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Cadherin-Fc Chimeric Protein-Based Biomaterials: Advancing Stem Cell Technology and Regenerative Medicine Towards Application

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Additional information is available at the end of the chapter

1. Introduction

'Stem cell' – the term was first coined by Russian histologist Alexander Maksimov in 1908 to herald the existence of special cells those have capacity to generate blood cell. Stem cells are the core materials of regenerative medicine and tissue engineering. Although there are multiple types of stem cells available based on their origin and functionality; however, scientifically they can be classified into four well-defined classes– (1) embryonic stem cell (ESC), (2) adult stem cells (ASC) for example, muscle satellite cells are muscle-specific adult stem cell, (3) induced pluripotent stem cell (iPSC), and (4) pathological stem cells (PSC) for example, cancer stem cells (CSC) [1]. Out of these 4 types, ESC and ASCs are true physiological stem cells, iPSCs are engineered stem cells and PSCs are conditional stem cells. Among them, ESC and iPSC are being considered true pluripotent stem cells, which have the capacity for unlimited self-renewal and differentiation into all the specialized cell types of the body. Therefore these cells have been considered the most favorable cells for using in regenerative medicine and tissue engineering [2,3,4,5,6,7,8].

Stem cells need a special environment for their survival, maintenance and growth. During the early stage of establishing the culture methodologies for stem cells, it was realized that they need support from other cells for example, mouse embryonic fibroblast (MEF). Co-culture methodology with gamma-irradiated MEF cells used as feeder-cells and enriched culture media with fetal bovine serum (FBS) were successfully utilized for establishing *in vitro* stem cell culture [9,10]. However, using a second non-related cell type (although growth restricted) is not suitable for differentiation studies – particularly, for 3D cell culture. Later, the MEF layer

was successfully removed from the culture system by introducing MEF-conditioned media (MEF-CM) that has made the protocol more suitable for experimentation targeting regenerative medicine but not up to the desired standard due to the presence of xenogeneic agents in the system [11,12,13]. MEF-CM is enriched cell culture media with MEF-secreted molecules that functions as a depot for the necessary cytokines for the healthy maintenance of stem cells. However MEF-CM alone were not adequate to upkeep ESC and iPSC survival and growth thereby suggesting that MEF cells are not only providing necessary nutrients and cytokines, in addition they are also backing as physicochemical supports through the ECM to these cells. However, technically it remains elusive to point out the essential factors, required to maintain stem cell culture, present in the MEF-CM due to the inconsistency in expression and secretion of biological factors between experiments and batches. Moreover, it has been shown that not only proliferation of these cells but the secretion of necessary biomolecules and deposition of ECM components were also directly related to the gamma-irradiation [11,12,13]. Such factors directly influence properties of stem cells in culture, and instigate restriction for application of relevant protocols for regenerative medicine and tissue engineering. Therefore suitable cell-recognizable biomaterials are highly desired to overcome the dependency of cell-based basal supports for stem cell culture.

Matrigel was one of the first biomaterials that was effectively applied as plate-coating materials for *in vitro* culture of human ESC and iPSC with the aid of MEF-CM as culture medium [14,15]. This was a significant advancement in stem cell technology to make stem cells free from undesirable feeder-layer cells. Matrigel is a product from decellularization of Engelberth-Holm-Swarm (EHS) mouse sarcoma cells, and a cocktail of laminin, collagen IV, entactin, heparin sulfate proteoglycans, and known and unknown growth factors with variable compositions [16,17,18,19]. It closely resembles the embryonic basement membrane in consistency and activity as well as providing a biologically functional complex [17,19]. However, Matrigel is not a defined material with high purity and incorporated with substantial lot to lot variation in constituents both in qualitative and quantitative measures. It has also been reported contaminated with Lactate Dehydrogenage Elevating Virus, and has raised additional concerns for safe application of this material in stem cell culture [20]. Such kinds of issues are strongly demanding a more defined culture condition under good manufacturing practice (GMP) for safe application of stem cell protocols or methodologies if the ultimate objective is to employ stem cells in regenerative medicine or tissue engineering.

The individual components of Matrigel provide specific functional queues to ESCs and iPSCs. For example, ESC exhibits normal growth when cultured on laminin-coated plate, which was not observed on either fibronectin- or collagen IV-coated surface [21,22,23,24]. It was also reported that specific laminin isoforms have distinctive effects on stem cells; for instance, laminin-111, -332, -511 support adhesion and proliferation of stem cells but isoforms -211 and -411 of laminin do not [22]. The information suggested that designing a defined matrix for stem cell culture requires special biomaterials that can deliver concurrent supports for cell adhesion, proliferation and differentiation. In fact, effective stem cell culture condition with high pluripotency was occasionally achieved in spite of introducing several synthetic and semi-

synthetic biomaterials alone or as a blend as cell-culture substrate, and therefore, designing such a biomaterial remains a challenging but ultimately rewarding task.

Pioneering work from our laboratory introduced Fc-chimeric protein in stem cell technology approximately a decade ago, and over the years we and others have established multiple Fc-chimeric proteins as significantly favorable cell-recognizable biomaterials in stem cell technology. These works with varieties of Fc-chimeric proteins spanning from ECM component protein [for example, E-cadherin (ECad)] to cytokine [for example, hepatocyte growth factor (HGF)] have shown tremendous potential to overcome the major barriers in stem cell technology, namely defined condition for stem cell culture, selective differentiation to the target lineages, convenient purification of the desired cells etc., for the application of stem cell technology targeting to regenerative medicine. In this article we will focus on ECad-Fc and NCad-Fc chimeric proteins as novel cell-recognizable biomaterials in stem cell technology towards application in regenerative medicine.

2. Rationale for using protein as biomaterials

An ideal chemically defined xenogeneic-agent free stem cell culture system might be consists of chemically known matrix for plate coating that would provide structural basal support to the stem cells and defined media that is supplemented with highly pure recombinant proteins as functional cytokines. The system should essentially be free from serum or feeder-cells or any other animal products. Even though it is very demanding however, designing and preparing a completely defined stem cell culture system is highly challenging. One worthwhile goal is to design a defined plate-coating material that can successfully replace Matrigel. Since stem cells are essentially dependent on cell-cell or cell-surface interaction for survival, which are mainly mediated by extracellular matrix protein (ECM), a cell-recognizable biomaterial should preferably mimic ECM protein(s).

Such kind of biomaterials can either be employed as a scaffolding molecule that may provide structural support of the growing cells, or as functional effector molecules that can target cellular signal recognition machineries like cell surface receptors or channels to trigger or maintain signaling cascades necessary for survival, proliferation, and differentiation of experimental cells [25]. To act as an artificial ECM the biomaterial under consideration should mimic the physicochemical and biological properties of native components of ECM to facilitate targeted functionalities of cell for example, adhesion, proliferation, differentiation, etc [26]. Similarly, the candidate effector molecules should have physicochemical signature of the comparable native molecules for recognition as functional substrate to endogenous receptors or channels of experimental cells. Synthetic biomaterials have limitations for providing perfect biochemical structural motif for effective recognition by the cellular recognition machineries to execute necessary cellular function, and therefore are generally not efficient enough for practical applications for *in vivo* condition. Moreover, many of these synthetic biomaterials are

not biologically compatible at a desired level and may generate pathophysiological compli-
cations in the long term in the body.

Proteins are native elements of cells and natural ECM scaffolds [27] and therefore recombinant
proteins could be one of the best candidates to design superior biomaterial for application in
regenerative medicine and tissue engineering. Recent progress in biochemistry, molecular
biology, bioinformatics, and engineering provides the prospect of expressing and purifying
desired recombinant protein with high yield (g/L is achievable) in large scale [28], which can
eventually be applied (directly or with modification) as novel, simplified, and bio-active
macromolecules in regenerative medicine and tissue engineering [29,30,31]. Such proteins can
be generated from a genetic template by natural cellular read-out process namely,
DNA>RNA>protein that ensures excellent uniformity and reproducibility of the designed
biomaterial depending on cellular conditions, where the production is executed. The native
biological production process confirms high degree of reproducibility, which is not realistic
by traditional chemosynthetic or mechanosynthetic processes. On the contrary, protein science
has its own negative issues for example, highly efficient expression system for the desired
protein, convenient purification of the target protein, proper folding of the purified protein,
stability of the functional protein, mode of application of experimental protein etc. Chimeric
protein technology has long been considered one of the potential methodologies to overcome
many of these issues including higher productivity, better stability, and efficient purification
of a target protein for bulk scale. Fc-chimeric protein is one such engineered protein that was
introduced in 1989, and has been showing great promise for comparatively convenient
production efficiency of chimeric protein with functional integrity and long-term stability, and
therefore successful applcation in diverse fields of biomedical sciences [32,33,34]. An illustra-
tion of Fc-chimeric protein is shown in Fig. 1 with ECad-Fc as a model.
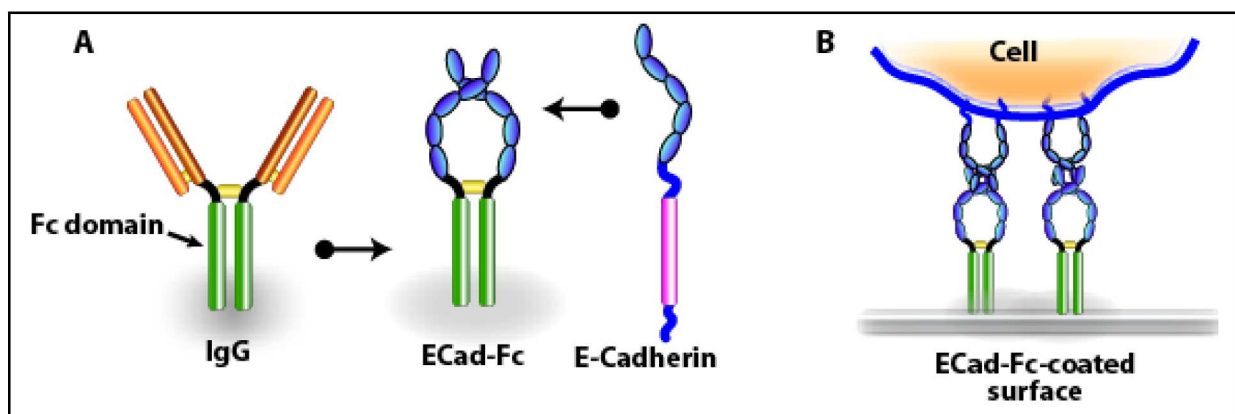
Figure 1. Schematics of Fc-chimeric protein, and its molecular function. (A) Functional domain of target protein is
fused as N-terminal with the Fc domain of IgG. ECad is shown here as an example. (B) Plasma-membrane localized
ECad dimer can interact with apposing ECad dimer and form high affinity binding that makes cell-cell and cell-surface
adhesion.

3. Cadherins in cell biology

The cadherins is a large family of single transmembrane proteins with more than 100 members. Out of these we will be focusing on epithelial cadherin (ECad) and neural cadherin (NCad) in this report. They are the member of classical cadherin family, and both of them are glycosylated in their extracellular domain. They have the ability to function as adhesion molecules for the relevant protein-expressing cells. Generally cadherin forms homophilic dimer, and the dimeric forms of cadherins take part in Ca^{+2}-dependent coupling from apposing cells that mediates cell-cell adhesion. These single transmembrane-domain plasma membrane-resident proteins are not only necessary for cell-cell adhesion but also involved in indispensible signaling cascades, which are critical for the development-to-homeostasis-to-demise of cells and organisms.

The extracellular N terminal region of ECad consists of 5 structural domains, which are the signature motifs for ECad and are responsible for the homophilic binding between two neighboring as well as apposing molecules, while the C-terminal intracellular region of ECad interacts with several intracellular proteins such as β-catenin/Armadillo and p-120 catenin [35,36,37]. The p-120 catenin is associated with the targeted transport and stabilization of the adhesion complexes on the plasma membrane. Beside, β-catenin interacts with α-catenin, which in turn initiates actin filament formation *via* interaction with formin at the adherens junction [38,39,40,41,42]. However, how cadherin-catenin complexes are connected with cytoskeletal components *e.g.*, actin is not clearly known.

ECad has been shown linked with many early-to-late developmental and differentiation processes *in vivo* and *in vitro* systems including ESCs, MSCs, iPSCs, and whole embryo [43,44,45,46,47]. ECad knock out mouse was reported embryonic lethal [48,49], which is a direct evidence of its critical importance in stem cell biology and regenerative medicine. Our lab first envisioned the application of ECad as a novel cell-recognizable biomaterial little over a decade ago while Nagaoka *et al.* endeavored to improve the differentiation and maturation efficiency of hepatocyte in an *in vitro* system [50]. The idea was conceived from the fact that Fc domain of IgG can bind directionally with an appropriate surface *via* hydrophobic interaction, and the fused protein stretches out directionally to offer interaction with a suitable partner [51]. At that period, several reports suggested that ECad is indispensable for tissue morphogenesis, and is also required for maintenance of matured tissues. Awata et al. showed that ECad-mediated cell-cell interaction is necessary for hepatocytes to maintain their differentiated phenotypes by forming 3D spheroid structure, or multi-layer cell aggregates [52]. Further it was reported that high cell density culture of fetal liver cells [53,54], which most likely is an ECad-dependent characteristics, enhanced hepatocyte maturation in culture. These findings suggested that cell-cell interaction may directly influence hepatocyte maturation as well as maintenance of differentiated phenotypes. There was, however, no substantial information regarding the role of ECad in the relevant processes, and to reveal the answer it was essential to have a suitable tool or methodology that can expedite cell-cell interaction analysis in a controlled manner. ECad-Fc was designed and deployed as a novel biomaterial in the regenerative medicine field

to address this issue; after a decade, it has been proven to be a suitable material for stem cell technology and regenerative medicine.

4. ECad-Fc as a cell-recognizable biomaterial

As a biomaterial, ECad-Fc was first applied as plate-coating materials for hepatocyte differentiation experiments [50]. It was observed that differentiated hepatocytes can efficiently adhere with the cell culture plate coated with ECad-Fc. The adhered cells demonstrated comparable molecular characteristics e.g., low DNA synthesizing activity and maintenance of tryptophan oxygenase (TO) expression like those of spheroid-form hepatocytes. As well, the hepatocyte cultured on ECad-Fc-coated plate supported the differentiation of hepatocytes in culture. These results suggested important roles of ECad-Fc matrix for the maintenance of differentiating hepatocytes. This was the first report of ECad-mediated matrix dependability, as a biomaterial, for any cell type in regenerative medicine. After a while, Nagaoka *et al.* published the landmark report regarding the application of ECad-Fc cell-cooking plate (since target cell can be obtained on such type of biomaterial-coated plate without additional cell purification method therefore named so) as a defined matrix for successful maintenance of murine stem cells without any feeder layer in 2006 [55]. This report signified the alluring potential of ECad-Fc as a biomaterial for practical application in stem cell technology and regenerative medicine.

Xenogeneic-agent free stem cell culture method is extremely critical if the objective of the relevant protocol is to apply the relevant products in regenerative medicine. Since MEF secrets many unidentified molecules, which are potential xenogeneic elements for human subject therefore feeder-cell-based early methodologies are not considerable for applying in regenerative medicine. Matrigel is also produced from mouse carcinoma tissue and ill-defined therefore causing serious known and unknown hazards of xenogeneic contamination in experimentations. An immunogenic sialic acid (NeuGc) has been identified in a co-culture experiment for human ESCs applying MEF and animal derivatives as serum replacement [24,56]. This is specifically worrying as such kind of non-human sialic acid can initiate immunogenic processes in human triggering complete graft rejection and consequential complexities. Non-human animal-derived products also can be a possible cause for mycoplasma contamination, which can directly infect the cells in culture and either damage them totally or can change their properties, and thereby directly or indirectly initiate complicacies for regenerative medicine protocols. Human feeder-cells and serum have been recommended for culturing human ESCs to evade xenogeneic compound in experimental system for regenerative medicine. However, this is associated with a high risk of microbial contamination, for example retroviral components, and hence are not as suitable for *in vivo* application. Therefore it is a prime importance to establish completely defined human stem cell culture system for safe application of relevant products in regenerative medicine.

5. ECad-Fc is a unique defined matrix for ESC and MSC

The study of Nagaoka *et al.* [55] revealed that murine ESCs can maintain their pluripotency on ECad-Fc-coated surface for extended culture periods (Fig. 2). Cells cultured on such type of substratum were later successfully used to generate germline-competent chimeric mouse [57]. Consistent with the findings, a separate study using mouse mesenchymal cell lines STO and NIH3T3 stably expressed with ECad as feeder-cell showed higher level of stem cell marker expression with standard colony-forming phenotype compare to the cells cultured on normal MEF-feeder-cell layer [58]. A number of feeder-free culture methods for ESCs have been reported where ESCs grow with their standard tightly-bound colony phenotype [4,11,13,22,24,56,59]. This type of tight colony formation generates heterogeneous cell population within a colony, which potentially affects homogenous accessibility of cytokines to these cells as well as creates heterogeneous niches. As a result stem cells in a colony differentiate heterogeneously and produce various kinds of cells as contamination with the desired type of cells, a major drawback that regenerative medicine has to overcome. In this respect, ECad-Fc matrix drives murine stem cells out of the colony to form a normal monolayer of cells, where stem cell resides as single cell condition [55]. This is a ground breaking technology that provides an exciting solution for overcoming the inherent colony forming phenotype-linked cellular heterogeneity. Biochemical analyses revealed that these cells bear all the signatures of pluripotent stem cells, and can form all three germ layers in a teratoma forming assay, and as mentioned earlier can generate germline-competent chimeric mouse. Additionally, they require lower amounts of LIF for maintenance of pluripotency, reducing costs related to ESCs culture. The monolayer-type single cell ESCs was also associated with higher proliferation ability and greater transfection efficiency compared to the colony-forming cells cultured on other substratum. Such improved proliferation ability could be extremely helpful for quick amplification of iPSCs on ECad-Fc substratum, which could mean shorter waiting periods for patients to receive cell therapy. The higher transfection efficiency of stem cells on ECad-Fc cooking plate could be exploited for targeted delivery of desired extracellular cargo for example, transgene products or drug molecules, into these cells for better outcomes.

This type of cooking-plate technology, where ECad-Fc provides basal support to the cells, and other immobilized factors for example, LIF-Fc [57] which satisfy specific needs, can be very advantageous for (1) ensuring undifferentiated state of stem cell in culture, (2) cost reduction associated with cytokines, and (3) hassle-free working condition without the necessity of regular media change, which is a standard time-consuming practice for stem cell culture.

The single-cell phenotype seen for ESCs was also observed for other stem cells for example, mouse embryonal carcinoma cells F9 and P19 but not for differentiated cells for example, NMuMG mouse mammary gland cells, MDCK kidney epithelial cells and isolated mouse primary hepatocytes [60]. This result indicated that ECad-Fc-mediated cellular migratory behaviors are most likely specific for embryonic stem cells. Reportable that ECad-facilitated cell-cell adhesion is often rearranged during initial stages of embryogenesis to control cell migration, cell sorting, and tissue function, which is suggesting a close cooperativity of stem cell maintenance, proliferation, and differentiation with ECad [39,48,49,61,62]. However, there
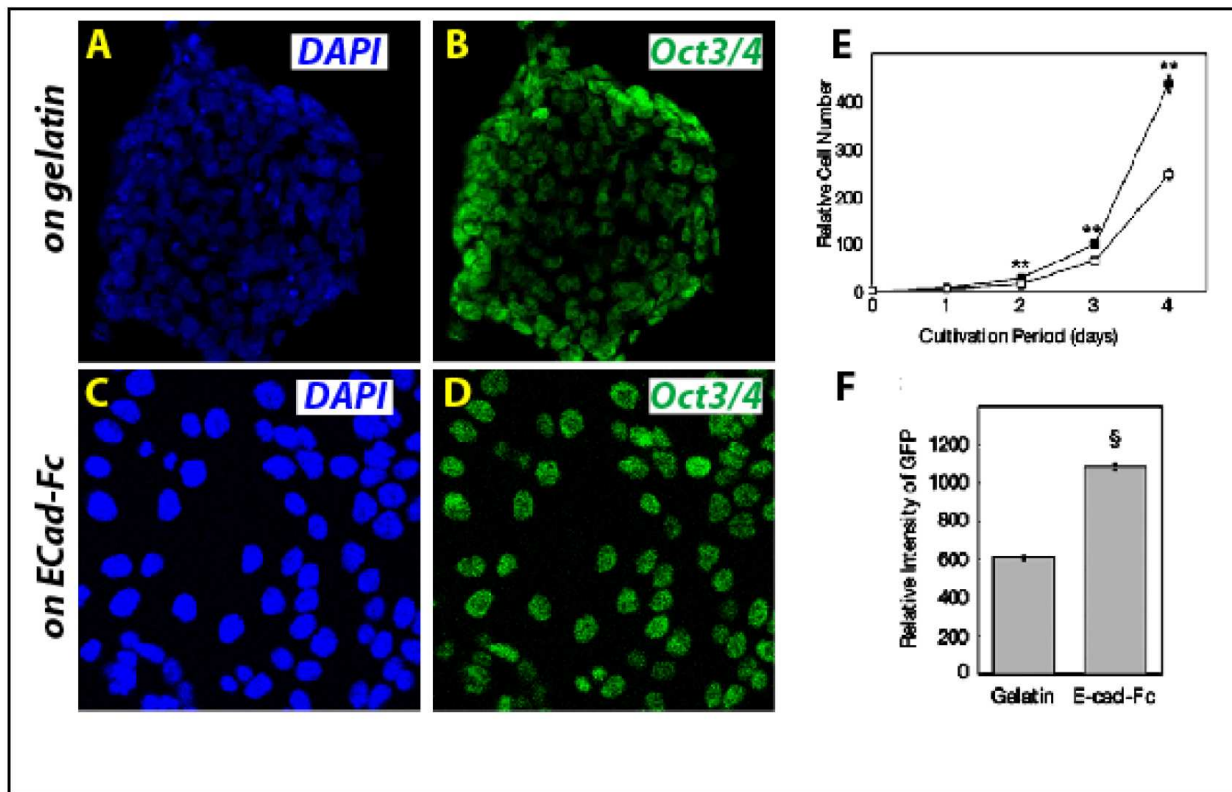
Figure 2. ECad-Fc is a defined matrix for culturing monolayer of iPS cells. Mouse EB3 cells were successfully cultured on ECad-Fc-coated surface that showed monolayer phenotype (C and D) compare with compact colony phenotype (A and B) for general protocol, which was significantly advantageous for faster growth (E), and higher transfection efi-ciency (F).

is no such suitable system to explore the necessary signaling pathways to address these questions. Nevertheless, since ESC does not form colony on ECad-Fc cell-cooking plate therefore this can be a perfect tool for obtaining single cell model system of stem cells to investigate relevant signaling pathways necessary for stem cell maintenance, proliferation, and differentiation. Our recent study successfully exploited this single-cell phenotype for monitoring cell cycle properties of stem cells on cell-cooking plate (unpublished), indicating the importance of this system for cell biology experiments designed to reveal their individual characteristics. The findings could be invaluable for regulating stem cells for desired applica-tion in regenerative medicine.

Most of the stem cell innovations, comprising generation of ESCs and iPSCs, were primarily established in mouse model, and then applied in human models. Similarly, ECad-Fc cell-cooking plate technology was first developed and established for murine stem cells [55,57]. Thereafter, ECad-Fc cooking-plate was successfully applied for human ESC culture following similar methodologies with additional consideration for mild enzymatic treatment during the cell dissociation and seeding steps [56]. A strong protease cocktail Accutase (Millipore) was used for murine ESC culture; however, Accutase treatment was found detrimental to human ESCs, which was recuperated by using enzyme-free proprietary preparation named, Cell Dissociation Buffer (Life Technologies). It is reportable that the human ESCs were cultured on

ECad-Fc cooking plate with a completely defined media named mTeSR1 (Stemcell Technologies), and that made the culture method completely defined and xenogeneic-agent free, which is a significant achievement in regenerative medicine. The stem cells cultured on ECad-Fc cooking-plate were practically identical to those cultured on Matrigel-coated plate including cell morphology, proliferation rate, preservation of undifferentiated phenotype, and ability of differentiation into multiple cell types in embryoid bodies as well as in teratoma assay [56]. Interestingly, contrasting with the single-cell phenotype for mouse ESCs, human ESCs produced normal colony forming phenotype on ECad-Fc cooking-plate. The mechanism underlying the difference for this observation was not completely understood though.

Human and mouse ESCs have been shown to demonstrate significant disparities in expression of cell surface markers, transcription factors, cytokines, and proteins in them. The difference was evidently recognized by the fact that mouse ESC can be maintained in undifferentiated state with the addition of LIF devoid of feeder-cell but human ESC cannot [14]. It has been shown that the inhibition of Rho-ROCK signaling pathway generates cell scattering in human ESCs suggesting direct connection between cell scattering and signaling pathways [63]. While both mouse and human ESCs express ECad, however, it appears there are diverse additional factors involved to define ECad-mediated activities in these cells and additional investigations are required to reveal the complete molecular circuitry associated to this phenomenon.

MSC is a type of ASCs, and can be collected from donor by satisfying approved ethical issues. These cells have been considered as potential starting materials for regenerative medicine and tissue engineering. They must be expanded *in vitro* before dispensing for specific applications to accomplish anticipated therapeutic effects. MSCs also need xenogeneic agent-free culture method for maintaining their differentiation potency over the culture period. ECad-Fc cooking-plate technology was effectively applied for this reason as well [43]. The cultured MSCs on human ECad-Fc (hECad-Fc) matrix exhibited superior attachment on culture plate compare with standard tissue culture plate and gelatin-coated plate. The MSCs cultured on hECad-Fc showed comparable level of CD 105 and significantly greater level of β-catenin and ECad expression. It has been reported that β-catenin enhances the activity of Oct-4, which is one of the principal Yamanaka factors that plays critical function during the regulation of self-renewal of ESC [45,64], on conjecture it can be suggested that MSCs maintained on ECad-FC cooking-plate might preserve superior stem-ness compare to the MSCs maintained on tissue culture-treated plate and gelatin-coated plate, and therefore possess greater applicability for regenerative medicine.

6. ECad-Fc in directed differentiation and *in-situ* cell sorting of stem cell

Targeted differentiation of stem cells and enrichment of desired cell for example, hepatocytes, from the pool of differentiated cells are very important steps towards use of the cells for regenerative medicine. Functionally matured hepatocytes derived from stem cells can be a potential remedy for various hepatic diseases. There have been several hepatic differentiation protocols reported from ESCs using orthodox techniques including embryonic body (EB)

formation, and clustered colony formation on gelatin- or feeder-cell-coated plates [52,54]. However, these protocols come with many drawbacks, for example, heterogeneous cell population, spontaneous differentiation, xenogeneic contamination, inefficient conversion to hepatocytes, requirement for enrichment of target cell population etc. Our group has effectively applied ECad-Fc as a cell-recognizable plate-coating materials that facilitated good quality mouse ESCs in culture with superior proliferative activities and single-cell phenotype. Similarly, the cell-recognition property of such Cadherin-Fc chimeric protein was exploited for the possibility of facilitated differentiation of ESCs to specific cells for example, hepatocytes and neural cells [29,30,50,65]. Remarkably, ECad-Fc substratum favored progressive differentiation of ESCs to cells with features of definitive endoderm, hepatic progenitor cells, and finally phenotypical as well as functional hepatocytes-like cells [30,50]. The ECad-Fc-coated substratum stimulated selective hepatocyte differentiation in association with ectopic hepatocyte-producing cocktail resulting around 55% hepatic endoderm cells devoid of neuroectoderm and mesoderm markers [30]. High level of (approximately 98%) ECad and developing-hepatocyte marker α-fetoprotein (FTP) were co-expressed in these cells. Since these differentiating hepatocytes express high level of ECad on the plasma membrane therefore ECad-Fc was employed for on-site one-step enrichment of *de novo* hepatocyte-like cells. Practically, 92% albumin expressing cells were successfully harvested on ECad-Fc cooking-plate without any harsh enzymatic treatment or mechanical cell sorting, which are usually detrimental for cells [30]. Therefore the technology can be successfully applied for quick and stress-free cell purification, which will be useful in regenerative medicine.

The enhanced differentiation and cell-recognizable properties were also observed with ECad-Fc and NCad-Fc-based mixed biomaterial cooking-plate for neural cells [65], and is discussed in detail under NCad-Fc section. Such kind of ECad-Fc and NCad-Fc hybrid cooking-plate can be applied for either generation of large number of homogeneous cell population, which can be applied for therapeutic evaluation, or for analyzing the signaling pathways related to nerve generation at a single cell level.

7. ECad-Fc is a superior matrix for iPSC

iPSCs are commonly derived from somatic cells by ectopic and forced expression of common transcription factors Oct4, Sox2, and Nanog along with protocol-dependent treatments with cocktails of some other transcription factors, and even miRNA or small molecules [10,66,67,68,69,70,71,72,73]. Despite the existence of many protocols for generating iPSCs, the required time and efficiency of iPSC generation is still not practical for application of the technology to a mass scale. As per recent published information, depending on protocol, it may take somewhere between 2~4 weeks to get a 1% conversion of cells to iPSCs. During the reprograming process, starting cells experience mesenchymal-to-epithelial transitions (METs) as a natural requirement [74]. This fact was further proved by the findings that MET happens during the initial stage of reprograming process [71,74,75]. Recent evidence further suggested significant functional roles of ECad and other cell adhesion molecules in METs.

ECad interacts with cytoskeletal components *via* various intracellular molecules for example, α-catenin, β-catenin, and p-120 [38]. ECad-mediated signaling was found associated with cytoskeletal remodeling processes through Rho activation [41,63,76]. ECad has been established as an essential factor for maintaining typical colony-forming phenotype of ESCs and iPSCs. Recent studies, remarkably, revealed that forced expression of ECad can significantly enhance the effectiveness of relevant iPSCs-generation protocol [45]. A separate study revealed that ECad expression was enhanced upon treatment with small molecules resulting in enhanced efficiency for the relevant iPSC-generation protocol [77]. This enhanced productivity for iPSCs was successfully reproduced by the application of N terminal extracellular domains of ECad, which suggested that the phenomenon is mainly mediated by the extracellular functional domains of this protein [77]. Most importantly, ECad was sufficient to generate iPSCs with only three Yamanaka factors –KLF4, SOX2, and c-MYC from murine fibroblasts without OCT4 [45]. This study indicated that the spatial and mechanical input exerted by ECad has a critical role in driving cell fate. However, it is not clearly understood how ECad can compensate for OCT4. Since many studies showed that where it was possible to skip other factors of Yamanaka-cocktail for reprograming of somatic cells to iPSCs but OCT4 was hardly indispensable [10,70,78], further studies are warranted to determine the underlying mechanism. One potential explanation might be that ECad and KLF4 together initiated an early MET process of the experimental cells, and then SOX2 and KLF4 operated co-operatively to propel pluripotency genes to induce initiation of reprogramming [74]. The hypothesis is favored by the fact that cells those already express ECad, for example keratinocytes, can be reprogrammed more effectively and quicker because the MET process is not required [71]. Since the extracellular domain of ECad is adequate to produce ECad-mediated influences related to the reprogramming of somatic cells to iPSCs we have therefore assumed that ECad-Fc could significantly enhance the reprogramming efficiency. Our preliminary observation suggested that indeed co-transfection of ECad-Fc-expressing plasmid with Yamanaka factors enhanced reprograming efficiency of mouse fibroblast (unpublished). Enhanced reprogramming efficiency was further witnessed while the Yamanaka-cocktail-transfected starting cells were cultured on ECad-Fc-coated plate compare to gelatin-coated plate. However, further experiments are necessary for providing detail quantitative and qualitative information for these observations. Nonetheless this finding is highly promising regarding enhanced and efficient generation of iPSCs using a biomaterial as substratum.

The protocols for generating ESCs or iPSCs as well as differentiation to target cells from these cells require cell isolation step either by mechanical process or in combination with enzymatic treatment [79]. These types of methodologies require skilled labor, specialized instrumentation, additional time and cost, and distinct morphologic and phenotypic features. Several protocols have been described recently for enzyme-selective passage of specific cells; however, they are not globally applicable and very often appeared with unwanted cells. Enzymatic treatment also caused karyotypic anomalies compared with manual passaging [66,80]. FACS protocol has been applied for cell sorting based on surface marker recognition. However, relevant protocols need enzymatic treatment, application of foreign molecules, and mechanical processes involving severe stress on experimental cells [81,82], which are highly unfavorable for cells. ECad-Fc cooking-plate, advantageously, neither needs any kind of mechanical sorting

nor any harsh chemical or enzymatic treatment. The experimental cells can selectively and strongly make homophilic binding with ECad-Fc matrix in a Ca^{+2}-dependent manner subjected to the differential expression pattern of ECad in them during the transformation process. The cells with no or low level expression of ECad cannot and does not firmly bind with ECad-Fc substratum and can be washed off with suitable buffer thus offering a unique, robust, and stress-free cell enrichment system. Such a protocol ensures quicker, cheaper and convenient cell enrichment system for *in vitro* culture without risk of additional contamination and cellular alteration, and therefore, is highly advantageous for application in regenerative medicine and tissue engineering to achieve desired therapeutic effect with minimal adverse consequences.

8. NCad in cell biology

N-cadherin (NCad) or neural cadherin is also known as Cadherin-2, which is encoded in human by *CADH2* gene [83,84]. Like ECad, it is also a cell-cell adhesion molecule composed of five extracellular cadherin domains, a transmembrane domain and a highly conserved cytoplasmic region. NCad can exist either as strand dimers or in an alternate monomeric form [85]. NCad typically forms homotypic homophilic interactions between two neighbouring cells for example, Sertoli cells and spermatides, and also heterotypic homophilic and heterophilic interactions, such as interaction between N- and R-cadherin in transfected L cells [86]; such interactions are Ca^{+2} dependent [87], and can be reversed by withdrawing Ca^{+2} from the system.

During embryogenesis cells undergo an epithelial-mesenchymal transition (EMT) initiating upregulation of NCad and the downregulation of ECad in the mesoderm [88]. It has been suggested that NCad expression is essential for morphogenesis of the mesodermal germ layer during gastrulation [89]. NCad expression pattern has been found complementary to that of ECad in epidermal ectoderm [88,90,91]. NCad expression has been detected in mesoderm and notochord in the early phase of embryonic development, which is later also evident in neural tissue, lens placode [92], some epithelial tissues, myocardium of heart [93], epiblast of skeletal muscle [94], endothelial cells, osteoblasts, mesothelium, limb cartilage, and primordial germ cells [95,96].

NCad is found to be present in the early hematopoietic progenitor CD34+CD19+ cells, and it was proposed that NCad plays critical role for the hematopoietic cell differentiation as well as the early retention of this subpopulation in bone marrow [97]. During skeletal muscle forma-tion mesodermal precursors exit from the cell cycle, and differentiate into myoblasts that terminally differentiates into multinucleate myofibers [98]. Cell cycle arrest and the expression of skeletal muscle–specific genes are the critical checkpoints for this developmental process [99]. All the epiblast cells undergoing skeletal myogenesis express the skeletal muscle-specific transcription factor MyoD, among them only the cells expressing NCad but not ECad can differentiate into skeletal muscle [94]. NCad function-perturbing antibodies showed that it plays a significant role in interaction between myoblasts in myotube formation and in myofibrillogenesis [100,101,102]. NCad is also found to be involved in myoblast migration in limb bud [103].

Cartilage is formed from the vertebrate embryonic limb by a highly synchronized and systematic event of cell commitment, condensation and chondrogenic differentiation of mesenchymal cells to chondrogens, and by the production of cartilaginous matrix. SOX9, an essential transcription factor for chondrocyte differentiation and cartilage formation, binds to the SOX9-binding motif in NCad promoter [104] that facilitates expressing of NCad gene products to play necessary roles in cellular condensation [105]. Prolonged expression of NCad due to the missexpression of wnt7a stabilizes NCad-mediated cell-cell adhesion resulting inhibition of chondrogenesis from mesenchymal chondrogenic culture [106]. The level of NCad mRNA was found increases during osteoblast differentiation and decreased during adipogenic differentiation thus suggesting their involvement in relevant differentiation processes [107]. NCad expression is increased in osteoblasts by BMP-2, FGF-2 and phorbol ester (e.g., PMA) in PKC-dependent manner, whereas factors like TNFα and IL-1 reduce the expression of NCad [108].

Migratory cell populations, also known as neural crest cells, are pluripotent cells those originate from dorsal part of neural tube and play important roles in embryonic development and pathophysiological conditions. These cells express NCad when they are associated with neural tube; however, NCad expression is down-regulated after EMT process and the relevant cells started to migrate over long distance, and finally transform into different types of tissues and cell populations, such as peripheral nervous system, cartilage, bone and melanocytes. Slug plays here important roles in down-regulating NCad that leads to a loss of cell-cell adhesion and allowing the cells to migrate. The dorso-ventral migratory cells re-express NCad during dorsal root and sympathetic ganglia developmental steps and promotes cell aggregation; thereafter, only dermal melanocytes express NCad [109,110]. This observation is suggesting critical involvement of NCad in the development of relevant tissues.

Several proteins can interact with NCad *via* intracellular and extracellular domains and influence subsequent signaling pathways. The functions of NCad in controlling neurite outgrowth, synaptic plasticity and guidance in synapse formation have been proposed [111]. These functions may involve interaction with other membrane bound molecules, such as fibroblast growth factor receptor (FGFR), which was confirmed by blocking the FGFR by pharmacological inhibitor [112]. NCad directly interacts with FGFR *via* HAV epitope of FGFR with IDPVNGQ epitope of EC4 of NCad [112], and this interaction between NCad and FGFR can be of both ligand dependent and independent [113] suggesting wider cooperative functional significance of this duo in relevant development and physiology.

EMT of squamous epithelial cells ectopically expressed specific amino acid sequences of EC4 of NCad induces motility. The cell motility behavior and adhesion is independent to each other, as antibody against the aforementioned relevant amino acid sequence of NCad inhibits cell motility but the cell-cell adhesion phenomena was uninterrupted [114]. The influence of NCad mediated cell migration is cell type specific, as it was found that NCad can inhibit LM8 mouse osteosarcoma cell migration but it did not have any significant effect on the movement of MDA-MB-435 cells [115]. The cytoplasmic domain of NCad form complexes with various types of molecules, such as p120, β-catenin, α-catenin and GAP-43, and regulate various cytoskeletal dynamics. All of these interactions are critically involved in tissue-to-animal development,

morphogenesis and maturation, and is suggesting the possibility of exploiting this gene product for regenerative medicine.

9. NCad-Fc as biomaterial in regenerative medicine

NCad-Fc was introduced by Lambert et al. in 2000, and the study revealed that NCad-Fc not only induced the recruitment of NCad on the plasma membrane but also other components of the cadherin/catenin complex. This work for the first time demonstrated that NCad-Fc can mimic natural cell-cell contact formation and signal transduction [116]. Pioneering work from our lab has introduced NCad-Fc as cell-coating biomaterials for stem cell culture. NCad-Fc protein was collected from 'pRC-NCFC' plasmid, which was constructed by inserting the N terminal extracellular domain of mouse NCad into pRC/CMV (Invitrogen) plasmid [29]. The expression and purification methodologies of NCad-Fc are similar like ECad-Fc and have been described in details in relevant publications [50,55,117]. Over recent years our laboratory work revealed significant advantages of NCad-Fc in neural differentiation from stem cells. Early work was performed with mouse embryonic carcinoma cell P19 and neural stem cell MEB5 because of their easy management over the ESCs. It was observed that culturing these cell lines on NCad-Fc substratum can maintain the undifferentiated state and scattering morphology compare with other control substratum such as gelatin, fibronectin, laminin or poly-L-ornithine. P19 and MEB5 cells were differentiated effectively to neural lineage on this defined matrix in presence of retinoic acid supplemented with insulin-transferrin-selenium commercial preparation (ITS, Invitrogen). Interestingly, P19 cells showed higher level of *Neurog1* expression on NCad-Fc-coated surface compare with gelatin-coated surface. Additionally, MEB5 differentiated on NCad-Fc matrix, compared to fibronectin-coated surface, showed complete neuronal differentiation phenomena and significantly higher expression levels of neural markers, such as *Neurog1* and *MAP2*. These results clearly suggested the superiority of NCad-Fc substratum over the other experimental substratum for neuronal differentiation process.

Later, the findings were extrapolated to MEF-dependent mouse embryonic stem cell ST1 and mouse iPSCs to evaluate whether the effect is restricted to specific pre-committed cell lines or it is globally applicable [65]. Since during EMT conversion ECad is downregulated and NCad is upregulated therefore a hybrid matrix of ECad-Fc and N-Cad-Fc was designed to exploit the stage-specific cadherin switching phenomenon. The concept was that, initially the ESCs and iPSCs would bind to ECad-Fc through cell-resident ECad, however, during and after neuroectoderm formation cadherin switching will cater for cellular NCad in place of ECad that would bind to NCad-Fc. The cadherin switching was experimentally confirmed in house during neural differentiation protocol (Fig. 3A), where Dkk-1, a Wnt signaling pathway antagonist, and LeftyA, a Nodal signaling pathway antagonist were used for triggering neural differentiation. Specific markers for primitive ectoderm, primitive neural stem cells, neural stem and progenitor cells were checked. Along with, promisingly, the efficiency of neural progenitor differentiation from mouse ESCs on cadherin-Fc chimeric matrix was significantly higher compare to the cells cultured on other standard substratum as evaluated by the higher

level of expression of neural progenitor marker Nestin gene products. Furthermore, the differentiated cells exhibited greater levels expression of βIII-tubulin (Tuj1) (Fig. 3B), microtubule associated protein 2 (MAP2), Pax6, and tyrosine hydroxylase but not GFAP, which is a marker of glial cell, signifying the presence of a lineage confined to neural cells.
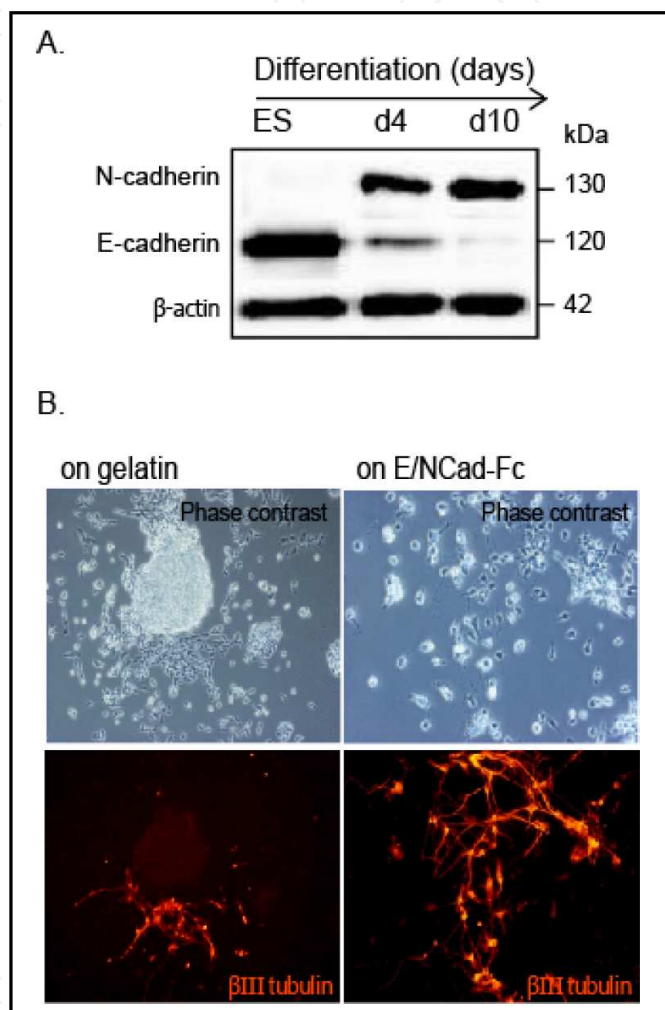
Figure 3. NCad-Fc, and ECad-Fc promote directed differentiation of target lineage from iPSCs. (A) Western blot data revealed ECad to NCad switching occurs during neuronal differentiation. The expression level was normalized using house-keeping gene, β-actin. (B) βIII-tubulin expression was significantly higher on E/NCad-Fc matrix compared to gelatin.

Culturing of ESCs and iPSCs on ECad-Fc and NCad-Fc hybrid substratum not only developed scattered cell morphology as reported for ECad-Fc substratum but higher cell proliferation rate and enhanced differentiation efficiency were also noted. Along with these phenomena significant higher degree of homogeneity and enhanced differentiation efficiency were also observed, which is a remarkable advantage for harvesting target neuronal cells from *in vitro*

system that can later be applied for regenerative medicine protocol. Although EB-based protocols are being relatively well-practiced for neural differentiation, however, the inconsistency of the embryoid body (EB) size and shape, and the asynchronous distribution of growth factors throughout the EBs give rise to heterogeneous products. Besides, monitoring cell morphology during differentiation process for EB-based differentiation protocols is inconvenient. Culturing ESCs or iPSCs in scattered single cell condition, on the contrary, can effectively overcome these issues. Interestingly, this blend of Cadherin-Fc matrices maintained a complete homogeneous cell population for murine ESCs and iPSCs for several passages. Highly homogeneous population of primitive ectoderm and neural progenitor cells were routinely generated on such a hybrid-type cooking-plate [65]. Enriched population of neuroectoderm progenitor cells can be obtained within 4 to 6 days by using E/NCad-Fc based monolayer-forming ESCs and iPSCs culture protocol and standard neurogenic cocktail treatment, which is a great advantage for quick generation of the target cells for application in regenerative medicine.

Some cells release 90 kDa fragment of soluble NCad (sNCad), and NCad-Fc was used to mimic sNCad response on neurite development [118]. Application of NCad-Fc by Doherty *et al.* with cerebral neurons showed that NCad-Fc initiated neurite outgrowth in a FGF receptor depended manner [111,119] suggesting that NCad-Fc can be utilized for controlling FGF receptor signaling pathway to facilitate relevant neuronal development events. Using mouse E12.5 ventral spinal cord explants as a convenient model Marthiens *et al*. showed that the axons formed contacts along the axon-shaft by long filopodia-like processes on NCad-Fc matrix [120]. They further showed that growth cones preferentially interact with cad-11 or NCad-Fc when progressing on this substratum whereas it differs on laminin. This study proved direct involvement of cadherin-11 and NCad in peripheral nervous system establishment from embryonic tissues [120].

Not only for neuronal population related regenerative medicine, NCad-Fc also showed potentials for application in other tissues as well, for example myogenesis related issues. Charrasse *et al*. used NCad-Fc to mimic NCad binding effect for myogenic differentiation [121]. They showed that NCad-Fc based NCad–dependent cell–cell adhesion triggers RhoA GTPase activity, which is essential for myogenic differentiation. Activity and expression of SRF, a transcription factor that binds to the promoter regions of muscle-specific genes [122,123] and controls the expression of MyoD, is controlled by RhoA. In turn, MyoD binds to the promoter region of skeletal muscle activating genes in mesenchymal cells and convert them to skeletal myoblasts [124,125,126]. These findings demonstrated that N-cadherin–dependent adhesion event that regulates the RhoA/SRF pathway to trigger myogenesis can be harnessed by NCad-Fc matrix and therefore such technology is holding great promises for using in relevant regenerative medicine protocols.

10. Conclusion

To design an efficient biomaterial capable of maintaining and stewarding specific cell phenotypes critical for the development, homeostasis, differentiation, and regeneration of tissues,

the material must have a high degree of selective recognition property to the desired cells. As well, such a biomaterial should be devoid of unexpected stimulation characteristics to the cells that can be hazardous to them or to the desired results of the protocols. Being the intrinsic component of cellular milieu, proteins are highly desirable molecules to be used in regenerative medicine and tissue engineering technology. Their 3D conformation made them perfectly fit in the cell-biology and ensuring that only specific function to the experimental cells has been achieved. The natural homeostasis properties of cells can adequately remove these proteins once they are used up without exerting any unnatural effect or stress to the cells. Expressing and purifying large protein with proper 3D conformation is extremely challenging therefore mimetic peptide technology has been becoming popular. These small peptide sequences represent small functional domain of the relevant proteins, albeit not with the native 3D structure of the parent protein molecule. While most cases they are being generated using artificial synthetic technology in test tubes, however, their purity, reproducibility and yield are major concerns for their confident application in stem cell technology. Additional limitations for mimetic peptides are (1) the restricted size of desired peptides, and (2) inability to provide native post-translational modifications, most of which are critical for proper biofunctionality of the relevant molecule. Therefore mimetic peptides cannot and do not behave identically as their natural parent protein. On the contrary, Fc-chimeric proteins can be generated with high degree of reproducibility with identical molecular properties using the natural cellular readout process from the DNA template. The additional stability of the target protein instigated by the presence of Fc domain is significantly advantageous for higher yield of the tailored chimeric protein. The intrinsic property of Fc domain to form homodimer is beneficial to keep the target chimeric protein in soluble form. On the other hand, the natural affinity of Fc domain to bind with Protein A or Protein G is a technical boon for convenient purification of the target protein without fusion of any secondary bait to the amino acid sequence, which often create complex situation for getting rid of them at the later stage of the processing to harvest only the desired designed protein. Directional binding of Fc domain with the polystyrene or hydrophobic surface and catering the functional protein outwards is also an intrinsic benefit for using this class of chimeric proteins for obtaining higher functional efficacy of the applied biomaterials. Since the specific homophilic interactions between cadherins mediate cell attachment therefore specific cadherin isoform-expressing cells can be purified by using the relevant cadherin-Fc biomaterial as surface-coating materials. For example, iPS cells express high level of ECad and neuronal cells express NCad therefore, by employing these matrices in different time points of differentiation protocol, the target cells can be purified *in situ* without the necessity of any harsh enzymatic or mechanical treatments. Some of these chimeric proteins are commercially available for application and some are in pipeline, which can be obtained from our laboratory under proper regulatory affairs. Collectively, Fc-chimeric protein-based biomaterials provide distinct advantages for overcoming many existing challenges in stem cell technology and significantly advancing the regenerative medicine and tissue engineering field towards practical application.

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