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IMMOBILIZATION OF N-CADHERIN PEPTIDE ON POLY(ETHYLENE TEREPHTHALATE) FOR STEM CELL CULTURE

Miss Suttinee Poolsup

A Thesis Submitted in Partial Fulfillment of the Requirements

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ศุทธินี พูลทรัพย์: การตรึงเพปไทด์เอ็น-แคดฮีรินบนพอลิเอทิลีนเทเรฟทาเลตสำหรับการเพาะเลี้ยงเซลล์ต้นกำเนิด (Immobilization of N-Cadherin Peptide on Poly(ethylene terephthalate) For Stem Cell Culture) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ.ดร.วรวีร์ โฮเว่น, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ผศ.นพ.ดร.นิพัญจน์ อิสรเสนา ณ อยุธยา, 69 หน้า.

ในงานวิจัยนี้นำพอลิเอทิลีนเทอเรฟทาเลท (พีอีที) ซึ่งเป็นพอลิเมอร์สังเคราะห์ชนิดหนึ่งที่นิยมนำมาประยุกต์ใช้เป็นวัสดุทางการแพทย์ มาใช้เป็นวัสดุรองรับการตรึงเพปไทด์เป็นวงที่เลียนแบบมาจากโปรตีนเอ็นแคดฮีริน เพื่อสร้างสิ่งแวดล้อมสำหรับการเพาะเลี้ยงเซลล์ต้นกำเนิด การทดลองเริ่มจากการดัดแปรพื้นผิวพีอีทีด้วยปฏิกิริยาไฮโดรลิซิสทำให้มีหมู่คาร์บอกซิล จากการวิเคราะห์ด้วยโทลูอิดีนบลูโอเอสเซ พบว่าภาวะในการทำปฏิกิริยาไฮโดรไลซิสที่ดีที่สุดทำให้ได้ความหนาแน่นของหมู่คาร์บอกซิลเท่ากับ 8.31×10^{-7} มิลลิโมล/ตารางเซนติเมตร จากนั้นทำการตรึงเพปไทด์โดยใช้เอ็น-ไฮดรอกซีซักซินิไมด์และเอ็น(3-ไดเมทิลแอมิโนโพรพิล)-เอ็น'-เอทิลคาร์โบไดอิไมด์ไฮโดรคลอไรด์เป็นรีเอเจนต์คู่ควบ จากการทดลองพบว่าเซลล์ต้นกำเนิดที่เป็นเซลล์ประสาทของหนูสามารถยึดเกาะบนพื้นผิวพีอีทีที่ตรึงด้วยเพปไทด์ได้ ผลจากการศึกษาการเจริญแขนงประสาทจากนิวโรสเฟียร์ของเซลล์ประสาทของหนู การแสดงออกในระดับยีนผ่านวิถีที่ควบคุมการส่งสัญญาณภายในเซลล์ที่ขึ้นกับปริมาณเบต้า-แคดทีนินภายในเซลล์ การตอบสนองของเซลล์ต้นกำเนิดที่เป็นเซลล์กระจกตาของมนุษย์ แสดงให้เห็นว่าแผ่นฟิล์มพีอีทีที่ตรึงด้วยเพปไทด์ดังกล่าวมีความสามารถในการรักษาคุณสมบัติของความเป็นเซลล์ต้นกำเนิดของทั้งเซลล์ประสาทและเซลล์กระจกตาได้ อีกทั้งยังส่งผลต่อการแสดงออกของยีนที่เกี่ยวข้องภายในเซลล์ แสดงให้เห็นถึงศักยภาพของวัสดุดังกล่าวในการประยุกต์สำหรับการเพาะเลี้ยงเซลล์ต้นกำเนิด

สาขาวิชา เทคโนโลยีชีวภาพ ลายมือชื่อนิสิต .....

ปีการศึกษา 2553 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

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5172582223: PROGRAM IN BIOTECHNOLOGY
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SUTTINEE POOLSUP: IMMOBILIZATION OF N-CADHERIN PEPTIDE ON POLY(ETHYLENE TEREPHTHALATE) FOR STEM CELL CULTURE. THESIS ADVISOR: ASSOC. PROF. VORAVEE P. HOVEN, Ph.D., CO-ADVISOR: ASST. PROF. NIPAN ISRASENA NA AYUDHAYA, M.D., Ph.D., 69 pp.

Poly(ethylene terephthalate) (PET), one of the most widely used synthetic materials in biomedical applications, was used as platform for immobilizing N-cadherin mediated cyclic peptide to generate microenvironment for stem cell culturing. PET surface was first modified by hydrolysis to introduce active carboxyl functionalities. Upon using an optimized condition for hydrolysis, carboxyl group density of 8.31×10^{-7} mmol/cm^2 was obtained as determined by Toluidine blue O (TBO) assay. The peptide immobilization was followed thereafter using *N*-hydroxysuccinimide (NHS) and *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride) (EDC) as coupling reagents. The specific adhesion via N-cadherin binding molecule and phenotype expression of mouse neural stem cell were observed. The cells can attach on the peptide-immobilized PET surface. The results from neurite outgrowth of mouse neuronal spheres, Wnt/β-catenin signaling activity as well as the expression of human limbal epithelial stem cells suggested that N-cadherin mimic cyclic peptide immobilized on the PET film has ability to maintain neural and limbal stem cell features as well as provide biological effects on the expression of specific gene within cells. These results demonstrated the potential of the cyclic peptide-immobilized PET films in stem cell culture applications.

Field of Study : Biotechnology Student's Signature .....
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LIST OF ABBREVIATIONS

ASCs	: Adult stem cells
AFM	: Atomic Force Microscopy
CAM	: Cell adhesion molecules
CM	: Conditional medium
EGF	: Epithelial growth factor
ESCs	: Embryonic stem cells
ECD	: Extracellular domain
ECM	: Extracellular matrix
FBS	: Fetal bovine serum
FGF	: Fibroblast growth factor
HSCs	: Hematopoietic stem cells
LESCs	: Limbal epithelial stem cells
MSCs	: Mesenchymal stem cells
NSCs	: Neural stem cells
PET	: Poly(ethylene terephthalate)
SCs	: Stem cells
SPR	: Surface Plasmon Resonance

CHAPTER I

INTRODUCTION

1.1 Statement of Problem

Stem cells are uniquely positioned at the foundation of potential regenerative medicine therapies because of their specialized ability to self-renew and to generate numerous differentiated cell types including progenitor and effecter cell populations. Their fate and function are governed by a combination of intrinsic determinants and signals from microenvironment or niche. Within the niche, stem cells are exposed to complex spatially and temporally controlled biochemical mixtures of soluble chemokines, cytokines and growth factors as well as insoluble transmembrane receptor ligands and extracellular matrix molecules [1-3].

Therefore, understanding these regulatory systems is the key biological components required for cellular therapies. This goal is to repair damaged organs and tissues *in vivo* as well as generating tissue constructs *in vitro* for subsequent transplantation. Recently, culturing stem cells *in vitro* in order to generate a number of stem cells, especially embryonic stem (ES) cells, to be high enough for practical clinical usage is still not successful. In the conventional method, stem cell research is carried out with cells cultured on feeder-cell layers that have been treated and coated on the surface-culture substrates. These feeder cells can be well supporting cells and provide nutrients into culture medium. However, these cells senesce after several passages thus limiting their continual use and the need to derive new feeder cells may result in batch-to-batch variation [4]. Also this system possesses a risk of contamination with retroviruses and other pathogens that could be transmitted to the human cells [5].

Creating a suitable microenvironment for their growth, differentiation and ultimately mimicking the stem cell niche is a key to the success of stem cell culture *in vitro*. Biomaterials are rapidly being developed to display and deliver stem cell-regulatory signals in a precise and near-physiological pattern. Biomaterial related

researches include conductive platform formed by protein-conjugated synthetic polymers that present bioactive ligands and respond to cells.

Chemical modification of material surfaces with immobilization of bioactive peptides or molecules is a promising strategy for directing and controlling cell-biomaterial interaction [6]. Once the cells are attached to the surface of the material, intracellular signals regulating proliferation and differentiation of cells are generated via interactions between specific receptors and cell signaling molecules adsorbed or exposed on the materials. These materials should be useful for the culture and/or preservation of embryonic stem (ES) cells and various other kinds of stem cells, including neural and limbal stem cells.

Recent work reported by Williams and coworkers have been demonstrated that cyclic peptides containing a tandem repeat of the individual motifs to function as N-cadherin agonists. HAVDI and INPISGQ sequences have been identified as functional binding motifs in extracellular domain1 (ECD1) of N-cadherin [7]. When presented to neurons as soluble molecules, the dimeric versions of the motifs stimulate neurite outgrowth in a similar manner to native N-cadherin. This novel N-cadherin mimic cyclic peptide should be invaluable for dissecting out those cadherin functions that rely on signaling as opposed to adhesion and clearly have the potential to be developed as therapeutic agents for the promotion of cell survival and axonal regeneration.

Currently, a large number of synthetic polymeric materials with various different properties are available for medical applications. Poly(ethylene terephthalate) (PET) is one of the most widely used synthetic materials. Because of its biocompatibility, excellent fiber- and film-forming properties and affordable material, it has found in biomedical application as a material for artificial blood vessels, tendons, hard tissue prostheses, cell culturing substrates and many others. As are most synthetic polymers, PET is relatively inert and hydrophobic without functional groups able to take part in covalent peptide immobilization. To overcome this drawback chemical modifications have been attempted to alter the surface properties of the material. Hydrolysis is a chemically modifying method of PET surface by alkaline solution to introduce carboxyl groups. Such the action increased the hydrophilicity of the polymer and created the anchor functionalities for subsequent reactions [8-9].

Herein, the covalent immobilization of N-cadherin mimic cyclic peptide to the carboxyl groups of modified PET film surface was subsequently performed in order to determine the applicability of the surface-functionalized PET films towards cell culture applications. This research aims to develop the appropriate microenvironment *in vitro* for culturing stem cells and studying cell responses. Using two stem cell types, there are neural stem cells and limbal stem cells.

1.2 Objectives

1. To introduce active site on poly(ethylene terephthalate) film surface for binding with biomolecules (N-cadherin mimic cyclic peptide).
2. To study biological function of the N-cadherin mimic cyclic peptide.
3. To determine stem cell properties on PET-immobilized N-cadherin mimic cyclic peptide.

1.3 Scope of Investigation

The stepwise investigation was carried out as follows:

1. Literature survey for related research works.
2. Hydrolysis of PET film surface to be used as a substrate.
3. Cyclization of N-cadherin mimic peptide via iodine oxidation reaction.
4. Immobilization of N-cadherin mimic cyclic peptide on carboxyl groups of PET film surfaces.
5. Characterization of carboxyl groups on PET film surfaces and N-cadherin mimic cyclic peptide concentration on gold-coated SPR disk attached with carboxyl groups used as a model representing substrate.
6. To test stem cell attachment and properties on N-cadherin mimic cyclic peptide immobilized PET film surfaces.

CHAPTER II

THEORY AND LITERATURE REVIEW

2.1 Stem Cell

Stem cells are defined functionally as cells that have the capacities to self-renew throughout the lifetime of an organism as well as the potential to differentiate into a variety of different lineages [10-11] (Figure 2.1). Self-renewal is the division of a cell to give rise to two daughter cells, at least one of which is identical to the cell from which they arose. Differentiation occurs when the stem cell becomes a more specialized cell type and loses the capacity for self-renewal. Stem cells can give rise to one or more distinct cell types [12]. Because of these special properties, stem cells have been positioned at the foundation of regenerative medicine therapies. The structure of differentiation is based on observations from developmental biology. Differentiation during embryogenesis begins with gastrulation when cells separate into three structural layers: endoderm, mesoderm and ectoderm. While together the three layers (or lineages) give rise to all the cells in the body, each layer proceeds through stages of differentiation to generate an independent subset of phenotypes. It has been found that the *ectoderm* includes skin and neural cells; the *mesoderm* includes cardiovascular, blood and skeletal cells; and the *endoderm* includes cells of the gastrointestinal tract. These layer developments are used to refer to the differentiation potential both *in vivo* and *in vitro* [13]. A totipotent cell, such as the fertilized egg, is capable of differentiating not only into all three lineages, but also yields the extra-embryonic cells that support fetal development. Pluripotent cells can differentiate into all three lineages. Committed to a specific lineage, multipotent cells are able to form more than one cell type, but usually within the same lineage. Differentiation of cells from one lineage to another is referred to as stem cell plasticity or trans-differentiation [14].

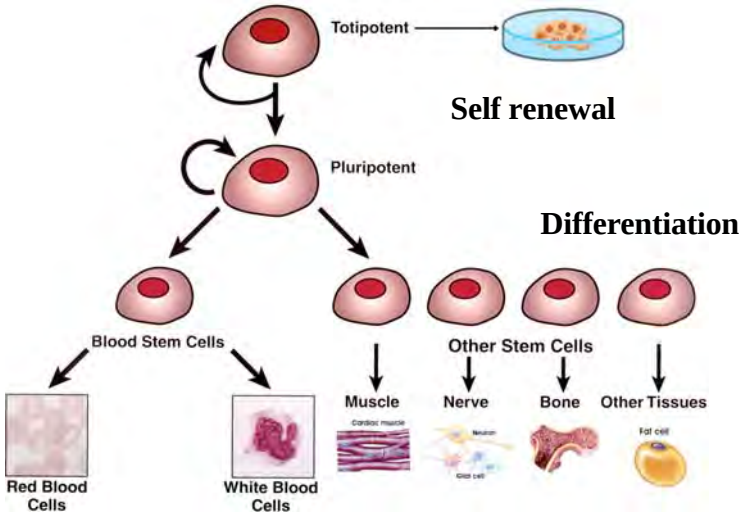


Figure 2.1 The hierarchical structure of stem cell differentiation [14]

2.1.1 Types of Stem Cells

Stem cells can be divided into two types by their sources.

2.1.1.1 Embryonic Stem Cells

Embryonic stem cells (Figure 2.2) are isolated from the inner cell mass of the blastocyst during embryological development. Their pluripotent nature gives them the ability to differentiate into any one of the three germ layers, as described above [15], [16]. The basic characteristics of an ES cell include self-renewal, multilineage differentiation *in vitro* and *in vivo*, clonogenicity, a normal karyotype and extensive proliferation *in vitro* under well-defined culture condition [17].

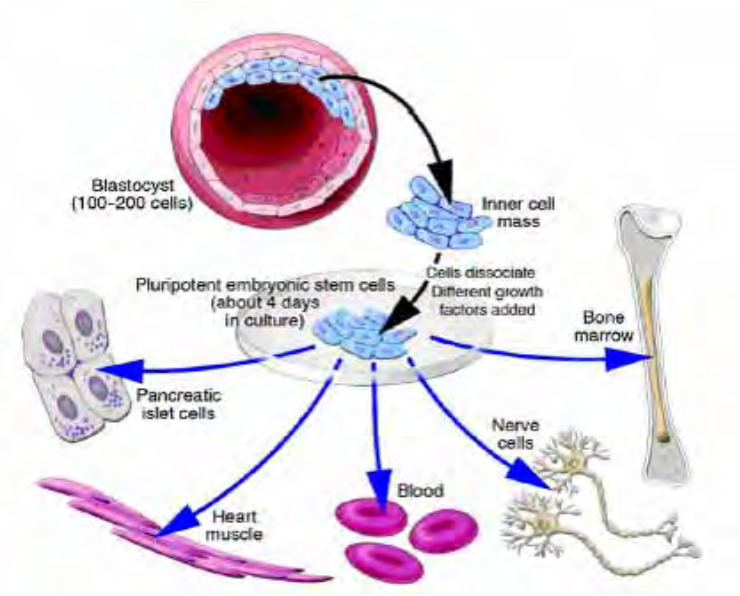


Figure 2.2 Embryonic stem cells [14]

2.1.1.2 Adult Stem Cells

Adult stem cells are those cells found in tissues after birth. These cells are often referred to as progenitor or multipotent cells since they have limited differentiation potential (Figure 2.3). Initially it was thought that adult stem cells were only located in a limited selection of organs and could differentiate into just those phenotypes found in the originating tissue. The field is still developing, however, and recent studies have identified stem cells in more tissues and indicate a greater range of potential than that originally believed. The basic characteristics of an adult stem cell are a single cell (clonal) that self-renew and generated differentiated cells. Stem cells have already been found in bone marrow, cord blood, adipose tissues, neural tissues, corneal tissue, etc. These stem cells, and the phenotypic lineages they have been shown to generate, are indicated in Table 2.1. According to these special properties, stem cells are attractive for use in cell-based therapies to treat debilitating diseases and tissue damages.

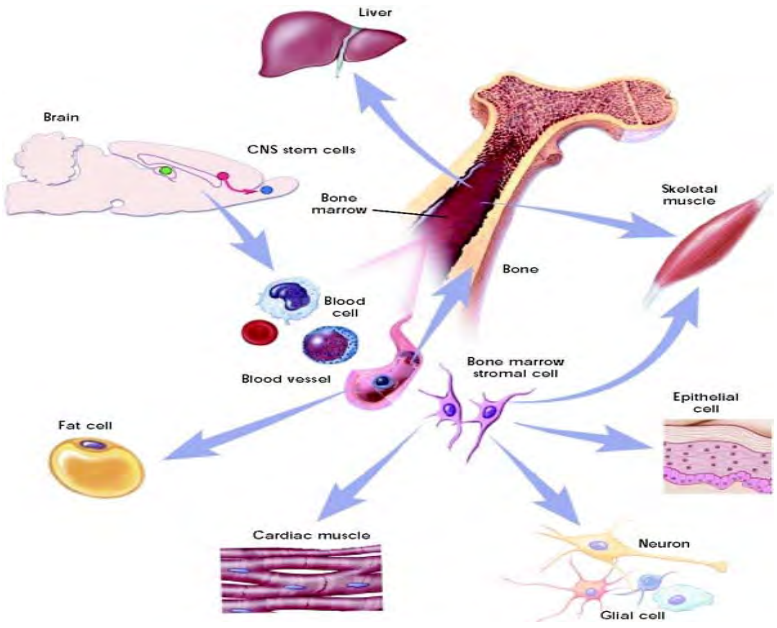


Figure 2.3 Adult stem cells [14]

Table 2.1 Differentiated cells derived from human adult stem cells [14]

Tissue source	Cell type	Derived cells
Blood	HSC	Blood cells
	EPC	Endothelial cell
Bone Marrow	HSC	Hepatocyte, blood cells
	EPC	Endothelial cell
	MSC	Adipocyte, cardiomyocyte, chondrocyte, endothelial cell, neuron, osteocyte, thymic cell
Fat	PLA	Adipocyte, chondrocyte, myocyte, neural progenitor, osteocyte
Umbilical Cord Blood	HSC	Blood cells
	MPC	Adipocyte, endothelial cell, blood cells, osteoblast

2.1.2 Stem cells Culture Method

Human ESCs were successfully grown in the laboratory for the first time in 1998 [18]. Under appropriate culture conditions, ESCs have demonstrated a remarkable ability to self-renew continuously. The culture conditions required to keep undifferentiated state of human ESCs. Conventionally, they were grown in petri dishes on a layer of mouse embryonic fibroblasts (referred to as “feeder cells”) in a medium containing serum. The feeder cells are inactivated, so they are not dividing and expanding, but they produce growth factors that sustain the ESCs. The feeder cells in the bottom of the culture dish provide extracellular matrixes (ECM) which they can attach. Also, the feeder cells release several nutrients into the culture medium. The mechanism of how feeder cells maintain the proliferation of undifferentiated ESCs is unknown [19]. However, the exposures of xenogenic products producing from mouse feeder cells have many problems in human clinical applications.

Researchers have found other alternatives to support hESCs culture. For instance, many studies have explored several alternative cell sources (e.g. human embryonic derived fibroblasts, foreskin fibroblast and adult bone marrow cells) [20-21] as feeders to support hESC culture and thereby limiting cross-species contaminations. Additionally, the use of animal-based products as well as feeder cells may introduce batch-to-batch variation [16]. Nevertheless, cell and tissue therapy required maintenance of large quantities of undifferentiated hESCs, and therefore usage of feeder cells may not be an optimal ECM for stem cells.

2.1.2.1 Stem cell niche

Generally, the ECM retains stem cells in the microenvironment or niche that comprises three effectors e.g. physical signals (e.g. laminin, fibronectin and collagen), soluble signals (e.g. growth factors, cytokines and chemokines) and proteins on the surfaces of neighboring cells. Accordingly, the ultimate decision of a cell to differentiate, proliferate, migrate, apoptosis or perform other specific functions is a coordinated response to the molecular interactions with these ECM effectors [2]. Moreover, the physicochemical environment including oxygen tension (pO_2) and pH, also contributes to the regulation of stem cell fates, however, these stem cells do not have a stable niche *in vivo* [22]. *In vitro*, these molecules can serve to influence stem

cell fate and/or to facilitate regenerative medicine applications. Soluble factors and coated substrates have been used in maintaining stem cells undifferentiated, as well as in promoting a particular differentiation pathway.

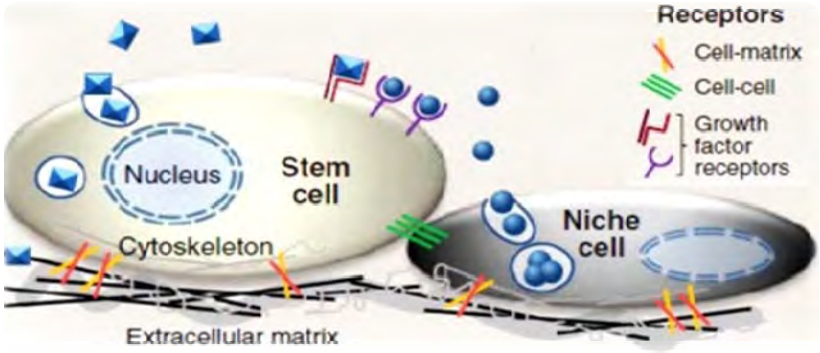


Figure 2.4 Stem cell niche [23]

In order to maintain the phenotype expression and differentiated functions of stem cells, the simulated natural environment of the biomimetic ECM support has to provide the appropriate signals to the attached cells. In recently, Xu and coworkers [24] reported the first successful feeder-free culture of hESCs on coated culture dishes with laminin, collagen and Matrigel with 100% MEF conditioned medium (CM) supplemented with growth factors. The ES cells cultured on these ECM-based biomaterials had a doubling time comparable to ESC grown on feeder layers. In contrast, cells grown on gelatin-coated dishes in MEF-CM showed poor survival. Additionally, all survival cells differentiated in the first passage. Although the above study was successful in expanding the hESCs in a feeder-free condition, it still required mouse conditioned medium. Other studies used various growth factors in culturing ES cells on culture dishes coated with natural ECM-based biomaterials and without conditioned medium [21-25]. Yao et al. [26] developed a chemically defined medium to eliminate influence of unknown components from growth factors. The use of cell-based ECM components, however, has the effect of pathogen transmission. Therefore, it would be advisable to use synthetic ECM-based support, which would offer numerous advantages such as risk free environment and control over biochemical and biomechanical properties. The stimulated natural environment of the biomimetic ECM support has to provide the appropriate physical, chemical signals as well as spatial cues

that are essential to mimic the natural tissue growth including cell adhesion, proliferation and differentiation [27]. However, the successful culture systems for stem cell expansion depend upon cell interaction with other cells and microenvironment via cell adhesion molecules (CAM).

Cell adhesion molecules (CAM) are trans-membrane glycoproteins in the cell surface that mediate binding to extracellular matrix (ECM) molecules or to counter-receptors on other cells. These molecules determine the specificity of cell-cell or cell-ECM interactions. The structures of cell adhesion molecules have several types (Figure 2.5) such as (i) the integrin family, (ii) seletins, (iii) the immunoglobulin (Ig) superfamily and (iv) cadherins. While the extracellular domain mediates adhesion, the intracellular domain proteins provide structural and functional linkages between adhesion receptors and the cytoskeleton [28]. Therefore, CAM has impact on cellular responses in terms of cell morphology and growth.

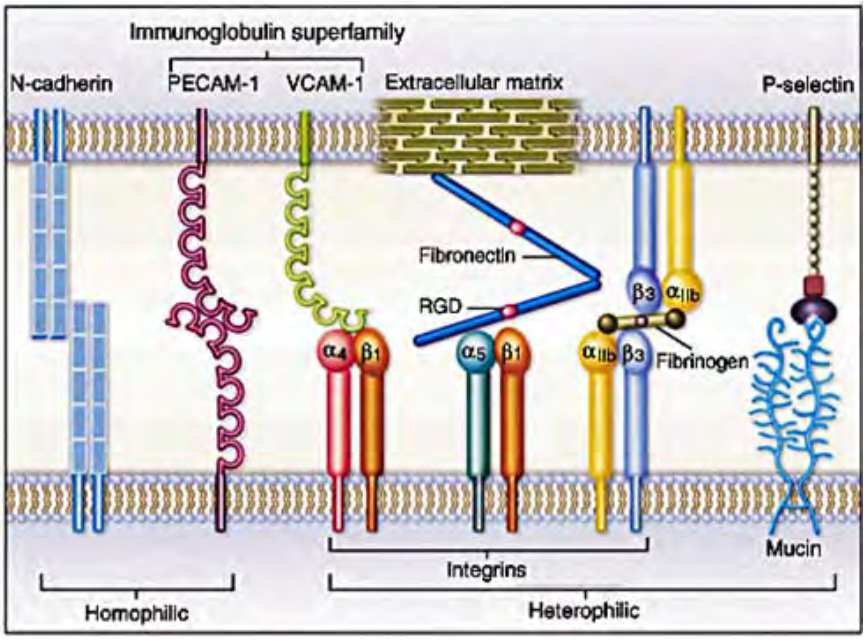


Figure 2.5 Homophilic and heterophilic interactions of cell adhesion molecule [29]

In 2001, Tsuchiya and coworkers [30] studied the effects of various cell adhesion molecules and extracellular matrices on the initial cellular adhesion properties of articular chondrocytes, ligament cells and mesenchymal stem cells. The investigation

aimed to develop optimal scaffolds for tissue engineering of articular cartilage and ligament. Fibronectin was shown to be the most potential to promote cell adhesion.

In 2008, Lu and coworkers [31] investigated the effect of β1 integrins on cell-matrix interactions. Human adipose tissue-derived stem cells (ASCs) were encapsulated in collagen type I gels for the induction of chondrogenesis. The effects of β1 integrins on chondrogenesis were analyzed by blocking ASCs with β1 integrins neutralizing antibodies. In addition, the effects of β1integrins on the gene expression of the RockI and RockII kinases, which are the most important effectors of prototype GTP ase Rho A. Cell shapes of ASCs were also analyzed in order to better understand the mechanisms by which β1 integrins regulate chondrogenesis.

In 2010, Zhao and coworkers [32] studied the effect of bone marrow alteration on hematological abnormalities in liver cirrhosis. Immunohistochemistry revealed increased expression of E-selectin, P-selectin and von Willebrand factor (vWF) in bone marrow sinusoidal endothelium (an interface between the peripheral blood and the hematopoietic compartment in bone morrow microenvironment) of the cirrhotic rats, which showed the same finding in specimens from patients. The result indicated that activation or injury of bone marrow sinusoidal endothelium mediated by E-selectin, P-selectin, and vWF might have role in pathogenesis of bone marrow changes during liver cirrhosis. The lesions of bone marrow sinusoidal endothelium might contribute to the hematological abnormalities in the end stage of liver disease in both a rat model and patients.

For an interesting type of CAMs, the cadherins constitute a large superfamily of cell-cell adhesion molecules that associated with morphogenetic processes. The classic cadherins are known as Ca^{2+}-dependent hemophilic binding molecules that can mediate cellular recognition in developing tissue. The cadherins were originally named for the tissue in which they are most prominently expressed, but most cadherins can be expressed in many different tissues such as E-cadherin (expressed in epithelial cells), VE-cadherin (expressed in epithelial cells that line in vascular tissue) and N-cadherin (widely expressed in nervous system) [33]. The formation of neural interaction and recognition are mediated by cell surface proteins defined as cell-cell adhesion molecules. N-cadherin can form and maintain neuroepithelial adherens-type junctions

as part of the synaptic junction. However, the precise roles of N-cadherin in neuronal interactions at developmental stage remain less clear [34].

Recently, the functions of N-cadherin involving in neural cell responses were investigated. The classical cadherins are composed of five extracellular domains, a single transmembrane domain, and two cytoplasmic domains. The first extracellular domain (ECD1) of these cadherins contains an evolutionarily conserved His-Ala-Val (HAV) motif and several lines of evidence suggest that this sequence is critical for function.

In 2000, Williams and coworkers [35] demonstrated that short cyclic HAV peptides can inhibit N-cadherin function. Interestingly, the nature of the amino acids that flank the HAV motif determine both the activity and specificity of the peptides. When turning the cyclic HAV peptide into an N-cadherin peptide by incorporating flanking amino acids that are specific to N-cadherin, it can substantially increase the antagonistic properties of the peptide. That opposite result was obtained when turning the peptide into an E-cadherin peptide. The peptides that contained flanking amino acids from E-cadherin had little or no effect on the N-cadherin response. In summary, a novel family of cyclic peptides containing the HAV motif has been developed as N-cadherin antagonists. The specific N-cadherin antagonists can be developed based on the incorporation of 1 or 2 flanking amino acids from native N-cadherin onto the HAV motif.

In order to develop N-adherin antagonists described in 2000, the same group of researchers demonstrated the feasibility of cyclic peptides (Figure 2.6) containing a tandem repeat of the individual motifs to function as N-cadherin agonists. They have identified the HAVDI and INPISGQ sequences as functional binding motifs in extracellular domain 1 (ECD1) of N-cadherin. When presented to neurons as soluble molecules, the dimeric versions of the motifs stimulate neurite outgrow in a similar manner to native N-cadherin. The response to the dimeric agonist peptides was inhibited by monomeric versions of the same motif and also by recombinant N-cadherin ECD1 protein. The responses were also inhibited by antibodies to a fibroblast growth factor receptor (FGFR) binding motif in ECD4 of N-cadherin and by a specific FGFR antagonist (PD17304). These data suggest that the peptides function by binding to and clustering N-cadherin in neurons and thereby activating an N-cadherin/FGFR signaling

cascade. The novel agonists will be invaluable for dissecting out those cadherin functions that rely on signaling as opposed to adhesion and clearly have the potential to be developed as therapeutic agents for the promotion of cell survival and axonal regeneration.

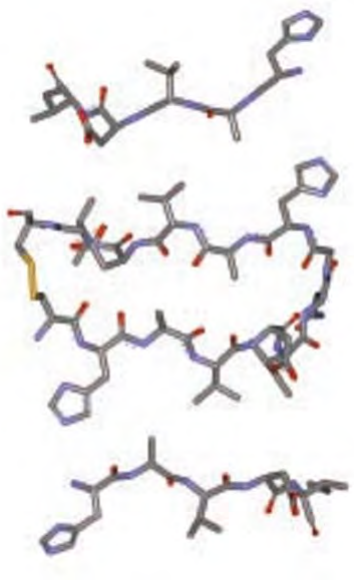


Figure 2.6 The structure above and below as a possible structure for the cyclic N-Ac-CHAVDINGHAVDIC-NH_2 peptide (middle) [7]

Moreover, in 2010, Burden-Gulley and coworkers [36] were interested in the function of a dimeric cyclic peptide from the above-mentioned research. They studied the effect of ADH-1 cyclic peptide, derived from the N-cadherin HAV site, which is an agonist of N-cadherin-mediated neurite outgrowth in order to generate a small molecule library of peptidomimetics to the HAV region of N-cadherin, which would be amenable to therapeutic use.

Besides N-cadherin can support axonal regeneration, they can be useful as cell surface marker in stem cell niches.

In 2007, Hayashi and coworkers [37] studied the mechanisms regarding the maintenance of corneal epithelial stem cells in their specialized niche. The immunofluorescence staining and flow cytometric analysis indicated that the expression of N-cadherin was localized only in limbal epithelial cells. N-cadherin positive cells showed significantly higher mRNA expression of the stem cell-related markers for ΔNp63, K15, Bmi-1 and ABCG2. Especially, ΔNp63 is a well-recognized stem cell

marker for both keratinocytes and corneal epithelial cells. Therefore, N-cadherin is a marker of epithelial stem cells in the corneal system. Interestingly, limbal N-cadherin positive cells maintained their expression of N-cadherin up to 1 day after seeding on 3T3 feeder cells but lost this expression over time in culture (Figure 2.7). These results suggest that culture conditions *in vitro* may allow for cells that proliferate and differentiate into transient amplifying (TA) cells *in vivo* or form colonies on feeder cells *in vitro*. This proliferative capability appears to be correlated with a down-regulation of N-cadherin expression.

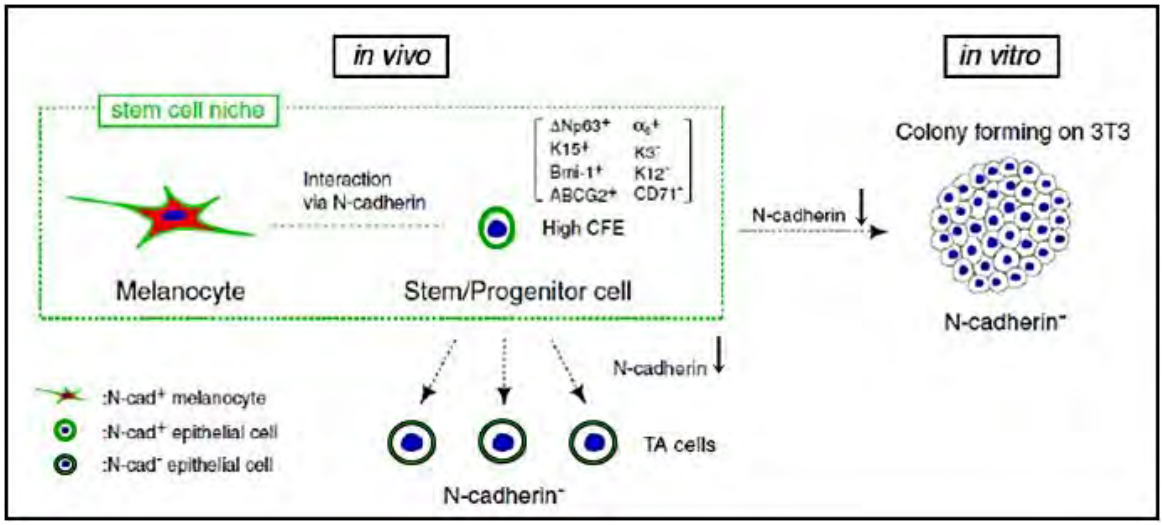


Figure 2.7 A model for epithelial stem cell niche [37]

In 2008, Haug and coworkers [38] examined how N-cadherin expression in hematopoietic stem cell (HSC) relates to their function. Bone marrow (BM) cells highly expressing N-cadherin (N-cadherin^{hi}) are not stem cells, being largely devoid of a Lineage^{-} Sca1^{+} cKit^{+} population and unable to reconstitute hematopoietic lineages in irradiated recipient mice. Instead, long-term HSCs form distinct populations expressing N-cadherin at intermediate (N-cadherin^{int}) or low (N-cadherin^{lo}) levels (Figure 2.8). The minority of N-cadherin population can robustly reconstitute the hematopoietic system, express genes that may prime them to mobilize, and predominate among HSCs mobilized from BM to spleen. The larger N-cadherin^{int} population performs poorly in reconstitution assays when freshly isolated but improves in response to overnight *in*

vitro culture. Their expression profile and lower cell-cycle entry rate suggest N-cadherin^{int} cells are being held in reserve. Thus, differential N-cadherin expression reflects functional distinctions between two HSC sub-populations.

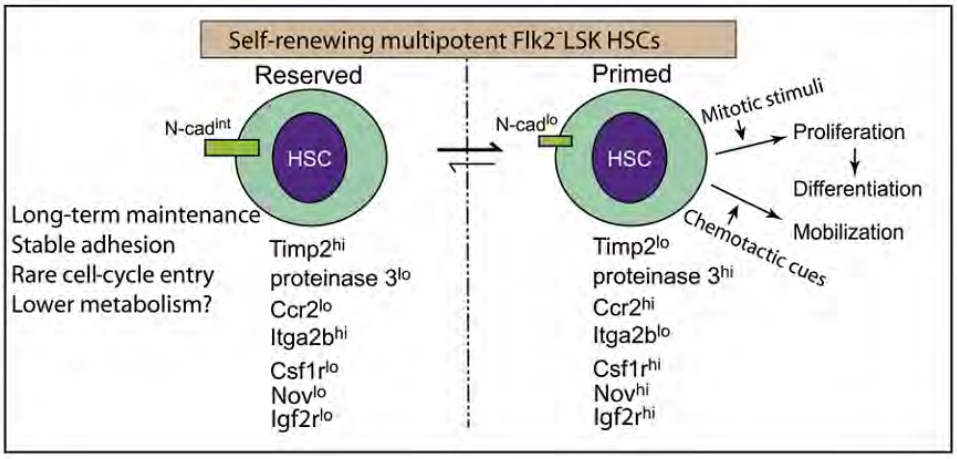


Figure 2.8 Model of reserved and primed HSCs [38]

Recent efforts have begun focusing on controlling the stem cell niche by engineering biomaterials and applying other crucial factors (Figure 2.9) [14]. The cultivation of mammalian cells *in vitro*, synthetic matrices like polymer materials are the most frequently used as substrates. To select appropriate polymers for cell culturing, it can help to understand the influence of these polymeric materials on viability, migration, proliferation and function of attached or adjacent cells. They provide a fully-defined microenvironment for stem cell propagation, as naturally-derived substrates may have undefined composition and batch-to-batch inconsistencies [39].

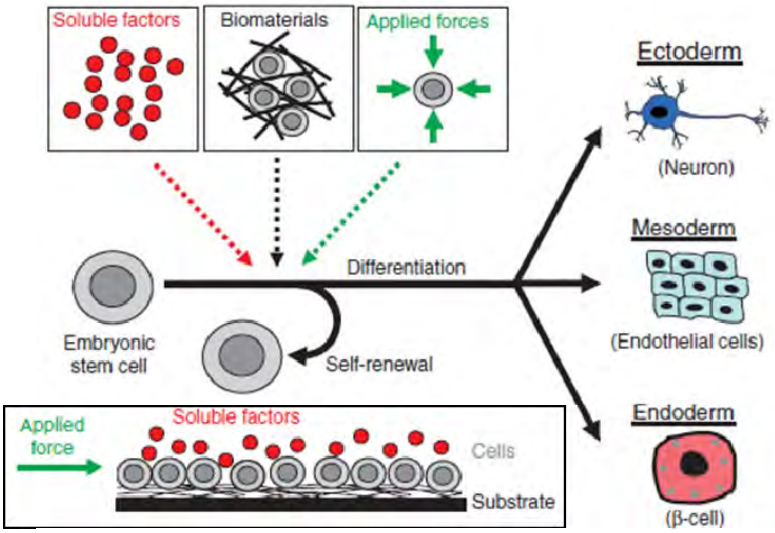


Figure 2.9 The factors in microenvironment that affect stem cell fate [14]

Because of the interactions between polymer surfaces and attached cells, polymer should be made more suitable for cells. Chemical modifications have been used in the biological modification of surface for increasing their hydrophilicity and functionality to immobilize biomolecules. Examples of the immobilized molecules include ECM ligands (e.g. laminin, fibronectin) to bind cell receptors [40] cell adhesion molecule that also improve their biocompatibility [41] and tethered synthetic oligopeptides (e.g. HAV, RGD and IKVAV) on the synthetic supports to promote cell adhesion and other cell responses [42]. Many polymers are currently used in biomedical application. Polyester such as poly(ethylene terephthalate); PET, has been used in several medical field since 1950 [43].

2.2 Poly(ethylene terephthalate) (PET)

Poly(ethylene terephthalate) (PET) is a synthetic polyester. Its high crystallinity and high melting point are responsible for its toughness as well as its excellent fiber- and film-forming properties and also it is an affordable material. Because of these properties, it has been used in many industrial, domestic applications. Due to its biocompatibility, it has also been used in biomedical engineering for surgical suture material, hard tissue prostheses, tendons and cell culture substrate (as Thermanox® or

Mylar®) [44]. As are most synthetic polymers, PET is relatively inert and hydrophobic without functional groups being able to take part in biomolecule immobilization. To overcome this drawback, modifications have been attempted to improve the surface properties of the material.

Many methods of modification of PET surface have been applied, among them are controlled, chemical breaking of ester bonds [45] surface grafting polymerization [46] and plasma treatment [47]. The first group of methods includes reaction of PET with low molecular weight substances containing hydroxyl and carboxyl groups thus incorporating corresponding functionalities onto the surface. Such action increased the hydrophility of the polymer and created the anchor functionalities for subsequent reactions. The main problem is to find the proper parameters of these processes, parameters that do not cause high degradation or significant decrease of the mechanical properties of the sample [9].

In 1998, Chen and coworker [48] developed optimized reaction conditions for ester-selective reagents to produce surface-modified PET containing active functional groups. Hydrolysis is one of the most effective modification method for PET surface involving chain cleavage of ester that leads to a surface mixture of alcohol and carboxylic acid groups with minimal degradation.

In 1999, Marchand-Brynaert and coworkers [49] reported the biological evaluation of RGD peptidomimetics that designed for covalent anchorage grafted on track-etched PET films. In order to promote cellular adhesion, the cell-attachment activity of peptidomimetic 3 was shown by culturing $CaCo_2$ cells.

In 2002, Pu and coworkers [50] studied the effect of ammonia plasma used for modifying PET and PTFE on expression of adhesion molecules in human umbilical vein endothelial cells (HUVEC). These were platelet endothelial cell adhesion molecule-1 (PECAM-1), intercellular adhesion molecule-1 (ICAM-1), Integrin avb3; vascular cell adhesion molecule-1 (VCAM-1), E-selectin, P-selectin and L-selectin were evaluated using flow cytometry and immunohistochemistry. The results have shown that the plasma treatment of PET and PTFE with ammonia improves the adhesion and growth of endothelial cells and slightly up-regulates the expression of adhesion molecules compared to the untreated material surfaces.

In 2005, Liu and coworkers [51] reported modification of PET surfaces by hydrolysis and layer-by-layer assembly of chitosan and chondroitin sulfate (CS) for enhancing endothelial cell adhesion and proliferation. The PET films incorporated with bio-macromolecules exhibit stronger ability to adhere endothelial cells and also to maintain the endothelial function, in particular for those assembled with larger number of chitosan/CS layers.

In 2006, Jiang and coworkers [52] investigated the *ex vivo* expansion of human umbilical cord blood (UCB) $CD34^+$ cells on PET surface immobilized with peptides containing the CS-1 binding motif (EILDVPST) and the RGD motif (GRGDSPC). The highest cell expansion fold was observed on the CS-1 peptide-modified surface. Moreover, all substrates surface immobilized with these peptides were more efficient in supporting in the expansion of human UCB $CD34^+$ cells than tissue culture polystyrene surface. The results suggested that covalently immobilized adhesion peptides can significantly influence the proliferation characteristics of cultured UCB $CD34^+$ cells.

In 2008, Jingrun and coworkers [53] studied an effective approach for developing blood compatible biomaterial. PET surfaces were modified by oxygen plasma-induced and ultraviolet (UV)-assisted acrylic acid (AAc) grafting polymerization. Then, the carboxyl (–COOH) groups acted as reacting sites to immobilize with gelatin and bovine serum albumin (BSA) on PET surfaces (PET-Gel-BSA). The results showed that the percent of platelet adhesion *in vitro* on PET-Gel-BSA film decreased than that on PET-Gel film and the cell density of human umbilical vein endothelial cells (HUVEC) was significantly higher than that on the control PET surface. Therefore, co-immobilization of albumin and gelatin molecules could improve the anticoagulation of the PET surface, and may enhance endothelialization.

In 2010, Boucher and coworkers [54] proposed a versatile approach based on the interactions between two de novo designed peptides, Ecoil and Kcoil, for oriented immobilization of epidermal growth factor (EGF) on PET films. After modification of PET surfaces by ammonia plasma treatment, K-coil peptides were covalently grafted on the film. Bioactivity of Ecoil-EGF captured on Kcoil-functionalized PET via coiled-coil interactions was confirmed by EGF receptor phosphorylation analysis following A-431 cell attachment. This study demonstrated that cell biological effects where tethered EGF

enhanced adhesion, spreading and proliferation of human corneal epithelial cells compared to EGF that was either physically adsorbed or present in solution.

Although there are a few literatures studying the appropriate microenvironment of cell cultivation using biomolecule-immobilized PET as substrate, none of them have studied the biological function of PET immobilized with N-cadherin mimic cyclic peptide. Their applications in maintaining stem cell properties in particular with neural and limbal stem cells, have not yet been evaluated.

CHAPTER III

METHODS AND MATERIALS

3.1 Materials

Poly(ethylene terephtharate)(PET) films (1.4 mm diameter) were purchased from Wako Pure Chemical Industry, Ltd (Japan). Iodine, Toluidine blue O, 11-mercaptoundecanoic acid and 11-mercapto-1-undecanol were purchased from Aldrich (USA). *N*-hydroxysuccinimide (NHS) and *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride) (EDC) were purchased from Fluka (Germany). Ethanol (EtOH), hydrochloric acid (HCl), methanol (MeOH) and sodium hydroxide (NaOH) were purchased from Merck (Germany). Acetic acid (CH_3COOH) was purchased from Carlo Erba (France), Dulbecco's modified Eagle's medium (DMEM)/Ham's F12 (1:1), DMEM/high glucose and fetal bovine serum (FBS) were purchased from Thermal Scientific (USA). Accutase, B27 supplement, L-glutamine, hydrocortisone, neurobasal medium, penicillin-streptomycin, sodium hydrogen carbonate ($NaHCO_3$), trypan blue dye, trypsin-EDTA and versene were purchased from Gibco (USA). Laminin, phosphate buffer saline, pH 7.4 (PBS) and primary antibodies, mouse anti-β-tubulinIII monoclonal IgG_{2b}, mouse anti-gial fibrillary acidic protein (GFAP) monoclonal $IgG_{1,}$ rabbit and anti-nestin monoclonal IgG, were purchased from Sigma (USA). Secondary antibodies, goat anti-mouse IgG_1, IgG_{2b} and mouse anti-rabbit IgG, were purchased from Invitrogen (USA). Mouse anti-β-catenin monoclonal IgG_1 was purchased from Santa Cruz Biot Inc. (USA). All reagents in Dual-luciferase assay were purchased from Promega (USA). Epithelial growth factor (EGF), fibroblast growth factor (FGF), heparin and insulin were purchased from R&D system (USA). N-cadherin mimic peptide (N-CHAVDINGHAVDIC-NH_2 sequence) was synthesized by Bio basic Inc. (USA). All reagents and materials are analytical grade and used without further purification.

3.2 Equipments

3.2.1 Contact angle measurement

Water contact angle were determined with Contact angle goniometer model Ramé-Hart 200-F1 equipped with a Gilmont syringe and a 24–gauge flat-tipped needle (Ramé-Hart, Inc., USA). The measurements were carried out in air at ambient temperature. The tested angle could be measured from a silhouette image of droplet shown on the screen. Dynamic advancing and receding angles were recorded while water was added to and withdrawn from the drop, respectively. The reported angle is an average of 5 measurements on different area of each sample.

3.2.2 Atomic Force Microscopy (AFM)

AFM images were recorded with Scanning Probe Microscope model NanoScope®IV, Veeco, USA. Measurements were performed in air using tapping mode. Silicon nitride tip with a resonance frequency of 267-295 KHz and a spring constant 20-80 N/m were used.

3.2.3 Scanning Electron Microscopy (SEM)

The morphology of cell adhesion was observed by a JEOL JEM-5410LV scanning electron microscopy (Japan). Fixed samples were dehydrated with ethanol. After being sputter-coated with gold, samples were examined.

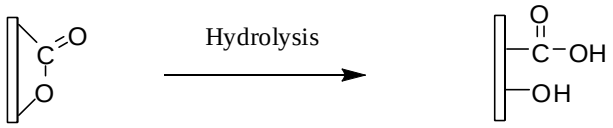
3.2.4 Optical Density Analysis by Luminometer

Optical densities of the samples in Dual-Luciferase Reporter assay were recorded with Glomax 20/20 Luminometer, Promega, USA.

3.3 Preparation of Surface-hydrolyzed Poly(ethylene terephthalate) Substrates

Poly(ethylene terephthalate); PET films were cleaned in an alcohol/water (1/1, v/v) solution for 1 h, rinsed with water and then air-dried at ambient temperature. The

cleaned PET films were subsequently soaked in aqueous solution of 1 M NaOH (aq) at 60 ºC for 1 h [48]. After being incubated for a given time, the substrates were taken out and then immersed in 0.1 M HCl (aq) for 15 min at ambient temperature to neutralize NaOH. Finally, the substrates were rinsed thoroughly with distilled water and dried *in vacuo* for overnight at ambient temperature to obtain the surface-hydrolyzed PET films.



PET substrate Hydrolyzed PET substrate

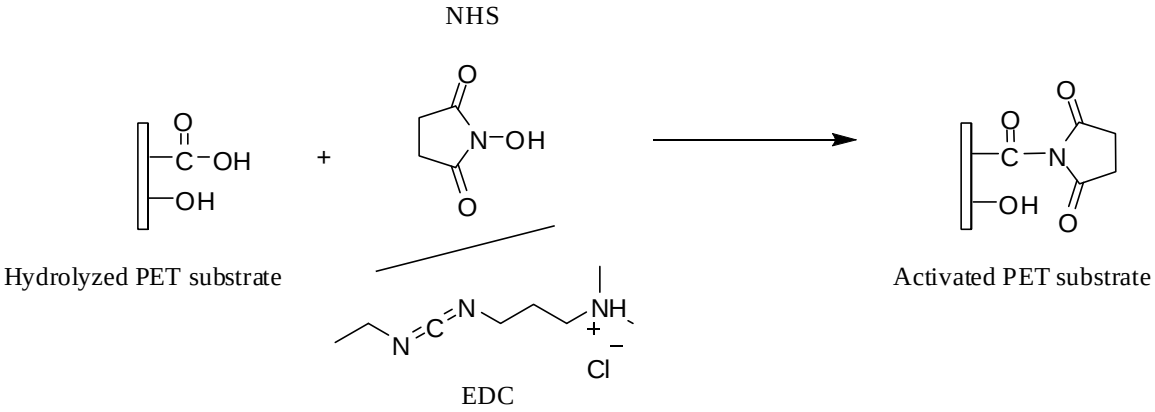
Scheme 3.1 The production of carboxyl groups on PET film surface by hydrolysis.

3.4 Determination of Carboxyl Groups on the Surface-hydrolyzed PET Substrate

A number of carboxyl (COOH) groups on the surface-hydrolyzed PET substrate were determined by a reaction with TBO according to a method of Liu *et al.* [51]. The substrate with an area of 1.54 cm^2 was immersed into a 0.5 mM TBO aqueous solution with a pH value of 10. The formation of ionic complex between the COOH groups and the cationic dye was allowed to proceed for 12 h at ambient temperature. The substrate was rinsed with 0.1 mM NaOH (aq) to remove the unbound TBO molecules. The bound TBO on the substrate was desorbed by incubation in 2 mL 50% acetic acid (aq) for overnight. The desorbed dye content was obtained by measuring the optical density of the solution at 633 nm with an UV-vis spectrophotometer (model Techna, specgene). The amount of carboxyl groups on the surface-hydrolyzed PET films was obtained from a calibration plot of the optical density versus dye solution having known concentration assuming that one carboxyl group reacts with one dye molecule.

3.5 Immobilization of Peptide to Carboxyl groups of the Surface-hydrolyzed PET Substrate

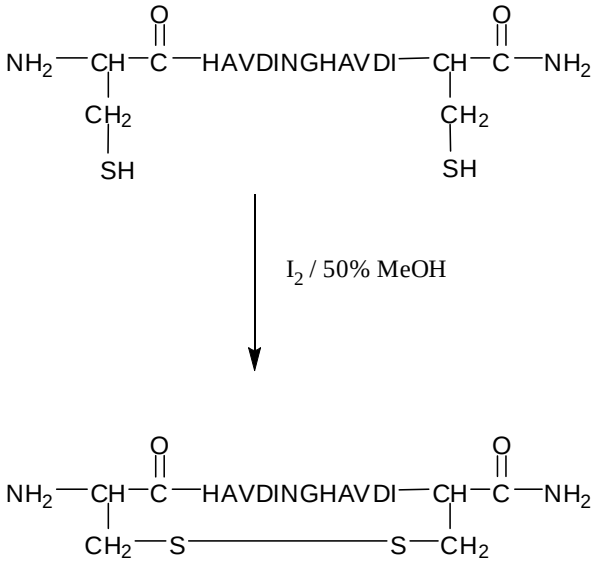
3.5.1 Activation of Carboxyl Groups of the Surfaced-hydrolyzed PET substrate



Scheme 3.2 Activation of carboxyl groups-exposed PET film surface

The surface-hydrolyzed PET films obtained from section 3.3 were immersed in an aqueous solution of EDCI (0.05 M) and NHS (0.1 M) and then stirred for 15 min. The activated PET substrates were removed from the solution and rinsed with deionized water and then dried *in vacuo*.

3.5.2 Preparation of N-cadherin Mimic Cyclic Peptide

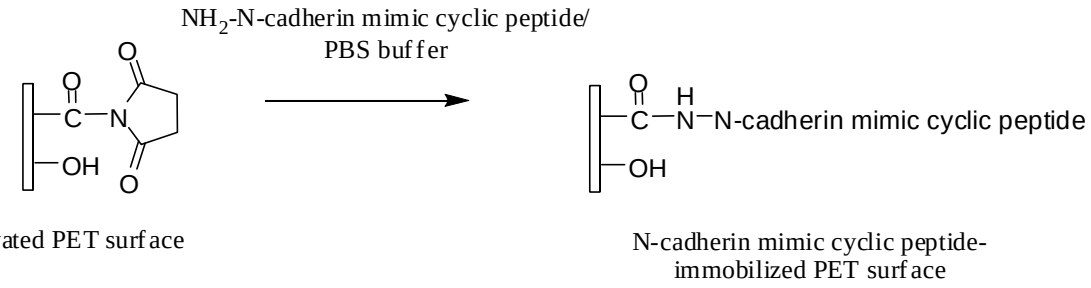


Scheme 3.3 Cyclization of N-cadherin mimic peptide molecule.

A synthetic N-cadherin mimic peptide has a sequence of N-CHAVDINGHAVDIC-NH_2. Two thiol groups of cysteine were oxidized by iodine. The peptide was first dissolved in 50% MeOH to prepare a 1 mg/mL peptide solution. The peptide solution was added dropwise to a mixed solution of 0.1M I_2 (1 mL) and 1M HCl (aq) (1 mL). The pH was adjusted to 8 with 1M NaOH (aq). The solution was then gently stirred for 30 min. Excess iodine in the peptide solution was removed by nitrogen blowing. The cyclized product appearing as white powder was obtained after lyophilization. Molecular weight of N-cadherin mimic cyclic peptide was analyzed by Microflex MULDI-TOF Mass spectrometer (Bruker Daltonics, Germany).

3.5.3 Immobilization of N-cadherin Mimic Cyclic Peptide on PET Substrates

To determine the effect of N-cadherin mimic cyclic peptide concentration and reaction time on the amount of immobilized peptide, surface plasmon resonance (SPR) technique was employed. A gold-coated SPR disk chemically modified by a mixture of 11-mercaptoundecanoic acid and 10 mM 11-mercapto-1-undecanol at ambient temperature was used as a model for surface-hydrolyzed PET substrate. After 24 h, the modified disk was rinsed with ethanol and dried with a light stream of nitrogen gas. SPR assays were processed using an Auto lab SPR obtained from the Eco Chemie (Netherlands) at 25 ºC. The carboxyl groups of the modified disk were activated by the aqueous solution of EDCI (0.05 M) and NHS (0.1 M) for 15 min before reacting with N-cadherin mimic cyclic peptide (obtained from the section 3.5.2) in PBS buffer.



Scheme 3.4 Immobilization of N-cadherin mimic cyclic peptide on the activated PET film surface.

The activated PET surfaces were immersed in a solution of N-cadherin mimic cyclic peptide in phosphate-buffered saline (PBS, pH 7.4) using the optimized peptide concentration identified by SPR. After that the immobilized PET surfaces were thoroughly rinsed with PBS buffer solution.

3.6 Evaluation of N-cadherin Mimic Cyclic Peptide Activity

The biological function of N-cadherin mimic cyclic peptide was evaluated in two stem cell types: mouse neural stem cells and human limbal stem cells.

3.6.1 Mouse Neural Stem Cell Culture

Mouse neural stem cells; mNSCs (obtained from Stem Cell Research Center, Faculty of Medicine, Chulalongkorn University) were cultured on 60-mm tissue culture dishes (Corning, USA) coated with 10 µg/mL of laminin for 2 h at 37ºC. The cells were cultured with neurobasal medium (serum-free medium) supplemented with B27 supplement, 1% penicillin-streptomycin, 2 µg/mL heparin. After filtration of mixed medium, 20 ng/mL EGF and 20 ng/mL FGF were sequentially added into the culture medium.

3.6.1.1 Cell Adhesion Assay

Unmodified PET films and PET films immobilized with N-cadherin mimic cyclic peptide were sterilized by 70% ethanol solution for 3-4 h in a 24-well tissue culture plate (Corning, USA). After that, all substrates were removed from the solution and rinsed with sterilized PBS solution. The unmodified PET film coated with 10 µg/mL laminin, an adhesive protein commonly used in neural stem cell culture, and incubated at 37ºC for 2 h were used as a positive control substrate. Then, these substrates were incubated with DMEM supplemented with 10% (v/v) FBS for overnight to inhibit unspecific attachment of cells to the substrates. After incubation, mNSCs were seeded in this well plate with a density of 2.5×10^4 cells/mL in the culture medium as described above (without EGF) and maintained at 37ºC in a humidified atmosphere of 5%CO_2 in air. After 8 h of incubation, the cultured substrates were washed once with PBS solution to remove the unattached cells before staining cells by immunofluorescence staining as described below in section 3.7. The primary antibodies

and their dilution used in this part were mouse anti-N-cadherin monoclonal IgG_1 (1:100) and mouse anti-β-catenin monoclonal IgG_1 (1:100). The secondary antibodies and their dilution were Alexa Flour 488(green)-conjugated goat anti-mouse IgG_1 (1:200). The cells were also counterstained with 4,6-diamidino-2-phynylindole (DAPI).

3.6.1.2 Luciferase Reporter Assay

Human embryonic kidney (HEK)293 cells (obtained from Stem Cell Research Center, Faculty of Medicine, Chulalongkorn University) were cultured in DMEM supplemented with 10% (v/v) FBS and 1% penicillin-streptomycin. The sterilized substrates were unmodified PET films, PET films immobilized with 0.1 and 0.5 mg/mL N-cadherin mimic cyclic peptide. Transient transfections of HEK293 cells were conducted on each substrate, in 24-well plates. A cell seeding density of 2×10^4 cells/mL was cultured overnight in growth medium. After 1 day of incubation, co-transfections were performed with Fugene reagent according to the user's manual (Roche, Germany) and each well received 2.0 μg reporter-coding plasmid or empty plasmid: 0.04 μg Lef-TK and 0.4 μg reporter construct. In this study, the reporter-coding vectors were transfected such as pcDNA-β-catenin and pcDNA-CDH2 plasmids. pcDNA empty plasmid was used as a control for transfection. Growth medium without antibiotic reagents was used as transfection medium. After 24 h of post-transfection, the growth medium was replaced with fresh culture medium. Cells were cultured for overnight and Luciferase activities were determined by use of the Dual-Luciferase Reporter Assay System (Promega, USA) according to the user's manual, followed by measurement on a luminometer (Promega, USA). Each condition was assayed in triplicate.

3.6.1.3 Neurite Outgrowth from Neurospheres

The cultured mouse neural stem cells were dissociated with accutase enzyme, collected by centrifugation, then resuspended in culture medium described above (without EGF) and cultured in 60-mm unattached-culture dishes (Corning, USA). After 4-5 days of culture, suspended cells formed neurospheres, and then spheres were selected to place onto the unmodified PET films, PET films immobilized with 0.1 and 0.5 mg/mL N-cadherin mimic cyclic peptide and coated with laminin. The neurospherers were cultured in the culture medium as described above (without EGF and FGF). After 4 days of culture, cells-attached substrates were removed from the

culture medium and the cells were stained with immunofluorescence staining method as described below in section 3.7. The primary antibodies used in this part were mouse anti-β-tubulinIII monoclonal IgG_{2b} (1:500), mouse anti-gial fibrillary acidic protein (GFAP) monoclonal IgG_1 (1:400) and rabbit anti-nestin monoclonal IgG (1:200). The secondary antibodies were FITC-conjugated goat anti-mouse IgG_{2b}, IgG_1 and mouse anti- rabbit IgG.

3.6.2 Limbal Epithelial Cell Culture

Limbal epithelial stem cells; hLSCs (obtained from Stem Cell Research Center, Faculty of Medicine, Chulalongkorn University) were cultured in limbal complete medium, 1:1 mixture of Dulbecco modified Eagle medium (DMEM) and Ham F-12 containing 10% fetal bovine serum, 1% Penicillin-Streptomycin, 2.5 mg/L amphotericin B, 1% L-glutamine, 0.5 mg/L hydrocortisone, 2.5 mg/ml $NaHCO_3$, 10 ng/mL EGF, and 5 mg/L insulin at 37ºC with humidified atmosphere of 5% CO_2 in air.. Culture medium was changed every 1-2 days. hLSCs were dissociated into single cells by using 0.05%trypsin-EDTA. Cells were observed under an inverted microscope (Nikon, Japan) before sub-cultivation for forming colony.

3.6.3 Preparation of 3T3 Fibroblast Feeder Cells

3T3 cell lines were purchased from American Type Culture Collection (ATCC). 3T3 were cultured in DMEM supplemented with 10% FBS, 1% Penicillin-Streptomycin and incubated at 37 ºC in humidified incubator with 5% CO_2 in air. Culture medium was changed every 2-3 days. Cells were sub-cultured by trypsinization when they reached 70-80% confluent cell density. For limbal epithelial stem cells culture, confluent 3T3 fibroblast cells were treated with 4 μg/mL mitomycin C (MMC) in culture medium and incubated for 2 hours at 37ºC humidified atmosphere of 5% CO_2 in air. Then cells were trypsinized and seeded into 6-well cell culture dishes at a density of 2.2×10^4 cells/mL to be used as feeder cells.

3.6.4 Limbal Epithelial Cell Colony Forming

Limbal epithelial cells cultured on cell culture dishes containing mitomycin C (MMC)-treated 3T3 feeder cells were trypsinized and seeded at 70-80% confluent cells onto the unmodified PET films in comparison with the PET films immobilized with 0.5

mg/mL N-cadherin mimic cyclic peptide. To study the effect of other factors on colony forming efficency, we were interested in two parameters: growth factors added into culture medium and cell density seeded onto substrates.

3.6.4.1 Effect of Growth Factor

Limbal epithelial cells were cultured on the substrates in 3T3 conditional medium. The conditional medium was divided into three conditions: untreated, treated with BMP7 as well as treated with bone morphogenetic protein-7 (BMP-7) and activin receptor-like kinase-5 (ALK-5) inhibitor. Colony formationof cells were observed and counted under an inverted microscope (Nikon, Japan).

3.6.4.2 Effect of Cell Density

First, limbal epithelial cells were cultured on the substrates in 3T3 conditional medium. After trypsinization, the cells were seeded at densities of 0.5, 1.0 and 2.0×10^4 cells/mL. Epithelial cells were sub-cultured when epithelial colonies were completely present on the substrates. Second, epithelial colonies were sub-culture until they cannot maintain epithelial-like colony (about 4-5 passages). Finally, the immunofluorescence staining was performed to evaluate the expression and the location of molecular marker that have been proposed to identify limbal epithelial stem cells or differentiated cells. ΔNp63α was used as an immunoreactive marker. Fluorescence images were obtained by Confocal microscope (Nikon, Japan).

3.7 Immunofluorescence Staining

The immunofluorescence staining was performed to evaluate the expression and location of different molecular marker. In brief, cells were fixed with 4% paraformaldehyde for 30 min at ambient temperature and absolute methanol solution for 20 min at -20ºC, respectively, and then permeabilized with 1X PBS supplemented with 0.3% Triton X-100 for 15 min, and blocked in blocking buffer (10% goat serum and 0.3% Triton X-100 in PBS) for 30 min at ambient temperature. After each step, cells were washed three times with PBS for 10 min each to decrease nonspecific antibody interactions. Next, the cells were incubated overnight at 4ºC with diluted primary antibodies in diluents (5% goat serum, 0.3% Triton X-100 in PBS). Finally,

cells were washed with PBST (PBS + 0.3% TritonX-100) supplemented with 0.1% BSA three times for 10 min each. Secondary antibodies, Alexa Flour 488(green)/546(red) conjugated goat anti mouse antibody or goat anti rabbit antibody, were also diluted in diluents at dilution 1:200 and incubated for 1 h at ambient temperature in dark. For nuclear staining, 0.1 μg/mL DAPI (Molecular Probes) was used and incubated for 5 min at ambient temperature. These immunostained cells were visualized by indirect fluorescence under the fluorescent microscope (Carl Zeiss, Germany).

CHAPTER IV

RESULTS AND DISCUSSION

In this chapter, the results can be divided into two sections. The first section concentrates on surface modification of poly(ethylene terephthalate) films via alkaline hydrolysis to generate carboxyl groups followed by N-cadherin mimic cyclic peptide immobilization. The second section is dedicated to evaluating biological functions of N-cadherin mimic cyclic peptide immobilized on the surface-hydrolyzed PET substrates towards two types of stem cells: mouse neural stem cells (mNSCs) and human limbal stem cells (hLSCs).

4.1 N-Cadherin Mimic Cyclic Peptide Immobilization on Surface-hydrolyzed Poly(ethylene terephthalate) (PET) Films

As depicted in Scheme 3.1, the ester bonds on the surface of PET were hydrolyzed in the basic aqueous solution of NaOH which led to the breakage of PET backbone and the formation of carboxyl and hydroxyl groups. The production of hydrophilic carboxyl/hydroxyl groups on the surface-hydrolyzed PET film can be monitored from a change in wettability of the film. After hydrolysis, the water contact angle (θ_A/θ_R) of the PET film was dropped from 71.8 ± 0.5º/27.1 ± 0.7º to 57.9 ± 0.8º /11.2 ± 1.7º (Table 4.1). It should be noted that the unmodified PET film surface usually possess a water contact angle in a range of 70-77º [44]. The presence of COOH groups on the surface of the hydrolyzed PET film was then determined by Toluidine blue O assay, a commonly known method for the determination of carboxyl group density [51]. The produced carboxyl groups on the surface of the hydrolyzed PET film can form a complex with toluidine blue O. The absorbance of the solution containing the desorbed complex was measured at 633 nm. The COOH content was obtained from a calibration plot of the optical density versus dye concentration (see Appendix D). The estimated carboxyl group density on the surface of hydrolyzed PET substrate is $8.31 \times 10^{-7} \pm 1.39 \times 10^{-7}$ mmol/cm^2.

Table 4.1 Advancing (θ_A) and receding (θ_R) water contact angles of PET films after stepwise modification.

PET film surface	Water contact angles (degree)	
	θ_A	θ_R
Pre-hydrolysis	71.8 ± 0.5	27.1 ± 0.7
Post-hydrolysis	57.9 ± 0.8	11.2 ± 1.7
After activation	64.7 ± 1.8	12.1 ± 0.7
After peptide immobilization	66.6 ± 1.7	16.3 ± 1.6

In this study, N-cadherin mimic cyclic peptide is of our particular interest. The peptide was obtained by cyclization of a linear peptide having a sequence of N-CHAVDINGHAVDIC-NH_2 via iodine oxidation (Scheme 3.3). As analyzed by mass spectrometry, formation of a disulfide bridge can be evidenced from a reduction of the molecular weight from 1466 of the linear peptide to 1464 of the cyclized one. The mass spectra of the peptide both before and after cyclization are displayed in Appendix B.

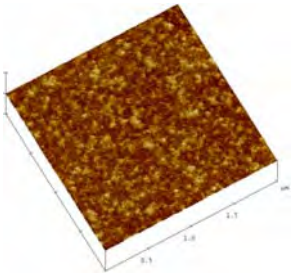
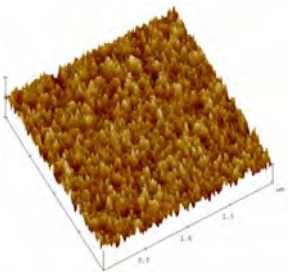
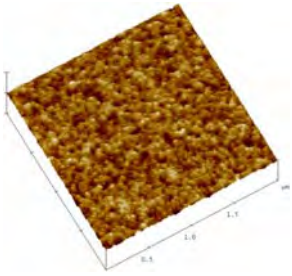
Generally, biomolecules are often immobilized on polymer surface via an amide bond formation between the carboxyl groups of the polymer and the amine groups of the biomolecule. To achieve the covalent attachment of N-cadherin mimic cyclic peptide to the carboxyl groups of the surface-hydrolyzed PET substrtae, a method of introducing reactive intermediate, *N*-hydroxysuccinimidyl (NHS) ester was used. The carboxyl groups of were first activated by a water-soluble carbodiimide, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS) to form NHS group (Scheme 3.2). The NHS group was then coupled with amine-terminated N-cadherin mimic cyclic peptide leading to amide bond formation (Scheme 3.4).

The carboxyl group activation and peptide immobilization were also determined by water contact angle measurements (Table 4.1). As expected, the water contact angle of the activated PET film was increased to 64.7 ± 1.8º/12.1 ± 0.7º indicating that the

hydrophilic carboxyl groups of the surface-hydrolyzed PET film have been replaced by hydrophobic *N*-succinimidyl groups. The water contact angles became slightly higher after the peptide immobilization.

The surface morphology was determined by atomic force microscopy (AFM). As demonstrated in Table 4.2, the roughness and topography of PET films were changed upon stepwise surface modification. It is not surprising to see that the surface of PET film became rougher with a roughness of 6.52 ± 0.40 nm after hydrolysis. After being immobilized with N-cadherin mimic cyclic peptide, the surface of PET film was slightly smoother than the surface-hydrolyzed PET films implying that there was a coverage of the peptide on the surface.

Table 4.2 AFM images of surface-modified PET films.

Sample	Cleaned PET film	Hydrolyzed PET film	N-cadherin mimic cyclic peptide-immobilized PET film
Morphology in 3D			
RMS roughness (nm)	2.17 ± 0.24	6.52 ± 0.40	5.18 ± 0.31

Surface plasmon resonance (SPR) is a well-recognized optical-based method that depends on the measurement of changes in the refractive index, which is proportional to the mass of the bound or adsorbed species on its surface. It is commonly used for studying interactions between biomolecules that are attached to the substrates and those

in solution. In our particular case, we chose to use SPR as a tool to determine an optimized condition in terms of time used for EDC/NHS activation and peptide concentration mainly because the activation and peptide immobilization can be monitored in real time. This helps minimize the use of the costly peptide. In this study, a gold-coated SPR disk modified with a mixture of 11-mercaptoundecanoic acid and 10 mM 11-mercapto-1-undecanol was used as a model for surface-hydrolyzed PET substrate. In principle, the SPR disk modified by such method should contain an equal amount of COOH and OH groups of which should be the case for the surface hydrolyzed PET film. It should be emphasized that the breaking of one ester bond of PET generates one COOH and one OH group. The measured SPR angle shifts during the activation and immobilization steps can be converted into mass uptakes, using a sensitivity factor of 120 mDegrees per 100 ng/cm^2 [55].

As shown in Table 4.3, there was no variation of SPR angle shift in the step of peptide immobilization upon increasing the activation time from 15 to 30 min indicating that 15 min was sufficient to obtain the highest degree of activation and that certainly had no impact on the amount of subsequently immobilized peptide. The amount of immobilized peptide was essentially the same for the surface-hydrolyzed PET substrates that were activated for different period of time. SPR signals as a function of time for SPR analysis are shown in Appendix C.

Table 4.3 SPR angle shift and the corresponding amount of immobilized N-cadherin mimic cyclic peptide on the modified gold-coated SPR disk as a function of EDC/NHS activation time using peptide concentration of 0.5 mg/mL and immobilization time of 15 min.

Activation time (min)	SPR angle shift (m°)	Amount of immobilized N-cadherin mimic cyclic peptide (nmol/cm^2)
15	60.5 ± 1.10	0.077 ± 0.004
30	62.4 ± 2.70	0.080 ± 0.007

The results shown in Figure 4.1 suggested the amount of immobilized N-cadherin mimic cyclic peptide can be varied as a function of N-cadherin mimic cyclic peptide concentration in solution. The peptide concentration of 0.1 and 0.5 mg/mL that yielded the amount of immobilized peptide of 0.020 ± 0.002 and 0.080 ± 0.005 nmol/cm^2, respectively were chosen for immobilization of N-cadherin mimic cyclic peptide on the surface-hydrolyzed PET films for further studies. It should be noted that the peptide immobilization was carried out overnight for the surface-hydrolyzed PET substrate.

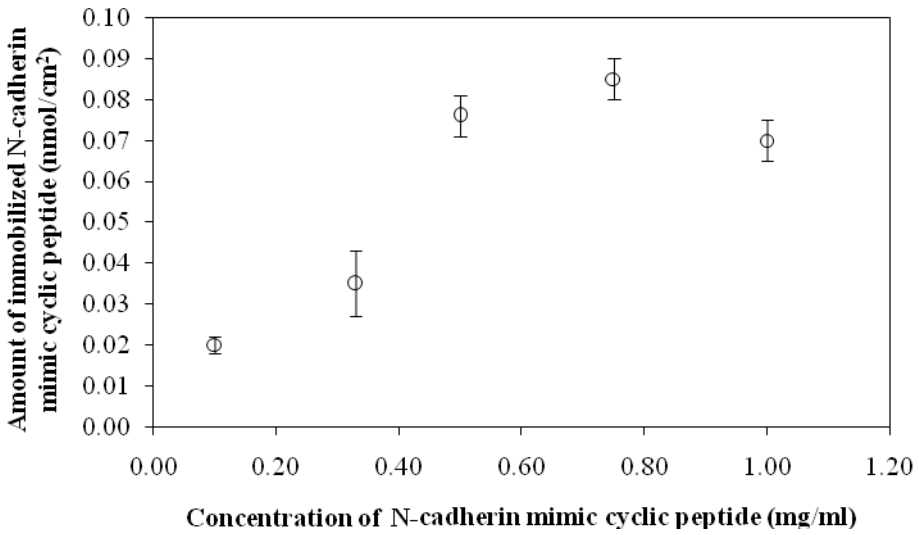


Figure 4.1 Amount of N-cadherin mimic cyclic peptide (nmol/cm^2) immobilized on the modified gold-coated SPR disk as a function of N-cadherin mimic cyclic peptide concentration using immobilization time of 15 min**.**

4.2 *In vitro* Stem Cell Responses to Surface-immobilized N-cadherin Mimic Cyclic Peptide

Previous studies have demonstrated that a soluble dimeric form of N-cadherin cyclic peptide, N-Ac-*CHAVDINGHAVDIC*-NH_2, functions as an agonist by stimulating specific transduction signaling known to initiate neurite outgrowth [7] and also promoting neural cell survival [56]. According to these properties, N-cadherin was a chosen molecule to develop a new artificial extracellular matrix (ECM) for maintaining

stem cell features. Furthermore, recent study has suggested that cellular responses on N-cadherin-adsorbed substrates are an important factor to clarify whether the stem cell can be maintained especially through cadherin-mediated interactions [57]. In this research, the surface-hydrolyzed PET films immobilized with N-cadherin mimic cyclic peptide were used as substrates for evaluating responses of two stem cell lines: mouse neural stem cells (mNSCs) and human limbal stem cells (hLSCs).

4.2.1 Adhesion of mNSCs

To examine specific activity of N-cadherin mimic cyclic peptide, mNSCs were cultured on unmodified PET film surfaces, PET films immobilized with cyclic peptide (0.1 and 0.5 mg/mL) and coated with laminin (10 μg/mL). The initial cell seeding density was 2.5×10^4 cells/mL. After washing with PBS solution at 8 h of incubation, the cell morphologies as visualized under bright field microscope are shown in Figure 4.2. mNSCs could attach on the PET film surface immobilized with both concentrations of the cyclic peptide (Figure 4.2c and d) and coated with laminin (Figure 4.2b). Since mNSCs could not attach on the unmodified PET film surface, cells still aggregate as neurospheres as a result of cell-cell interactions being presumably stronger than cell-material interactions (Figure 4.2a).

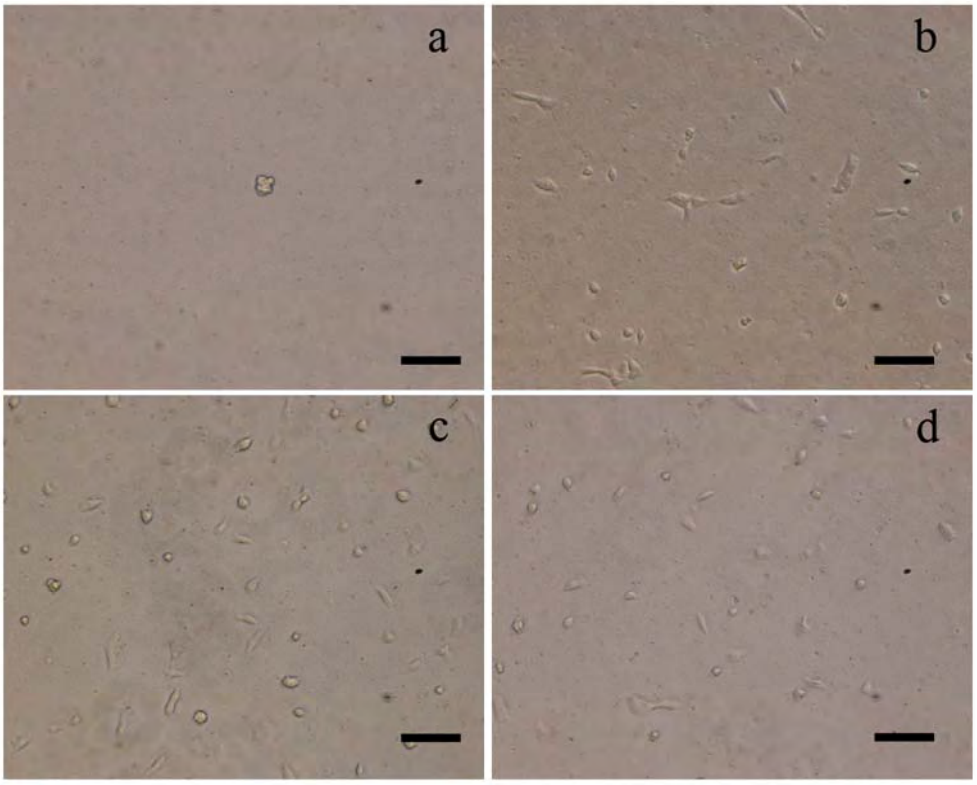


Figure 4.2 Morphologies of mNSCs on (a) unmodified PET film, (b) PET film coated with laminin, PET films immobilized with (c) 0.1 mg/mL and (d) 0.5 mg/mL N-cadherin mimic cyclic peptide,as observed by bright field optical microscope. Scale bar = 100 μm (10X).

The morphology of the attached cells on the modified PET film surfaces was further verified by scanning electron microscopy (SEM) as shown in Figure 4.3. The cell adhered on the 0.5 mg/mL N-cadherin mimic cyclic peptide-immobilized PET film surface seemed to be less spreading than that attached on the laminin-coated one.

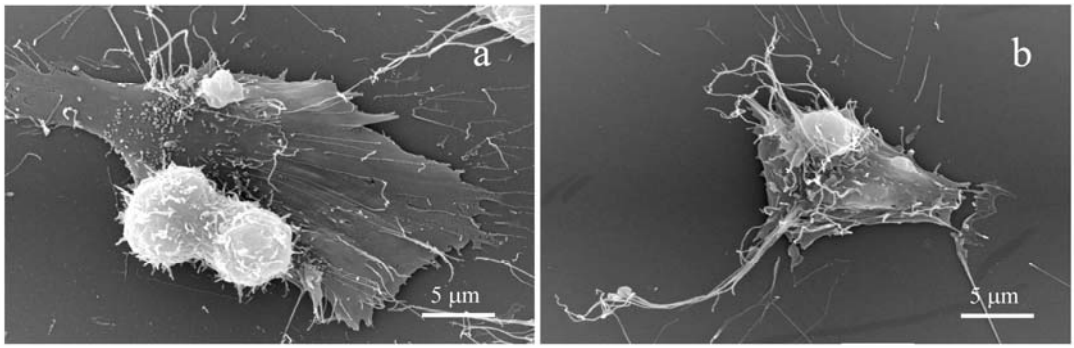


Figure 4.3 Morphology of mNSCs on laminin-coated PET film (a) and N-cadherin mimic cyclic peptide immobilized PET film (b) observed by SEM.

4.2.2 Neurite Outgrowth from mNSC Neurospheres

Neurite outgrowth from mNSC neurospheres was tested against N-cadherin mimic cyclic peptide both in the soluble form and the surface-immobilized form. For the test with the soluble peptide, mNCS neurospheres were cultured on the laminin-coated PET film surface. After 5 days of culture, migration of sphere-forming cells from their original spheres (12-15 spheres/well) was observed. The mNCS neurospheres shown in upper panel of Figure 4.4 (row a) were stimulated with 50 µM soluble form of N-cadherin mimic cyclic peptide added into the culture medium without EGF and FGF whereas those appeared in lower panel of Figure 4.4 (row b) were not stimulated. Originally, it was hypothesized that neurospheres induced with the soluble form of the cyclic peptide could exhibit greater axonal outgrowth than those were not induced. The results demonstrated in Figure 4.4 indicated that was not the case. Nevertheless, it was found that the induced neurospheres could differentiate into neuron-like cell more extensively than the non-induced neurospheres, as pointed out by the arrows. To verify the differentiated cell phenotype, indirect immunofluorescence staining was performed. The expressing markers of maintaining neural stem cell and neuron cell type were examined by immunoreactive anti-Nestin and anti-β-III-Tubulin, respectively. As visualized under fluorescence microscope (Figure 4.4), neural cells migrated from neurospheres, in both conditions and maintained in neural stem cell stage as can be realized from the red staining of anti-Nestin. The induced neurospheres could show a few neuron cell type which can be stained with green color of the

immunoreactive anti-β-III-Tubulin. Although the impact of soluble peptide on the neurite outgrowth cannot be realized from this investigation because most of the cells were in the form of astrocyte-like cell or glial phenotype that cannot generate long axonal outgrowth, the results indicated that this N-cadherin mimic cyclic peptide is cytocompatible and not toxic to the mNSC.

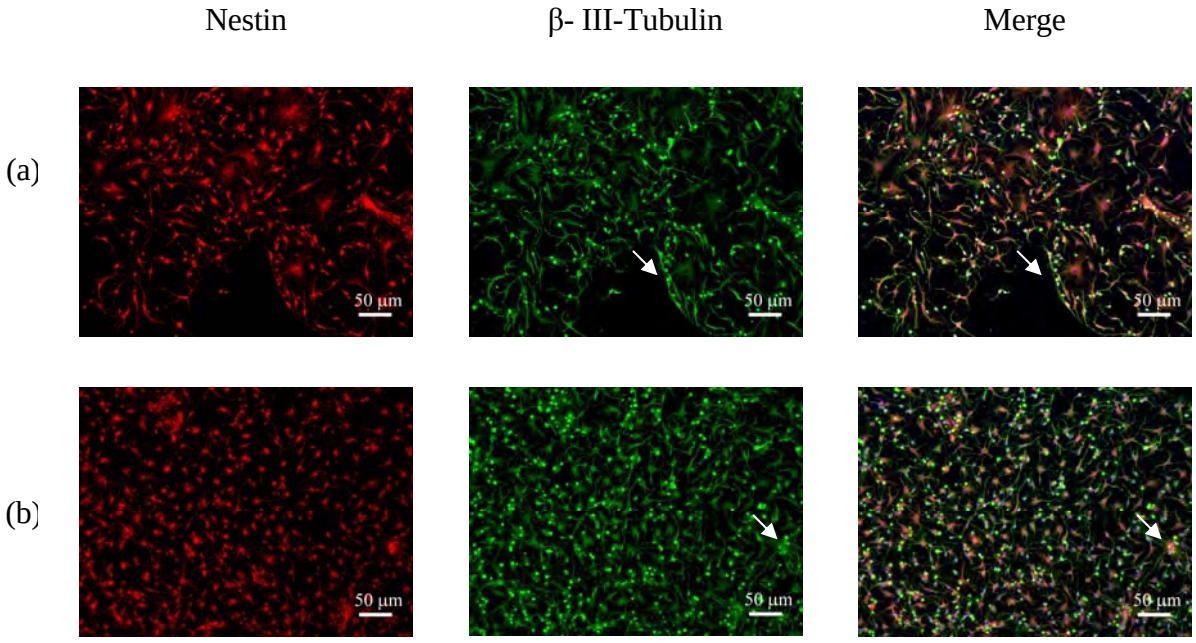


Figure 4.4 Fluorescence images showing differentiated cell phenotypes from mNSC neurospheres after 5 days culture on PET films coated with laminin with (row a) and without (row b), induction with soluble N-cadherin cyclic mimic peptide after immunoreactive staining with anti-nestin and anti-β-III-Tubulin.

For the test with the surface-immobilized N-cadherin cyclic mimic peptide, mNCS neurospheres were cultured directly on the surface-modified PET substrates. Migration of sphere-forming cells from their original spheres on different surfaces observed under bright field microscope is shown in Figure 4.6. The neural cells could migrate from neurosphere placed on the PET film coated with laminin as well as those immobilized with 0.1 and 0.5 mg/mL N-cadherin mimic cyclic peptide whereas the migration of cells were not observed on the unmodified PET film. Although the cell

migration on the PET film coated with laminin was greater than the peptide-immobilized PET films, the latter exhibited relatively longer axonal outgrowth than the former.

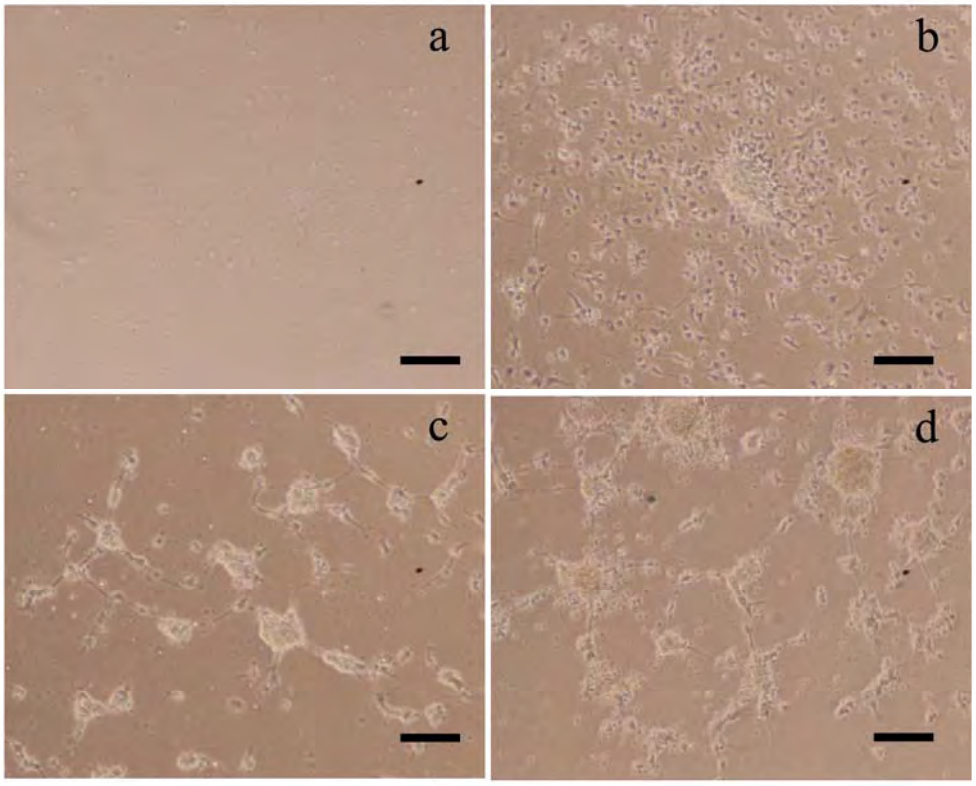


Figure 4.5 Migration of neurosphere-forming cells on various substrates after 3 days of culture: (a) unmodified PET film, PET films immobilized with (c) 0.1 mg/mL and (d) 0.5 mg/mL N-cadherin mimic cyclic peptide as observed by bright field optical microscope. Scale bar = 100 μm (10X).

The images observed in Figure 4.5 suggested that there was heterogeneous cell population at differential stages of neuronal and glial (astrocyte-like cell) development. Thus, the differentiated cells could not be directly identified from the evidence of cell morphology. The indirect immunofluorescence staining by specific antibodies was necessary. The expressing markers of maintaining neural stem cell, neuron cell type and glial cell type were examined by immunoreactive staining of anti-Nestin (red), anti- β-

III-Tubulin (green) and anti-GFAP (red), respectively, as observed under fluorescence microscope.

As shown in Figure 4.6, after 5 days of culture, all migrating cells from neurospheres cultured on the surface-modified substrates exhibited nestin-positive marker. This result indicated that process-bearing cells maintained the phenotype of neural stem cell. Though the NSCs could differentiate into neurons or glial cells under serum-free culture medium without EGF and FGF, the expression of positive cells for different markers on the different modified surfaces was varied. As shown in Figure 4.8, most migrating cells on all modified surfaces were weakly stained with anti-GFAP immunoreactive. It is then difficult to absolutely identify the glia cell type.

On the PET film coated with laminin (Figure 4.7 and 4.8; row a), the neuronal marker exhibited only in the area of neurosphere-forming cells. That is similar to the expression of cells cultured on the PET film surface-immobilized with 0.1 mg/mL N-cadherin mimic cyclic peptide (Figure 4.6 and 4.7; row b). Moreover, a few cells could migrate out from neurosphere and exhibited less axonal outgrowth than the cells cultured on the PET film surface-immobilized with 0.5 mg/mL N-cadherin mimic cyclic peptide. On this peptide immobilized surface (Figure 4.6 and 4.7; row c), cells could migrate out to lesser extent, but they exhibited longer axonal outgrowth as compared to the cells cultured on the PET surface coated with laminin. The neuronal marker not only expressed in the area of neurospheres, but also in the axon of a few cell.

The results above provided fundamental evidences about the effect of N-cadherin mimic cyclic peptide on neural stem cell behaviors. The neurospheres cultured on the PET film surface-immobilized with 0.5 mg/mL N-cadherin mimic cyclic peptide can generate axonal outgrowth to communicate with other surrounding neurospheres. It seemed that cell migrating and axonal outgrowth from the neurospheres are the principal approach for communication among neurospheres. Therefore, the underlying mechanisms controlling the interactions between NSCs and modified culture materials should be further investigated. Suitable culture system that can activate the axonal outgrowth of cells has to be identified.

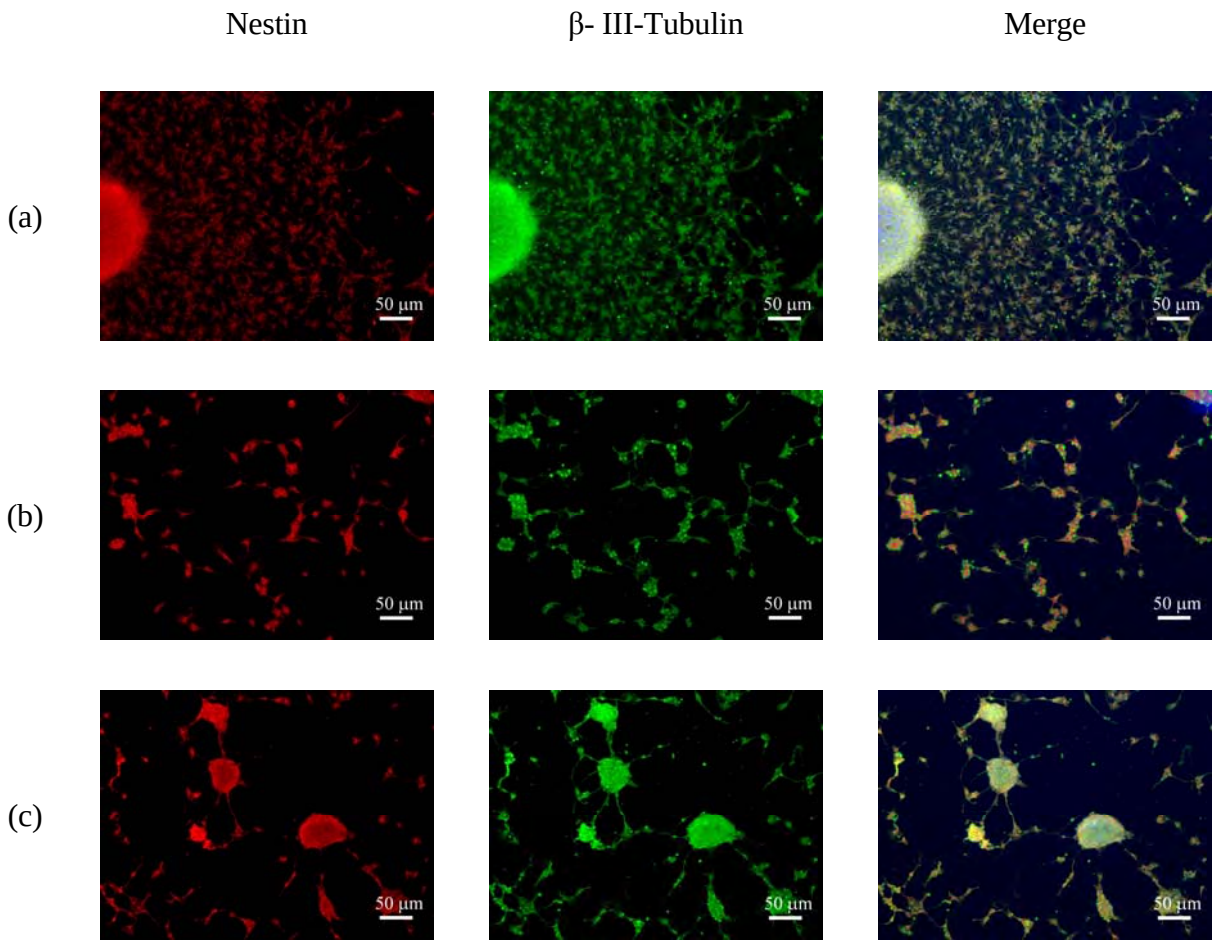


Figure 4.6 Fluorescence images showing differentiated cell phenotypes from mNSC neurospheres after 5 days of culture on PET film coated with laminin (row a), PET films immobilized with 0.1 mg/mL (row b) and 0.5 mg/mL (row c) N-cadherin mimic cyclic peptide,after immunoreactive staining with anti-nestin and anti-β-III-Tubulin. Scale bar = 50 µm.

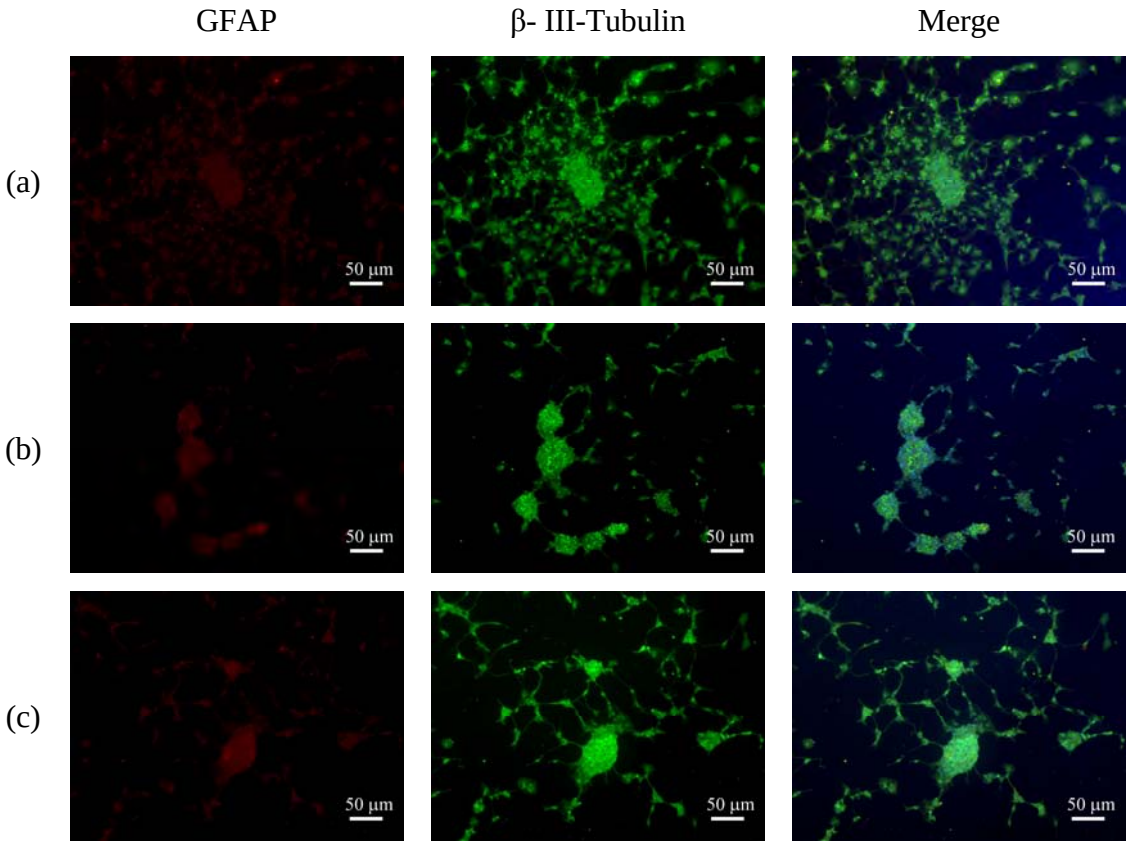


Figure 4.7 Fluorescence images showing differentiated cell phenotypes from mNSC neurospheres after 5 days of culture on PET film coated with laminin (row a), PET films immobilized with 0.1 mg/mL (row b) and 0.5 mg/mL (row c) N-cadherin mimic cyclic peptide after immunoreactive staining with anti-GFAP and anti-β-III-Tubulin. Scale bar = 50 µm.

4.2.3 Regulation of N-cadherin Mimic Cyclic Peptide in Wnt/β-catenin Signaling Pathway

As illustrated in Figure 4.8, β-catenin, a key effector of Wnt signaling pathway, interacts with intracellular domain of the N-cadherin [58] and also bind with α-catenin as well as p120 molecule at cell membrane. Whenever β-catenin is dissociated from the binding protein molecules, β-catenin free molecules will be present in cytoplasm. These free molecules can transport into nucleus to stimulate luciferase gene expression. On the other hand, the presence of N-cadherin molecules will inhibit β-catenin signaling activity resulting in a decreased luciferase expression level. To analyze β-catenin

signaling activity, we used a Dual-luciferase assay with human embryonic kidney (HEK)293 cells. As depicted in Figure 4.9, HEK293 cells co-transfected with pcDNA-β-catenin plasmid showed increased luciferase activity while the signaling activity was decreased in cells co-transfected with pcDNA-N-cadherin (CDH2) plasmid. Also, increasing amounts of extracellular domain N-cadherin by culturing transfected cells on PET films immobilized with N-cadherin mimic cyclic peptide could decrease luciferase activity as opposed to the unmodified PET film. As a result, the exposition of both intracellular N-cadherin and extracellular N-cadherin mimic cyclic peptide has an impact on the reduction of luciferase expression.

These results can support the biological function of N-cadherin mimic cyclic peptide that has the effect on related gene expression within cells.

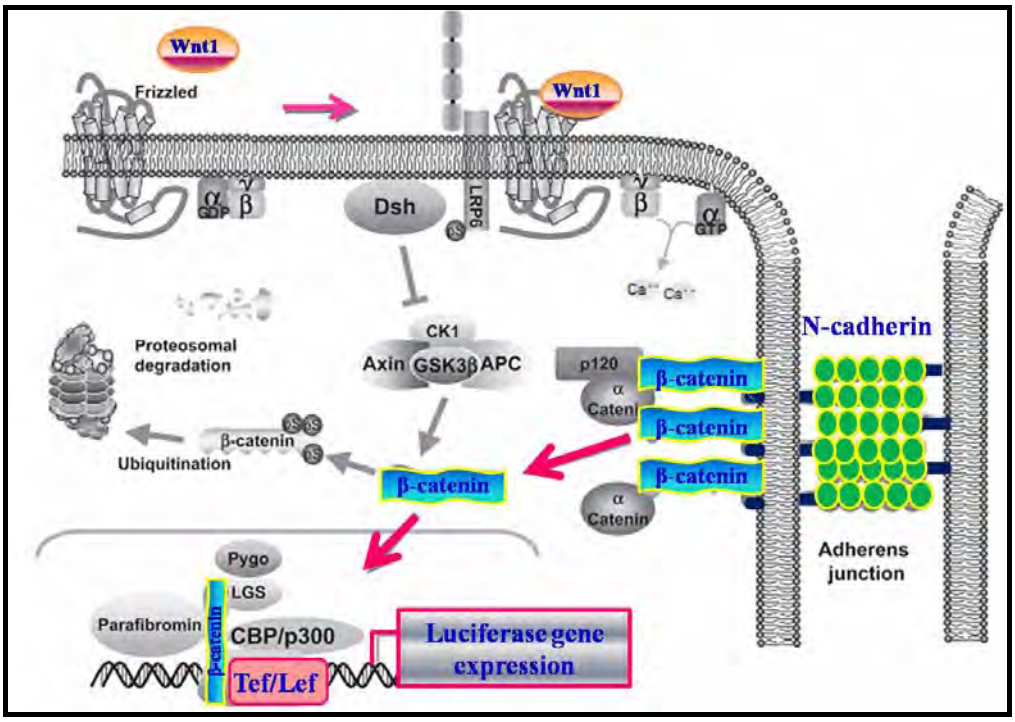


Figure 4.8 Activity of β-catenin in Wnt/ β-catenin signaling pathway [59].

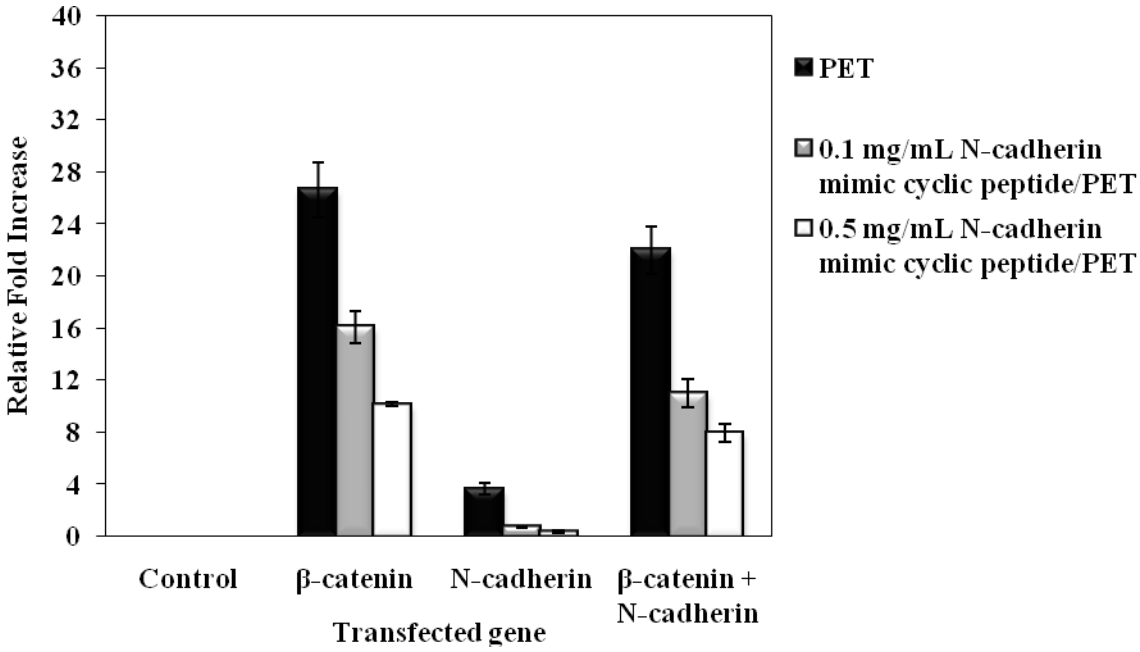
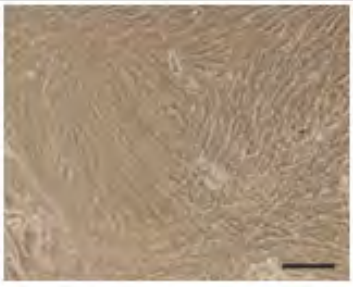
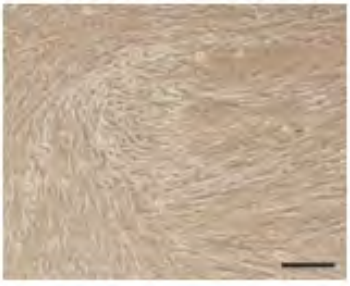
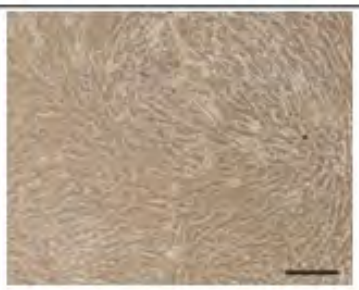
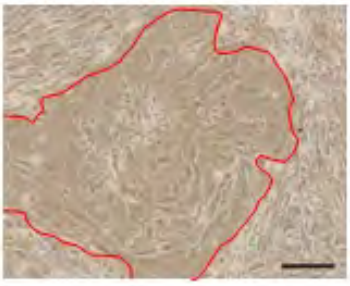


Figure 4.9 Dual-luciferase reporter assay of HEK293 cells transfected with designated gene on different surface-modified PET substrates.

4.2.4 Biological Responses of Human Limbal Stem Cells (hLSCs)

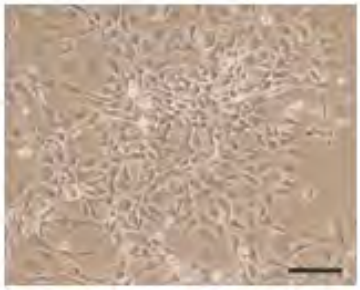
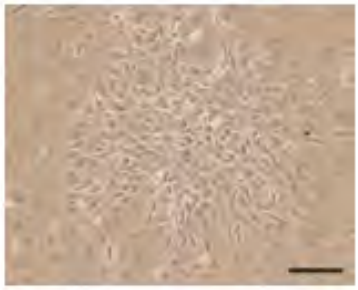
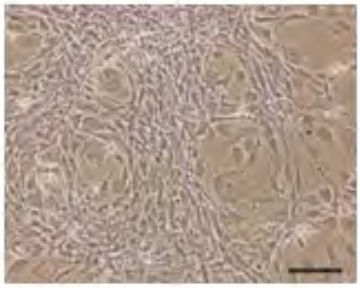
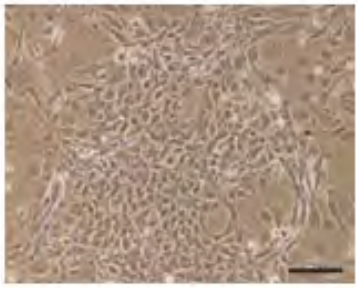
To assess the effect of N-cadherin mimic cyclic peptide on efficiency of epithelial-like colony formation, the first sub-cultivation or passage of hLSCs, that were cultured on mitomycin C (MMC)-treated 3T3 feeder, were dissociated into single cells and then seeded onto the unmodified PET films and the PET films immobilized with 0.5 g/mL N-cadherin mimic cyclic peptide. For the second passage, the hLSCs were cultured in the presence of growth factors which are BMP and ALK5 inhibitor in the culture system (Table 4.4). After 7 days of culture, the hLSCs, not treated and treated only with BMP-7, demonstrated that the migrating fibroblast-like cells grew entirely over the substrate surfaces whereas colonies of epithelial-like cells (marked with red line) could be observed for the cells treated with BMP-7 and ALK5 inhibitor on both substrates. ALK5 inhibitor seems to be an important factor in epithelial cell culture system. Thus, in the next passage, the hLSCs were treated with ALK 5 inhibitor for supporting epithelial-like colony formation.

Table 4.4 Clonal morphology of hLSCs cultured on different substrates at the 2nd passage, scale bar = 200 µm.

Culture system	Clonal morphology on substrate	
	PET	PET films immobilized with 0.5 mg/mL N-cadherin mimic cyclic peptide
Non-treated		
Treated with BMP7		
Treated with BMP7 + ALK5 inhibitor		

In the third passage (Table 4.5), the hLSCs with varied cell densities (0.5, 1.0 and 2.0×10^4 cells/mL) were seeded on mitomycin C (MMC)-treated 3T3 feeder cells. The limbal epithelial-like colony (marked with red line) could clearly form at a cell density of 2.0×10^4 cells/mL dissociated from both substrates. These results suggested that both the unmodified PET film and PET film immobilized with N-cadherin mimic cyclic peptide could efficiently support epithelial-like colony forming when cultured in suitable culture system.

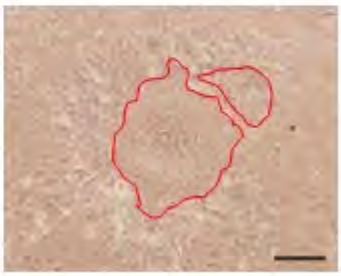
Table 4.5 Clonal morphology of hLSCs cultured on 3T3 feeder cells in the presence of 0.5 μM ALK 5 inhibitor at the 3rd passage , scale bar = 200 μm.

Cell density (cells/mL)	Clonal morphology of dissociated epithelial cells from each substrate on 3T3 feeder cells	
	PET	PET films immobilized with 0.5 mg/mL N-cadherin mimic cyclic peptide
0.5×10^4		
1.0×10^4		
2.0×10^4		

To further test the efficiency of the substrates, hLSCs from the third passage were dissociated and then seeded on the treated 3T3 feeder cells. As shown in Table 4.6, limbal cells in the fourth passage, which were cultured at a cell density of 2.0×10^4 cells/mL and treated with ALK5 inhibitor, were in the form of epithelial-like colony (marked with red line) only on the PET film immobilized with N-cadherin mimic cyclic peptide, but hLSCs cultured on the unmodified PET film were almost differentiated into

fibroblast-like cells as well as presented abortive colony. Next, we used immunofluorescence staining to further characterize colony formed on the peptide-immobilized PET film. In this examination, ΔNp63α was used as an immunoreactive marker which should appear red color if the stem cell properties are maintained. It was found that the colony formed contained p63 positive cells in their nucleus, as illustrated in Figure 4.10. Therefore, the expression of ΔNp63α in the epithelial-like colony was a crucial factor to confirm the potential of N-cadherin mimic cyclic peptide for maintaining limbal stem cell properties.

Table 4.6 Clonal morphology of hLSCs at the 4th passage seeded on growth-arrested 3T3 feeders at a cell density of 2.0×10^4 cells/mL and cultured in the presence of 0.5 µM ALK 5 inhibitor, scale bar = 200 µm.

Culture system	Clonal morphology of dissociated epithelial cells from each substrate	
	PET	PET films immobilized with 0.5 mg/mL N-cadherin mimic cyclic peptide
On 3T3 feeder cells		

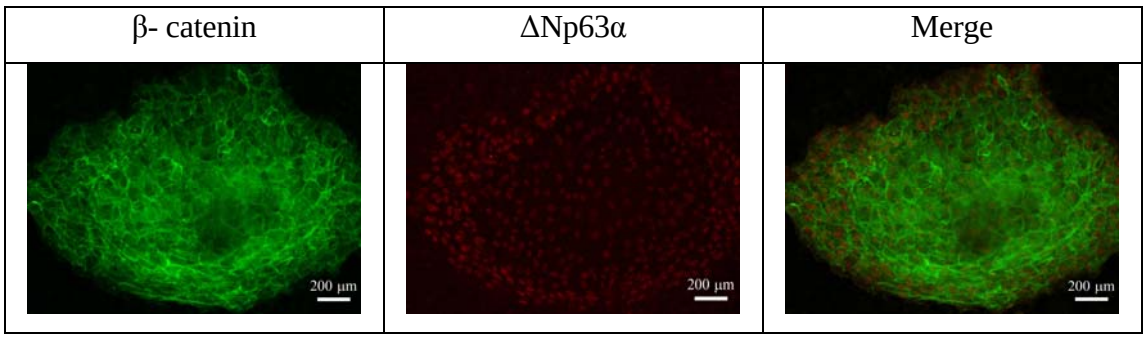


Figure 4.10 Expression of ΔNp63α (red color) in the colony of hLSCs cultured on the PET film immobilized with N-cadherin mimic cyclic peptide for the third passage and seeded on growth-arrested 3T3 feeders for the 4th passage.

CHAPTER V

CONCLUSION AND SUGGESTION

Poly(ethylene terephthalate) films can be successfully modified by alkaline hydrolysis to generate carboxyl groups on the surfaces. As determined by toluidine blue O assay, the carboxyl group density of the surface-hydrolyzed PET film was 8.31×10^{-7} mmol/cm^2. It has been demonstrated that the carboxyl groups of the hydrolyzed films are readily available for activation by EDC/NHS and subsequent attachment of N-cadherin mimic cyclic peptide. According to SPR analysis, the density of the immobilized N-cadherin mimic cyclic peptide relied on the concentration of the peptide. The optimize concentration at 0.5 mg/mL was chosen to be used for further studies.

The results from stem cell responses to N-cadherin mimic cyclic peptide suggested that the immobilized cyclic peptide has effects on supporting specific neural stem cell adhesion and maintaining their stem cell properties. According to the investigation on cell morphology, neural stem cells in the form of neurospheres could migrate and present axonal outgrowth from their original spheres as well as express neural stem cell marker (anti-Nestin immunoreactive) on the PET film immobilized with N-cadherin mimic cyclic peptide. Additionally, the immobilized cyclic peptide affected β-catenin molecules in Wnt/β-catenin signaling pathway that resulted in decreasing luciferase gene expression level. However, the extent of stimulation of neurite outgrowth on the PET film immobilized with the N-cadherin mimic cyclic peptide cannot be distinguished from that coated with laminin.

Although N-cadherin mimic cyclic peptide immobilized on PET film surfaces cannot be identified as a crucial factor for promoting neural growth, it has a significant influence on the efficiency of limbal epithelial colony forming. Moreover, limbal stem cells cultured on the PET film surface immobilized with the cyclic peptide can maintain stem cell properties as expressed by the limbal stem cell markers whereas the limbal cells cultured on the unmodified PET film cannot express these markers.

Therefore, it is expected that the N-cadherin mimic cyclic peptide can be used as an artificial ECM for maintenance of undifferentiated state of neural and limbal stem cells and a supporting factor for colony formation of limbal stem cells. It is interesting to utilize this cyclic peptide for developing culture system for limbal stem cells. To investigate efficiency of neurosphere forming of neural stem cells and expression of gene in limbal epithelial colonies are subjects of future studies.

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APPENDICES

APPENDIX A

Table A.1 Advancing (Θ_A) and receding (Θ_R) water contact angles of surface-hydrolyzed PET film versus hydrolysis time

Time (h)	Water contact angle (degree)	
	Θ_A	Θ_R
0	71.8 ± 0.5	27.1 ± 0.7
1	56.2 ± 0.7	13.2 ± 1.6
2	55.4 ± 0.7	14.6 ± 2.4
3	56.4 ± 1.0	14.0 ± 2.2
4	54.9 ± 0.6	12.9 ± 2.2

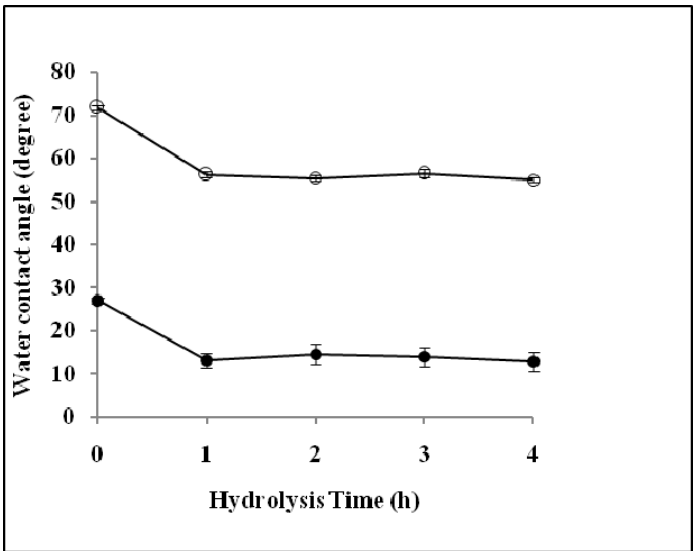

Figure A.1 Advancing (○) and receding (●) water contact angles of surface-hydrolyzed PET film versus hydrolysis time (1M NaOH)

Table A.2 Advancing (Θ_A) and receding (Θ_R) water contact angles of surface-hydrolyzed PET film versus NaOH concentration used in hydrolysis

Time (h)	Water contact angle (degree)	
	Θ_A	Θ_R
0	72.8 ± 0.5	27.1 ± 0.7
1	55.5 ± 0.2	13.2 ± 1.0
2	55.6 ± 1.0	11.7 ± 2.0
3	55.8 ± 0.8	10.5 ± 2.1
4	54.9 ± 0.6	11.5 ± 1.6

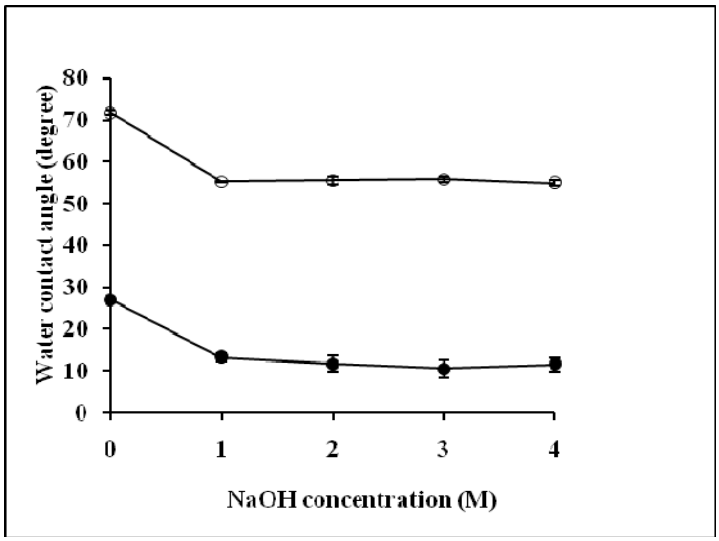

Figure A.2 Advancing (○) and receding (●) water contact angles of surface-hydrolyzed PET film versus NaOH concentration used in hydrolysis (reaction time = 1h)

APPENDIX B

Data corresponding in Chapter IV

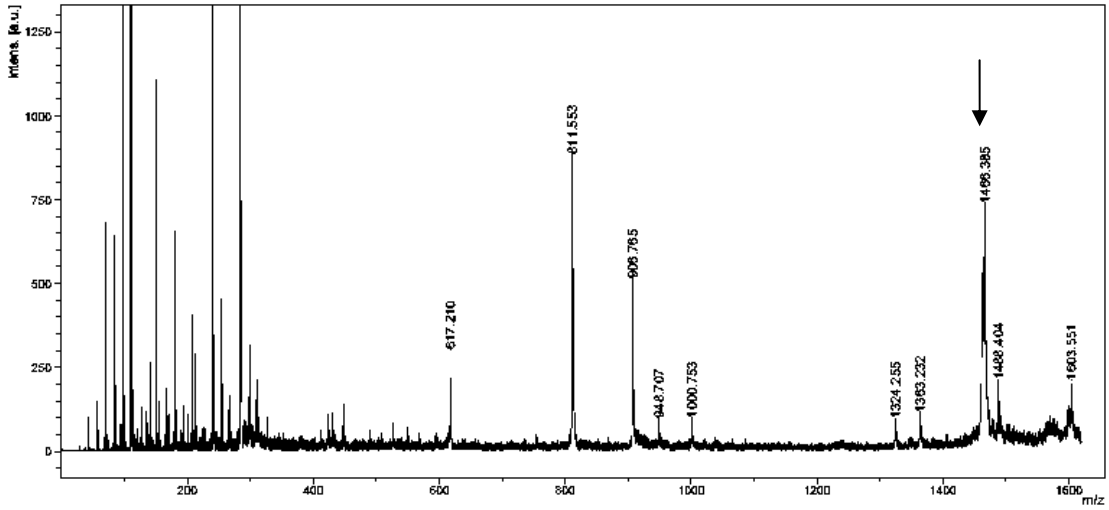

Figure B.1 Mass spectrum of N-cadherin mimic peptide before cyclization (MW = 1466)

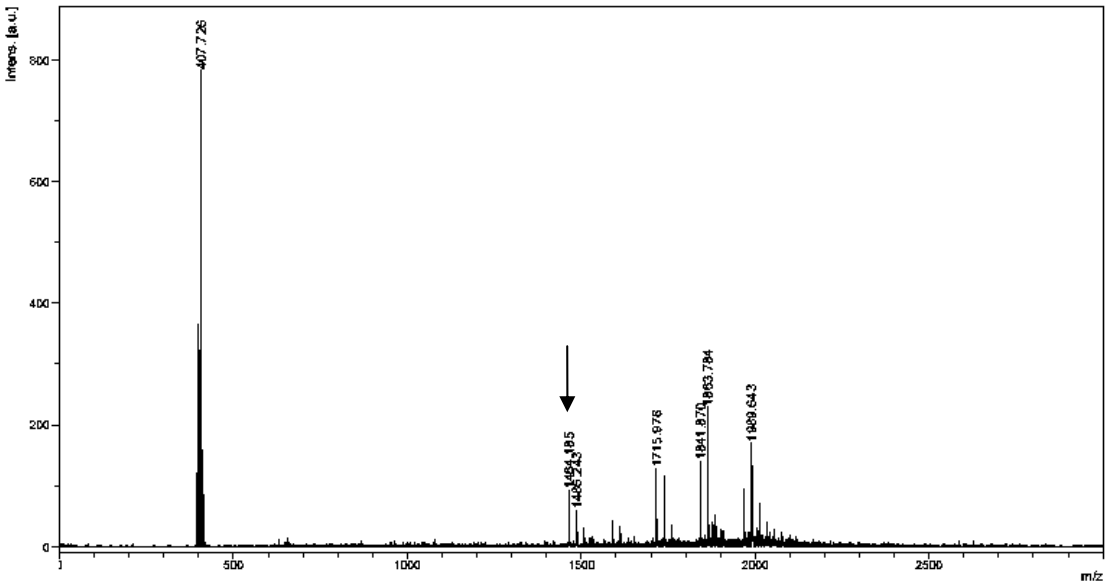

Figure B.2 Mass spectrum of N-cadherin mimic cyclic peptide after cyclization (MW = 1464)

APPENDIX C

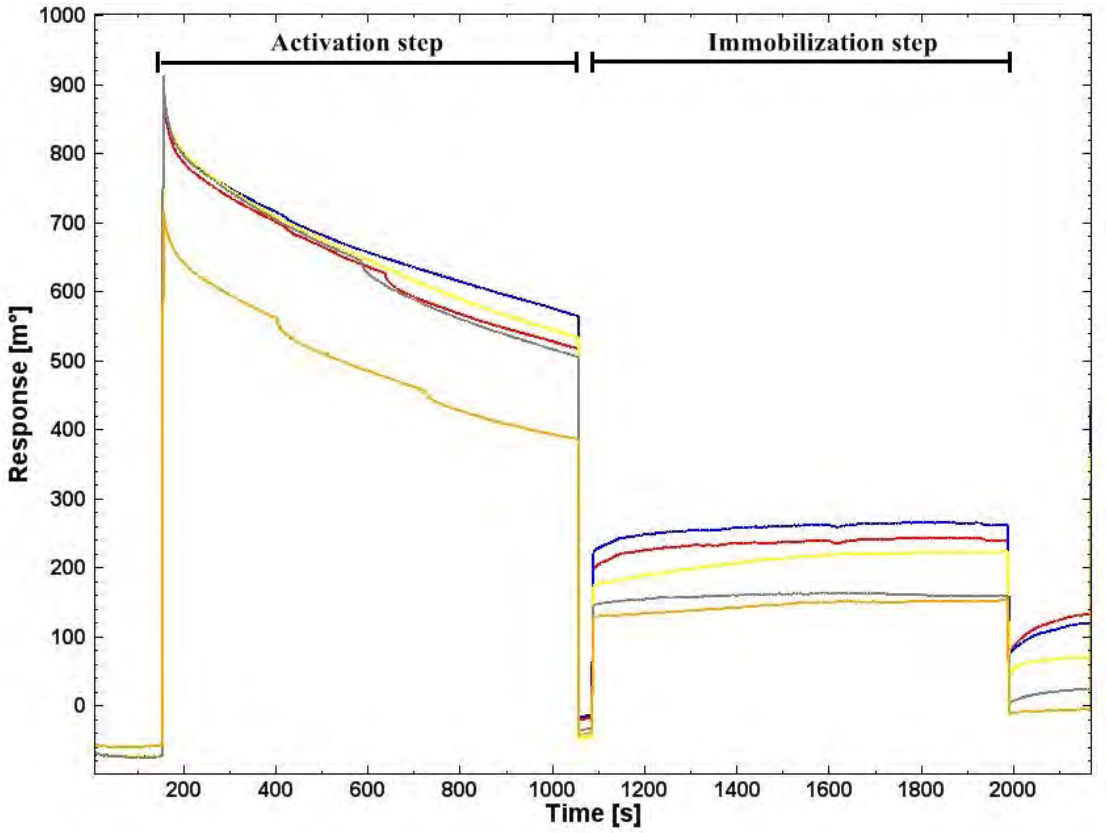

Figure C.1 SPR response of SPR disk immobilized with 1:1 mixture of 11-mercaptoundecanoic acid and 11-mercapto-1-undecanol after EDC/NHS activated and immobilized by N-cadherin mimic cyclic peptide

The measured SPR angle shifts during the activation and immobilization steps can be converted into mass uptakes, using equation C.1.

$$Amount\ of\ \ mass\ uptake\ (ng/cm^2) = \frac{SPR\ angle\ shift\ (m°) \times 100\ (ng/cm^2)}{120\ (m°)} \qquad (C.1)$$

Table C.1 Amount of N-cadherin mimic cyclic peptide immobilized on SPR disk immobilized with 1:1 mixture of 11-mercaptoundecanoic acid and 11-mercapto-1-undecanol determined by SPR as a function of N-cadherin mimic cyclic peptide concentration.

N-cadherin mimic cyclic peptide concentration (mg/mL)	**Amount of N-cadherin mimic cyclic peptide (nmol/cm^2)**
0.1	0.020 ± 0.002
0.3	0.035 ± 0.008
0.5	0.076 ± 0.005
0.7	0.085 ± 0.005
1.0	0.070 ± 0.005

Table C.2 Amount of N-cadherin mimic cyclic peptide immobilized on SPR disk immobilized with 1:1 mixture of 11-mercaptoundecanoic acid and 11-mercapto-1-undecanol determined by SPR as a function of N-cadherin mimic cyclic peptide concentration.

N-cadherin mimic cyclic peptide concentration (mg/mL)	**SPR angle shift (m°)**	**Amount of N-cadherin mimic cyclic peptide (nmol/cm^2)**
0.1	27.4	0.02
	18.4	0.02
	24.2	0.02
0.3	50.2	0.04
	43.7	0.03
	41.5	0.03
0.5	61.2	0.08
	60.4	0.08
	59.7	0.08
0.7	56.4	0.08
	63.1	0.09
	60.6	0.08
1.0	55.8	0.07
	56.0	0.07
	60.3	0.08

APPENDIX D

Toluidine blue O assay

Toluidine blue O assay is a method used for determination of the amount of carboxyl groups. The carboxyl groups of surface-hydrolyzed PET films can form a complex with toluidine blue o. The absorbance of the solution containing the desorbed complex was measured at 633 nm. The COOH content was obtained from a calibration plot of the optical density versus dye concentration which is displayed in Figure D-1.

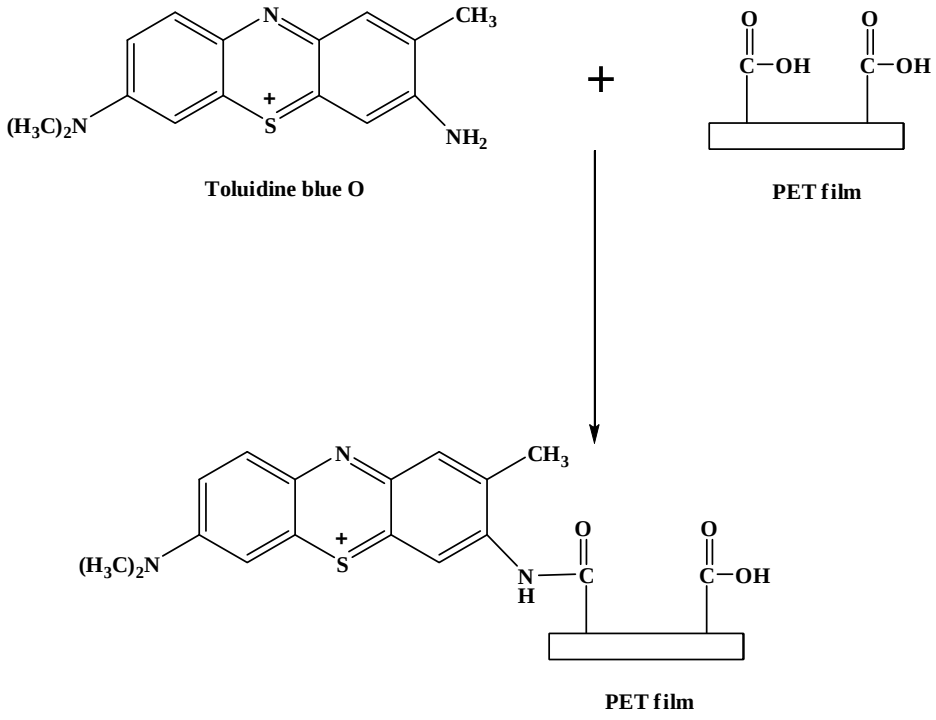

Figure D.1 Formation of toluidine blue O complex with carboxyl group.

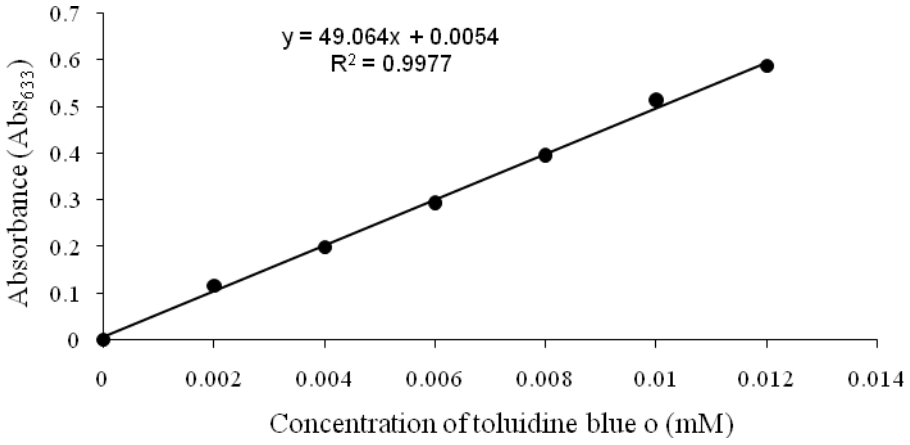

Figure D.2 Calibration curve of UV absorbance as a function of toluidine blue o concentration.

Dual-Luciferase Reporter Assay Protocal (Promega technical manual)

Materials: Luminometer

Siliconized polypropylene tube or small glass vial

Methods:

1.1 Preparation of Luciferase Assay Reagent II

- Prepare Luciferase Assay Reagent II (LARII) by resuspending the provided lyophilized Luciferase assay substrate in 10 mL of the supplied Luciferase Assay Buffer II. Once the substrates and buffer have been mixed, write "LARII" on the existing vial label for easy identification. LAR II is stable for one month at -20°C or for one year when stored at -70°C.

1.2 Preparation of Stop& Glow Reagent

- Prepare an adequate volume to perform the desired number of DLR™ Assays (100µL reagent per assay). Stop & Glo substrate is supplied at 50X concentration. Add 1 volume of 50X Stop & Glo substrate to 50 volumes of Stop & Glo buffer in a glass or siliconized polypropylene tube.
- Stop & Glo Reagent (Substrate + Buffer) is best when prepared just before use. If stored at 22°C for 48 hours, the reagent's activity decreases by 8%.

If necessary, Stop & Glo Reagent may be stored at -20°C for 15 days with no decrease in activity. It may be thawed at room temperature up to 6 times with ≤15% decrease in activity.

1.3 Standard Protocol

1.3.1 Predispense 100 µL of LAR II into the appropriate number of luminometer tubes to complete the desired number of DLR™ Assays.

1.3.2 Program the luminometer to perform a 2-second premeasurement delay, followed by a 10-second measurement period for each reporter assay.

1.3.3 Carefully transfer up to 20µl of cell lysate into the luminometer tube containing LAR II; mix by pipetting 2 or 3 times.
Do not vortex. Place the tube in the luminometer and initiate reading.

Note: We do not recommend vortexing the solution at Step 3. Vortexing may coat the sides of the tube with a microfilm of luminescent solution, which can escape mixing with the subsequently added volume of Stop & Glo® Reagent. This is of particular concern if Stop & Glo® Reagent is delivered into the tube by automatic injection.

1.3.4 If the luminometer is not connected to a printer or computer, record the firefly luciferase activity measurement.

1.3.5 If available, use a reagent injector to dispense 100µl of Stop & Glo® Reagent. If using a manual luminometer, remove the sample tube from the luminometer, add 100µl of Stop & Glo® Reagent and vortex briefly to mix. Replace the sample in the luminometer, and initiate reading.

Note: It is possible to prime auto-injector systems with little or no loss of DLR™ Assay reagents. Before priming injectors with LAR II or Stop & Glo® assay reagents, we recommend first purging all storage liquid (i.e., deionized water or ethanol wash solution) from the injector system. Priming assay reagent through an empty injector system prevents dilution and contamination of the primed reagent.

Thus, the volume of primed reagent may be recovered and returned to the reservoir of bulk reagent.

1.3.6 If the luminometer is not connected to a printer or computer, record the *Renilla* luciferase activity measurement.

1.3.7 Discard the reaction tube, and proceed to the next DLR™ Assay.

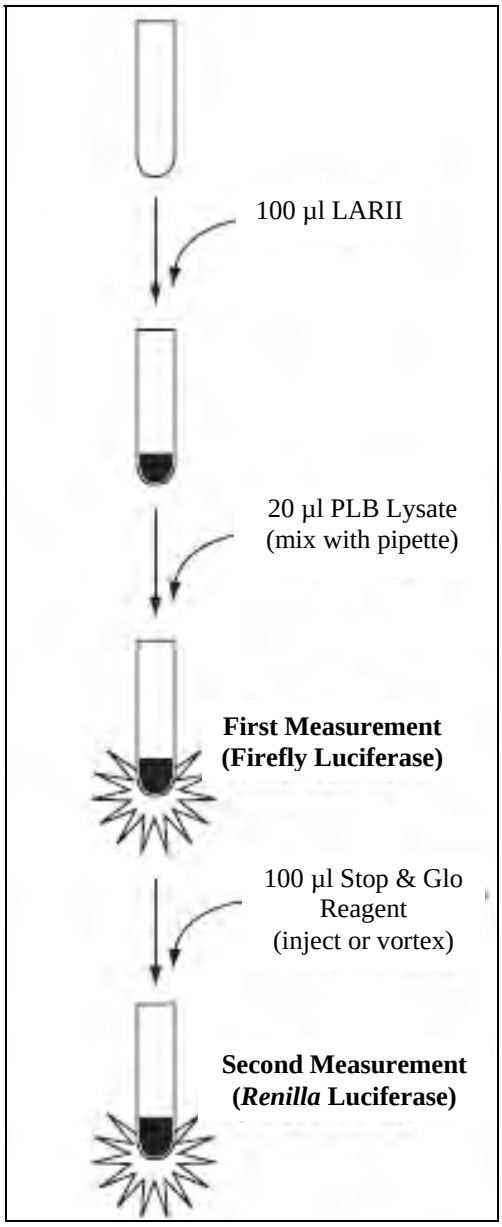

Figure D.3 Format of the DLR™ Assay using a manual luminometer or a luminometer equipped with one reagent injector (Promega technical manual).

Table D.1 The luciferase activity measurement of transfected HEK293 cultured on PET film

Transfected gene	PET			
	Control	β-catenin	N-cadherin	β-catenin+N-cadherin
Replication 1	0.83	24.4	2.91	21.0
Replication 2	0.75	17.6	2.88	13.7
Replication 3	0.77	19.6	3.12	15.1
Average	0.79	21.0	2.89	17.4
SD	0.05	4.81	0.02	5.14

Table D.2 The luciferase activity measurement of transfected HEK293 cultured on 0.1 mg/mL N-cadherin mimic cyclic peptide-immobilized PET film

Transfected gene	0.5 mg/mL N-cadherin mimic cyclic peptide/PET			
	Control	β-catenin	N-cadherin	β-catenin+N-cadherin
Replication 1	1.37	22.2	1.16	15.4
Replication 2	1.45	23.2	1.03	15.6
Replication 3	1.40	22.5	1.06	15.8
Average	1.41	22.7	1.10	15.5
SD	0.06	0.71	0.09	0.15

Table D.3 The luciferase activity measurement of transfected HEK293 cultured on 0.5 mg/mL N-cadherin mimic cyclic peptide-immobilized PET film

Transfected gene	0.5 mg/mL N-cadherin mimic cyclic peptide/PET			
	Control	β-catenin	N-cadherin	β-catenin+N-cadherin
Replication 1	2.32	25.0	0.85	21.0
Replication 2	2.03	19.2	0.92	13.6
Replication 3	2.18	22.4	1.02	17.9
Average	2.17	22.1	0.88	17.3
SD	0.21	4.08	0.05	5.23

Table D.4 The relative fold increase of luciferase activity compared to control (pcDNA3.1 plasmid)

Transfected gene / Substrate	Control	β-catenin	N-cadherin	β-catenin + N-cadherin
PET	1	26.7 ± 2.1	3.67 ± 0.4	22.0 ± 1.8
0.1 mg/mL N-cadherin mimic cyclic peptide/PET	1	16.1 ± 1.2	0.78 ± 0.1	11.0 ± 1.1
0.5 mg/mL N-cadherin mimic cyclic peptide/PET	1	10.2 ± 0.2	0.41 ± 0.1	7.98 ± 0.8

VITAE

Miss Suttinee Poolsup was born on July 11th, 1986 in Nakhonratchasima, Thailand. She received a Bachelor Degree of Science, Program in Genetics, Faculty of Science, Chulalongkorn University in 2007. Since 2008, she has been a graduate student studying Biotechnology as her program course at Faculty of Science, Chulalongkorn University. During her studies towards the Master's degree, she was awarded by Research Professional Development Project Under The Science Achievement Scholarship of Thailand (SAST) during 2008 – 2009.

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March 2011	Oral presentation at the 19th Science Forum 2011, Faculty of Science, Chulalongkorn University, Bangkok, Thailand. Oral Presentation Award in Session: Physical Science