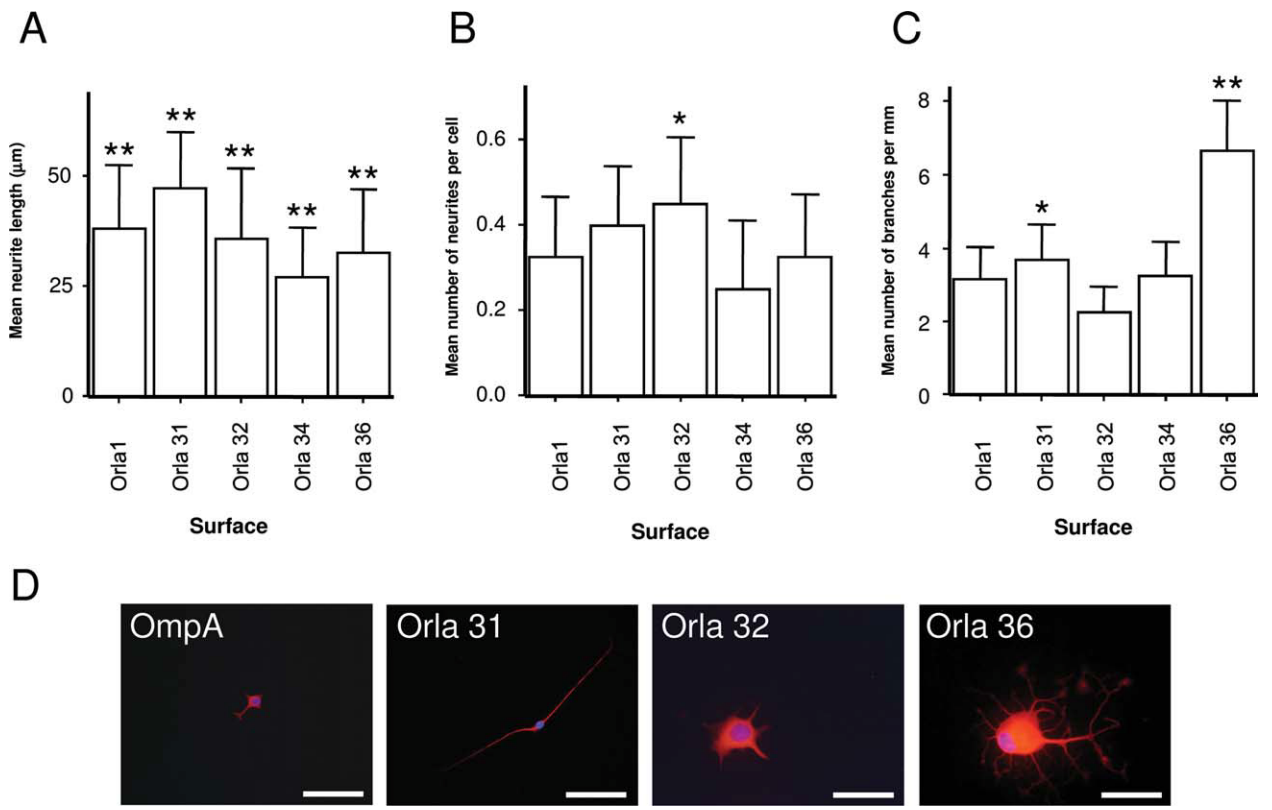

Figure 2. Regulation of neurite formation in response alternative ECM motifs. The mean neurite length (A), mean number of neurites per cell (B), and the mean number of branches/mm of neurite (C), were recorded from cultures of differentiating PC12 cells grown on different biomimetic surfaces. Values were compared with those of cells grown on control (OmpA-control) surfaces. Data represent mean values of 20 randomly selected single cells per surface for three repeats (+SEM). (D) Fluorescence micrographs of cultures grown on alternative biomimetic surfaces: OmpA-control, Orla 31, Orla 32, and Orla 36. Cells were stained with the nuclear marker, DAPI (blue), and the neuronal marker beta-III-tubulin (red). Scale bars: OmpA-control and Orla 31 : 100 µm; Orla 32 and Orla 36 : 25 µm. See Table I for types of motif used. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Neuritogenesis by differentiating PC12 cells is regulated by ECM functional domains

We have previously demonstrated that biomimetic surfaces identical to some of those described herein, promote the adhesion of PC12 cells on the culture surface.[18] PC12 cells are also known for their ability to differentiate and produce neurites in response to NGF and are regularly used as a model system to study neuritogenesis.[37] Furthermore, ECM proteins have previously been shown to affect PC12 cell growth and neural differentiation.[38–41] Here we report the effect of different ECM peptide domains on the development of neurites by PC12 cells following 10 days differentiation (Fig. 2). PC12 cells began to extend neurites within 2 days of exposure to NGF. Presentation of motifs from laminin, fibronectin, collagen I, and collagen IV were all found to increase neurite length when compared with the control synthetic surface. This is consistent with previous reports whereby exposure of PC12 cells to ECM molecules resulted in enhanced neurite outgrowth.[42–45] Similarly, we also found that no significant differences were detected between the lengths of neurites produced by the cells grown on surfaces displaying the motifs from either collagen I or laminin, which is consistent with previous reports.[42] However, our data for fibronectin motifs are not completely consistent with those of Paralkar et al. who showed that collagen IV and laminin produced similar levels of neurite outgrowth which were both more effective than fibronectin.[46] Collagen I has previously been shown to significantly increase the numbers of neurites formed by PC12 cells when compared with numbers induced in cultures grown on laminin.[42] In this study, although the collagen I motif did induce the mean number of neurites originating from the cell body, this was not a significant change when compared with control levels. On the other hand exposure to the collagen IV motif did significantly increase the number of processes formed per cell (Fig. 2). Clearly there are some inconsistencies between different published studies and some of the data reported herein. This is not surprising given the differences in the methods used to present the motifs on the surfaces. Accordingly, there is a

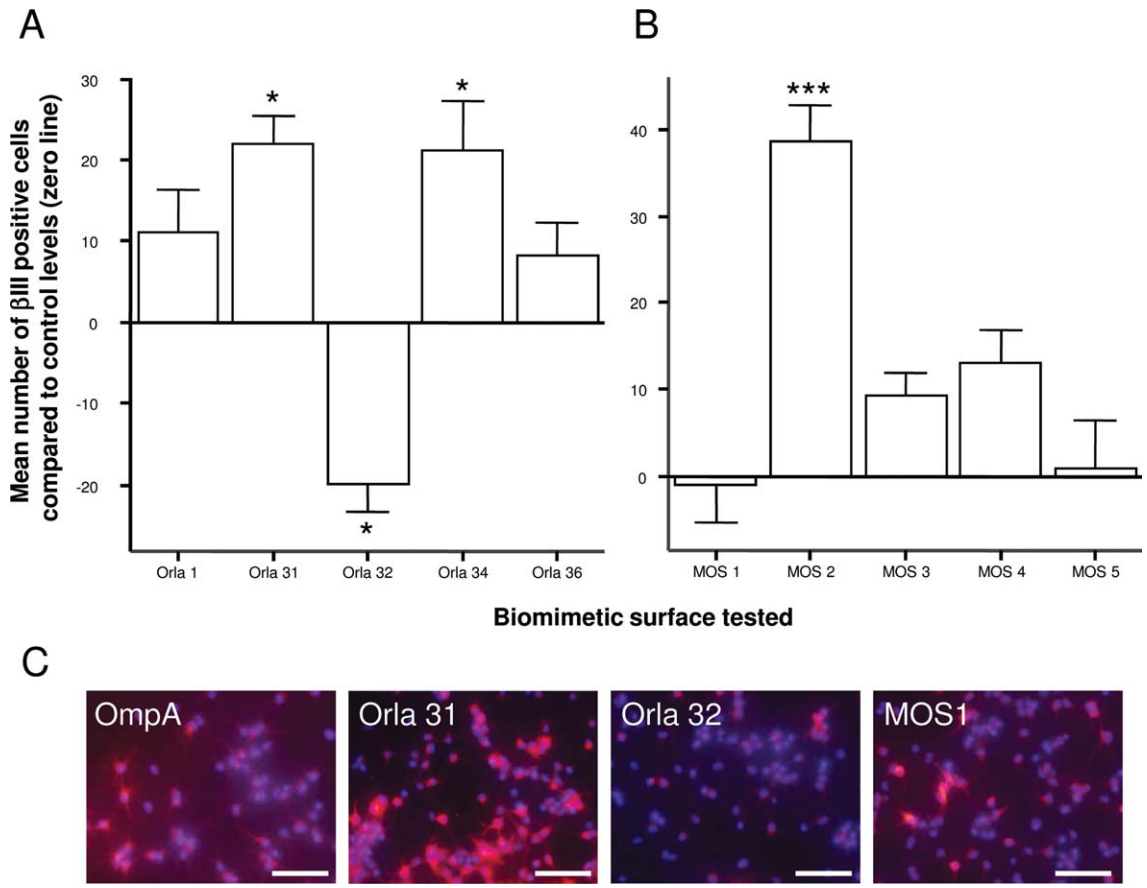

Figure 3. Influence of alternative ECM motifs on neural stem/progenitor cell differentiation and the induced expression of the neural marker, beta-III-tubulin. The mean number of beta-III-tubulin positive cells forming on control (OmpA-control) surfaces was compared with the number of beta-III-tubulin positive cells forming on biomimetic surfaces presenting a single ECM motif (A). Similarly, the number of beta-III-tubulin positive cells was recorded on surfaces presenting multiple ECM motifs and compared with control (OmpA-control) surfaces (B). Values represent the number of cells different to control (OmpA-control) levels (zero line) and were compared statistically to numbers of beta-III-tubulin cells identified on control (OmpA-control) surfaces. Data represent mean values of 20 randomly selected single cells per surface for three repeats (+SEM). (D) Fluorescence micrographs of cultures grown on alternative biomimetic surfaces: OmpA-control, Orla 31, Orla 32, and MOS1. All cells were stained with the nuclear marker, DAPI (blue), whereas neurons were labeled with beta-III-tubulin (red). Scale bars: 50 μm. See Table I for types of motif used. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

need to standardize this process and the presentation of functional ECM peptide domains in a controlled and consistent manner using well engineered synthetic surfaces, which will significantly improve reproducibility.

The local influence of surface molecules in determining distinctive growth patterns has been recognized.[47–49] To further investigate neuritogenesis, the number of branches per neurite formed was also recorded (Fig. 2). Peptide motifs for both collagen I and laminin induced significantly greater levels of branching per neurite, especially neurites grown on the laminin motif which appeared highly branched. Investigation of neurite branching has previously shown that laminin increases branching in comparison with fibronectin.[50] This is consistent with the data reported here whereby neurites growing on surfaces presenting the laminin motif produced significantly more branches when compared with cells cultured on surfaces expressing either of the fibronectin motifs.

Extracellular matrix influences the differentiation of neural stem/progenitor cells

Cell fate and the direction of cell differentiation are regulated by multiple factors including the interaction between cells and their surrounding ECM.[51] In this study, we investigated whether certain active ECM peptide domains influenced the differentiation of adult NSPCs to induce the formation of beta-III-tubulin positive cells, indicative of neural differentiation (Fig. 3). Each condition tested provided an adequate surface for adult NSPC adhesion with no significant variation in the number of cells attached

(data not shown). Motifs for collagen I and fibronectin PHSRN significantly enhanced beta-III-tubulin expression above control levels, whereas laminin and fibronectin RGDS also induced beneficial effects [Fig. 3(A)]. There are consistencies with these observations and earlier work on the effect of ECM proteins on neural development.[52]

The effect of ECM proteins on neuronal differentiation by neural stem cells (derived from the striatum of fetal rats) has been previously reported.[53] An array approach was used where either whole ECM molecules or artificial ECM proteins were tethered to a synthetic surface in the presence and absence of known growth factors. The authors showed that growth factors were more predominantly determinants for the specification of neural stem cells than matrix components, although the effects of growth factors were often influenced by the type of coimmobilized ECM. In the absence of growth factors, ECM proteins for laminin and fibronectin induced neuronal differentiation.[53] Using SVZ neurospheres, we also observed that motifs for both fibronectin RGDS and laminin had a moderate effect on the formation of beta-III-tubulin expression [Fig. 3(A)]. The differentiation responses to the fibronectin PHSRN and collagen I motifs were particularly significant. Indeed, collagen I has previously been recognized as a key component to enhance neural differentiation when used as a gel for three dimensional cell growth.[54] The advantage of presenting individual peptide domains, either alone or in combination, as enabled by the technology used in this study, allows the identification of those active sequences that are most important for the induction of neural differentiation in this cell system.

In contrast to collagen I, the collagen IV peptide motif significantly reduced the number of beta-III-tubulin positive cells formed by adult NSPCs [Fig. 3(A)]. This is contrary to the behavior of fetal cortical neurons whose differentiation is promoted in the presence of collagen Type IV.[55] Such results draw attention to the differences between the model systems where the response of developing cells to ECM proteins may differ as a consequence of their tissue origin. Alternatively, the effect of the whole collagen IV molecule is mediated by an alternative motif than tested herein. Furthermore, there are inconsistencies in the manner in which ECM proteins are represented in different studies, notably the use of either the whole ECM molecule adsorbed onto a solid surface (with different studies using alternative concentrations) or the presentation of the functional ECM peptide domain alone. The conformation of adsorbed whole molecules is likely to be variable and different to that where the active peptide motif is presented in a uniform and controlled manner as reported herein. This in turn is likely to influence the interaction of the cells with the surface and consequently the differentiation response.

Proteins of the ECM can possess several different functional domains and it can be difficult to determine which domain is important for a certain function within a particular cell growth system. This is further compounded when several domains are functioning simultaneously and presented in ways where the molecular conformation of such molecules is varied. To evaluate the function of different ECM peptide domains in combination, we produced biomimetic surfaces composed of a mixture of different motifs presented in a uniform fashion (Table I). When presented as single peptide motifs, differentiation responses to the collagen I and collagen IV motifs were approximately equal but opposite [Fig. 3(A)]. However, in combination, the effect of either functional domain appeared to cancel each other out resulting in no significant change when compared with control levels [Fig. 3(B), MOS1]. Similarly, when presented singly, the collagen I and fibronectin PHSRN motifs both induced neuronal differentiation which was significantly enhanced when the domains were copresented resulting in a synergistic effect (Fig. 3, MOS2).

In experiments where three or more motifs were presented, the outcomes were less predictable. The addition of the fibronectin RGDS motif to produce a surface in combination with the collagen I and collagen IV motifs (MOS3) will have reduced the relative amount of both types of collagen motif displayed. The reduction in the effective concentration of collagen IV motif, which had a negative effect on cells expressing beta-III-tubulin, combined with the positive influence of fibronectin RGDS and collagen I motif, resulted in an increase in beta-III-tubulin expression [Fig. 3(B), MOS3]. When three peptide motifs that individually produced positive effects on cell differentiation were combined on a single surface, the resulting effect although positive was not significantly different from the control surface [Fig. 3(B), MOS4]. Furthermore, when five peptide motifs were expressed in combination, it appeared that the positive effects of fibronectin RGDS, fibronectin PHSRN, laminin, and collagen I motifs did not significantly counteract the negative influence of the collagen IV motif [Fig. 3(B), MOS5].

In vivo, cells interact with the ECM which is composed of multiple types of colocalized protein. Given the complexity of these interactions, there are significant advantages to studying the function of ECM proteins and their active domains both individually and in various combinations. Earlier work studying rat neural stem cells demonstrated surfaces expressing the RGD peptide induce similar levels of neuronal differentiation to PLO/laminin surfaces.[56] Conversely, the laminin peptide IKVAV was found to

have no effect on neuronal development. Upon bringing the two conditions together and creating a surface comprised of the active RGD and the inactive IKVAV motifs, the effect on neuronal differentiation was decreased in proportion to the dilution of the combined positive and negative effects.[56] Our work is consistent with this observation in that when ECM motifs that elicit approximately equal positive and negative effects on cell differentiation are brought together, the cumulative effect amounts to no change relative to the control (MOS1). Although the additive effect of two motifs that each increased the number of beta-III-tubulin positive cells, resulted in significant enhancement of neural development (MOS2). On some occasions, however, the cumulative effects of individual ECM peptide motifs do not always translate during combination as would be predicted (e.g., MOS4 and MOS5). In agreement, studies of the differentiation of murine embryonic stem cells have shown that the summative effects of combined ECM molecules did not consistently correlate with their activities when presented individually.[57,58] It would appear, therefore, that in some instances, it is possible to create surfaces composed of ECM proteins that influence cell differentiation in predictable ways but on other occasions the combination of alternative ECM molecules introduces additional complexity.

In this study, we have demonstrated how biomimetic surfaces presenting the active peptide domains of various ECM proteins can be used to regulate neural differentiation *in vitro*. The presentation of individual ECM peptide sequences enables direct assessment of their function, whereas the production of surfaces containing more than one domain allows assessment of the combinatorial effects of different ECM peptides. The development of this technology will lead to the creation of synthetic ECM surfaces designed to interact with differentiating cells and will be of direct relevance to tissue engineering and regenerative medicine.

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