Development of Recombinant Human Vitronectin Protein as Coating Matrix Reagent for Human ES or iPS Cell Culture (Catalog No. 5121-0.1MG)

Introduction

Regenerative medicine offers the hope that cells for disease research and therapy might be created from readily available sources. To fulfill this promise, the available cells need to be converted into the desired cell types either in vivo or in vitro. Currently, there are two main approaches to accomplishing this goal: in vitro directed differentiation, which is used to push pluripotent stem cells, including embryonic stem cells or iPS cells, through steps similar to those occur during embryonic development; and reprogramming (also known as trans-differentiation), in which a differentiated cell is converted directly into the cell of interest without proceeding through a pluripotent intermediate. Both approaches require a unique cell culture system, in which, all chemicals or proteins are clearly defined for its quality and cost.

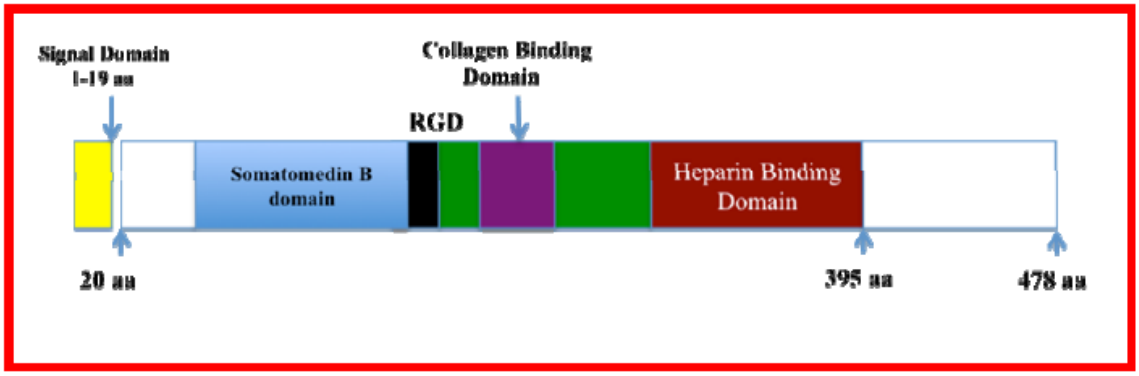
Stem cells are thought to reside within specific anatomical structure or "niches", where they receive signals that maintain them in an undifferentiated state and induce their self –renewal as needed. Currently, feeder cells or Matrigel are two commonly used culture methods for sustaining ES cell culture. Lot variation in feeder cell production and undefined Matrigel mixture protein extracts from Engelbreth-Holm-Swarm (EHS) mouse sarcoma cells makes research data which derived from those two culture systems un-relevant to protocol standardization for clinical application development.

To develop a chemically defined ES (or iPS) culture system, recombinant human vitronectin protein was developed as a stand alone coating matrix reagent based on recent publication(1) and manufactured in E.coli using a "temperature-shift – Matrix-buffer" inclusion body refolding technology. In this report, various parameters related to different iPS generation technologies, such as retroviral delivery, intracellular TF protein delivery derived iPS cell method (PiPS) and mRNA delivery based iPS system were tested for further development of recombinant human vitronectin applications in ES or iPS cell.

Review Structure of Recombinant Human Vitronectin (VTN)

Human Vitronectin (consisting of 459 aa) is also known as VTN, is a member of the pexin family. Vitronectin is an abundant glycoprotein found in serum and the extracellular matrix and promotes cell adhesion and spreading, inhibits the membrane-damaging effect of the terminal cytolytic complement pathway, and binds to several serpin serine protease inhibitors. N-terminal somatomedin B domain is a close-knit disulfide knot, with 4 disulfide bonds within 35 residues, and C-terminal 399-459 domain is dispensable for ES cells culture.

To maximally maintain VTN structure, several versions of recombinant VTN protein product were constructed, such as (Cat#: HRP-0299) with domains which including natural N-terminal to 398aa. this recombinant protein contains Somatomedin B and RGD domains. Another version of recombinant VTN protein product consists of 62 – 398aa domain (HRP-0323), and full-length VTN protein (Cat#: HRP-1056)



Protein Sequence of Recombinant human VTN (21-398aa)

MDQESCKGRCTEGFNVDKKCQCDELCSYYQSCCTDYTAECKPQVTRGDVFTMPEDEYTVYDDGEEKNNA
TVHEQVGGPSLTSDLQAQSKGNPEQTPVLKPEEEAPAPEVGASKPEGIDSRPETLHPGRPQPPAEEELC
SGKPFDAFTDLKNGSLFAFRGQYCYELDEKAVRPGYPKLIRDVWGIEGPIDAAFTRINCQGKTYLFKGS
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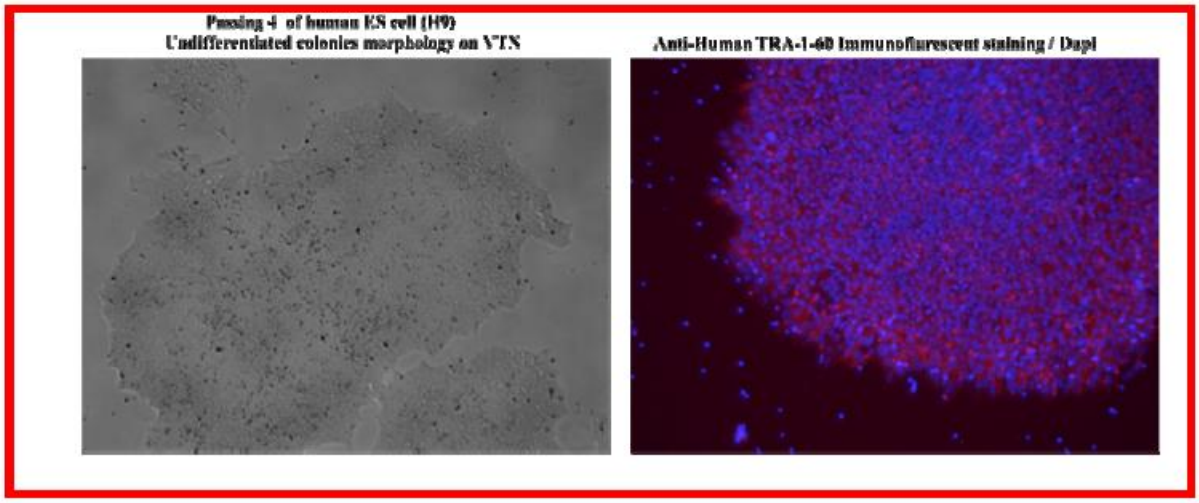
GSSLSAVFEHFAMMQRDSWEDIFELLFWGRTSAGTRQPQFISRDWHGVPGQVDAAMAGRIYISGMAPRP
SLAKKQRFRHRNRKGYRSQRGHSRGRNQNSRRPSR

Expression and Purification of Recombinant Human Vitronectin in E.coli

To achieve the goal of cost effective manufacture of recombinant human VTN, E.coli derived inclusion body refolding technology was selected for the production. Codon optimized human VTN cDNA fragment was synthesized and expressed in E.coli, inclusion bodies were highly purified using various washing buffers. After inclusion bodies were completely solubilized in 8M-urea buffer, protein samples were affinity column purified and refolded using our proprietary “temperature-shift” refolding technology. Final product is preserved in PBS buffer at 0.5mg / ml and stored at -20 °C.

Cultivation ES Cell Using Recombinant Vitronectin as Coating Matrix

To test recombinant VTN coating for human ES cell culture, 10μg VTN/well (6 well plate) was applied. After adding 1ml PBS / 10ug recombinant VTN / well and placed at 4 ℃ for overnight, removing PBS solution, the plate is ready for routine ES cell culture. Human H9 cells was maintained in Nutristem medium and passing at least 4 generations before checking its Immunoflurecent staining for TRA-1-61 marker. Human ES cells, which cultured on recombinant VTN coating plates, maintain excellent colony morphology, and easy enzymatic treatment for ES cell detachment. Typically, if H9 cell requires 7 - 8 min Accutase incubation when cultivated on Matrigel or feeder cells, it only requires 1 min Accutase treatment when recombinant VTN was used as coating matrix.



Easy Coating Protocol:

Coating recombinant human VTN protein for ES or iPS cell culture can be easily performed as following:

A: Add 1ml PBS buffer to a single well of 6 well plate, and mix well with 10ug recombinant VTN (20ul solution, 0.5mg / ml of VTN stocking solution), place culture plate at 4 ℃ for overnight.

B: Plates are ready for routine ES culture. (Remove coating PBS buffer before cell culture).

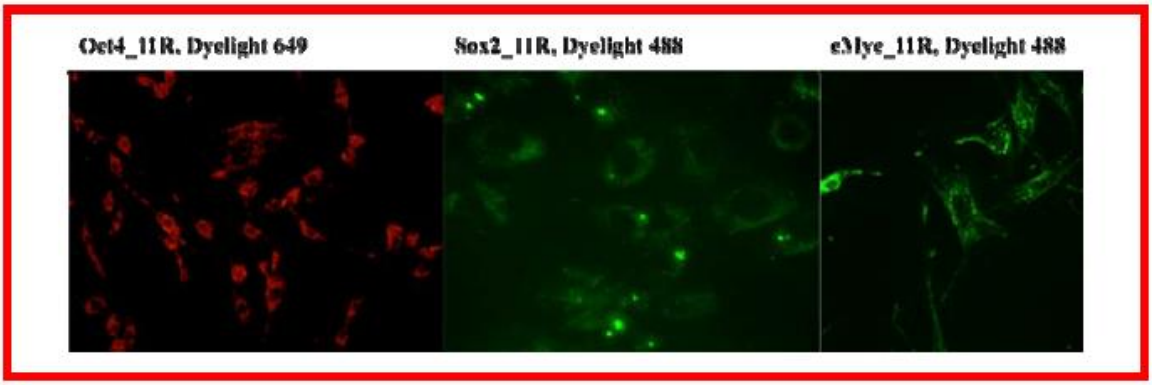
Note: Different culture plates such as CELLSTAR polystyrene dishes and BD Falcon hydrophobic surface have different binding capacities for various proteins. For CELLSTAR 6 well plate, 5ug recombinant VTN / well works well, but for BD falcon dishes, requires 10ug recombinant VTN / well for coating. (In general, 10 ng VTN /mm^2 for coating will be a good start concentration for testing).

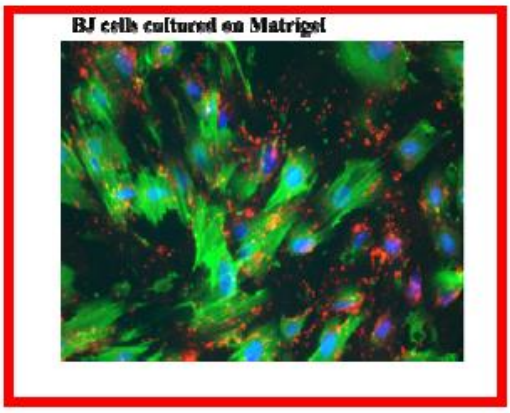
Additional Testing For Recombinant VTN Applications

For iPS generation, commonly used coating technologies dramatically affect gene, protein or RNA delivery efficiency. For retrovital or lentiviral based gene delivery systems, one round viral transduction was performed when primary target cells, such as human fibroblast still maintained on normal culture condition (non-coating plate), then shift to feeder cell or matrigel plate in the late stage of protocol. Both recombinant TF protein based and mRNA based nongenetic iPS generation systems heavily rely on daily 11R tagged protein loading or lipid mediated mRNA transfection. Coating condition wills dramatically effects protein or mRNA delivery efficiency.

A: For intracellular protein delivery

To test VTN coated plate versus Matrigel coated plate for 11R tagged recombinant TF protein delivery, Recombinant human Oct4_11R, Sox2_11R and cMyc_11R protein were directly labeled with immnuflurescent dye (DyLight 488, 649, Pierce Kit), human fibroblast cell (BJ) were seeded on either VTN or Matrigel coated 6 well plate. Incubation 2 ug of Oct4-11R, Sox2-11R or cMyc_11R for 1 hours at 37°C will have nearly 100% cell transduced with intracellular penetration (up-panel figure), but > 90% recombinant TF protein were nonspecifically binding to Matrigel and gives a low efficient for intracellular protein delivery.

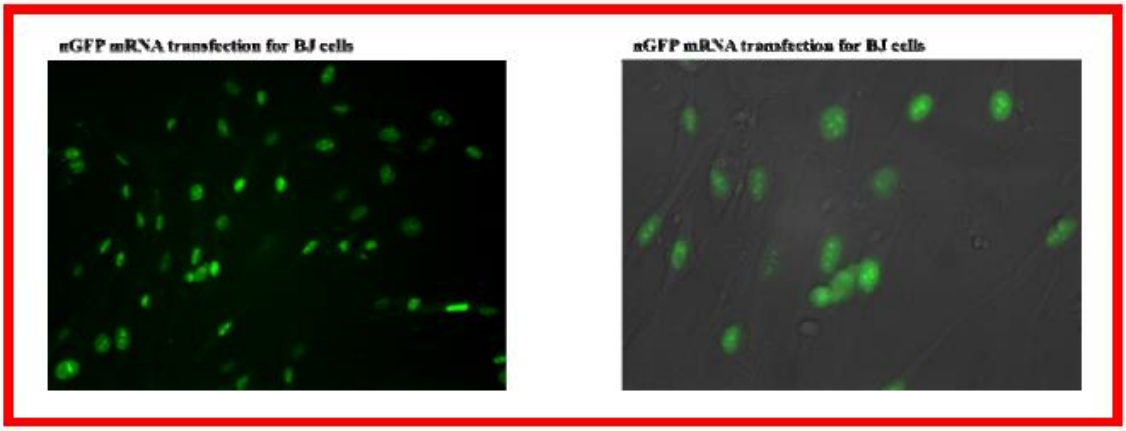


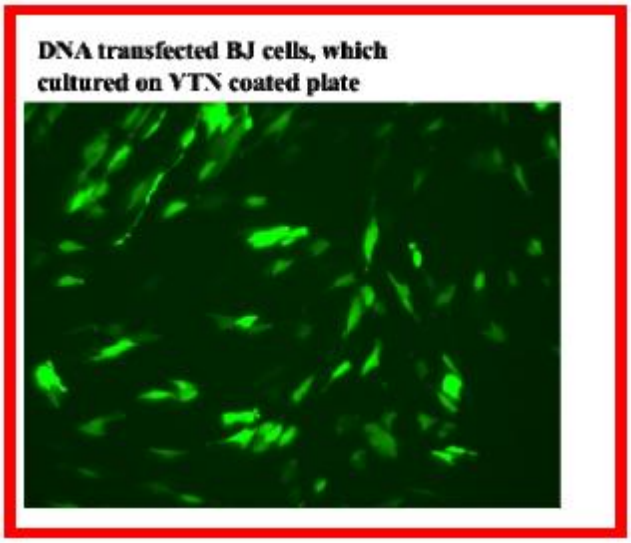


2ug of DyLight 649 labeled Oct4_11R were incubated with BJ cells, which cultured on Matrigel coated plate. Cells were fixed and staining with Invitrogen Alexa Fluor 488 phalloidin and DAPI. As data demonstrated in left figure, most of Ock4_11R (Red) were stick on matrigel, did not shown intracellular signal staining.

B: For mRNA delivery

Recently, direct delivery OSKML mRNA into human fibroblast has been demonstrated successfully for generating human iPS cell using feeder cell culture system. In this experiment, we are testing polymer derived mRNA transfection on human fibroblast cells, which cultured in recombinant VTN coated plate. Nuclear GFP (nGFP) mRNA (from Stemgent mRNA reprogramming Factor Set: Cat# 00-0067) was tested for human fibroblast transfection using either none coated or VTN coated plate (BD falcon 6 well plate, with 10ug VTN/well). Transfected with 100ng nGFP mRNA using Stemfect RNA transfection reagents (Cat# 00-0069), human fibroblast cells (BJ), which cultured on VTN coated plate had nearly double nuclear positive GFP cells when compared with non-coating control plate.





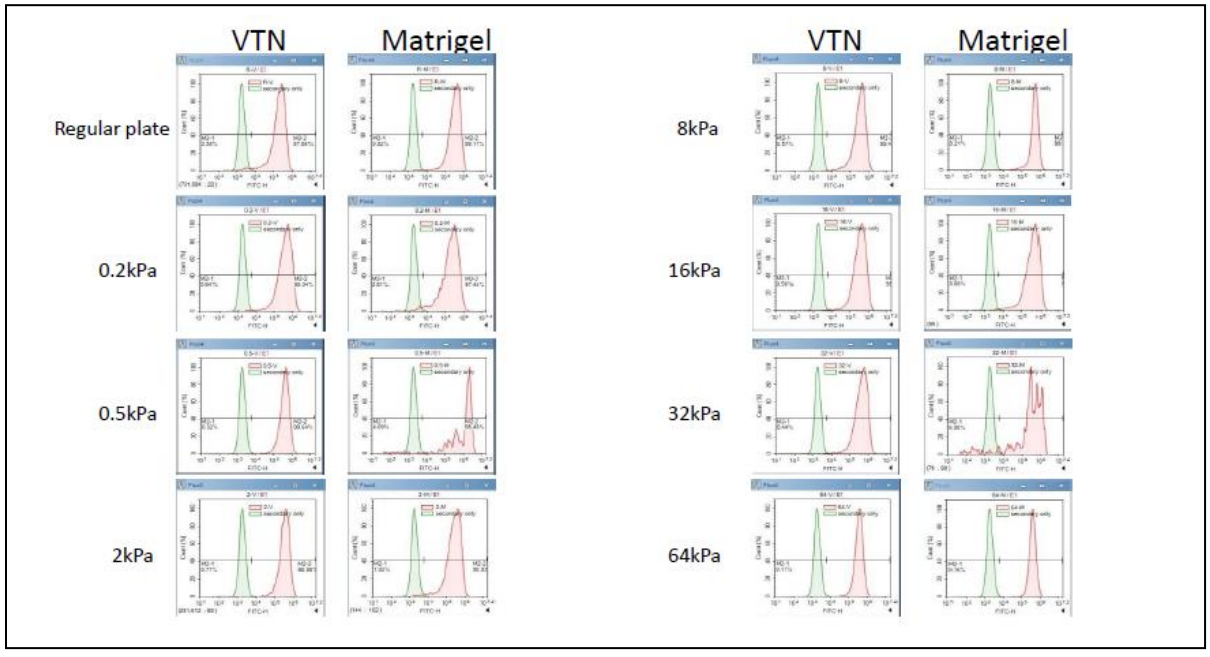
C: For DNA transfection delivery

Gene mutation correction will be performed in future for potential gene therapy using human ES orpatient-specific iPS cells in vitro. To test VTN coated plate for routine DNA transfection efficiency; polymer based DNA transfection, such as jetPEI was tested on human fibroblast cell in the present of 10% FCS culture medium. Following manufacture suggested protocol, as data demonstrated in right side figure, 2ug pCMV_GFP plasmid gives an excellent transfection efficiency, which is 20-30% better than non-coated control plate.

D : Utilizing vitronectin protein coating in 3D culture system

Chemically defined mechanobiological studies allow the characterization of cell response to mechanical stresses in details. Cells need to be supported by a material (such as Matrix Protein) with properties similar to the physiological environment. Silicone elastomers have been used to produce various in vitro scaffolds of different geometries for endothelial cell studies given its relevant mechanical, optical and surface properties. As recombinant human vitronectin protein coated cell culture plate nicely support human ES or iPS cells for long-term maintain in chemically defined condition, it provides a technical capability for studying human ES cell differentiation regulation under various stiffness for mechnobiological response along cell specific lineage differentiation pathway.

To explore recombinant human Vitronectin protein as coating matrix on silicone elastomers in 3D culture condition, use Matrigel as control, both materials was individually coated on 7 stiffness silicon plate set (24 well plate : Advanced BioMatrix Products, Cytosoft Set: 0.2kPa, 0.5kPa, 2kPa, 8kPa, 16kPa, 32kPa, 64kPa) in PBS buffer. Human H9 cells were seeded into each well in mTeSR1 medium. When the cells reach 30-40% confluency, switch to neural progenitor cell differentiation media (iXCells Biotechnologies products), Changing media daily for a total of 6 days. Finally, cells dissociate into single cells and fixed for immunostaining with anti-human Nestin antibody and subject the sample to flow cytometry for data analysis (Anti-Nextin antibody. R&D Inc, Cat#: MAB-1259-SP). Conclusion: Coating recombinant vitronectin protein on various stiffness silicon plates fully support human ES cell differentiation into neural stem cell in chemically defined medium.



Summary:

Human ES cells or iPS cells cultured on recombinant human vitronectin protein coated dishes not only maintain its self-renewal in long-term culture, but also benefit with easy enzymatic detachment treatment for routine cultivation. Enhancement of 11R tag recombinant TF protein and mRNA intracellular delivery makes recombinant vitronectin protein an excellent coating matrix for non-genetic human iPS generation applications. Further application of VTN in 3D culture condition with various stiffness might provide a unique mechanobiological study system for tissue regeneration research.