

## INTRODUCTION

Human induced pluripotent stem cells (iPSC) have great potential as a cell source for regenerative medicine, drug discovery and disease modeling. The quality and consistency of iPSC culture is critical for basic research and clinical therapy development. To improve reproducibility and safety, there have been significant effort in developing fully controlled iPSC systems such as using defined xeno-free media and feeder free substrates such as basement membrane extracts (BME). iPSC are anchorage dependent cells whose survival, pluripotency, and differentiation potential are critically dependent upon extracellular matrix (ECM). BME is a soluble form of the basement membrane that contains undefined ratios of ECM proteins. Proteins such as human recombinant vitronectin (VN) and fibronectin (FN) pose an attractive alternative to BME and have been shown to support iPSC culture. iPSC attach to the ECM via integrin receptors which are linked to focal adhesions and the cytoskeleton. Depending on the nature of the substrate, different integrin receptors may be recruited to promote iPSC adhesion and activate various survival, mitotic or differentiation pathways. It is critically important to understand the influences that diverse substrates may have on iPSC colony morphology or differentiation potential. Identifying and characterizing a chemically-defined surface for culturing iPSCs will be paramount for establishing consistency in stem cell cultures. In this work, we report differences in human iPSC colony morphology when cultured on different ECM substrates. Subsequently, iPSC colonies on VN, FN, or BME were characterized by assessing cellular identities, cytoskeletal structure, integrin/FA signaling, and differentiation potential.

## METHODS

**iPSC Maintenance:** hiPSC (IBJ6) were maintained in Bio-Techne's animal free iPSC media and passaged at 70% confluence using Versene™. Glass coverslips or 6 well plates were coated with Recombinant Human Vitronectin (R&D Systems, Catalog # 2308-VON, 10 µg/mL), Fibronectin (R&D Systems, Catalog # 1918-FN, 10 µg/mL), or Cultrex® Reduced Growth Factor BME, Type R1 (R&D Systems Catalog # 3433-010-R1, 1:40 dilution). Cells were passaged > 3 times prior to analysis via flow cytometry, immunocytochemistry, or differentiation.

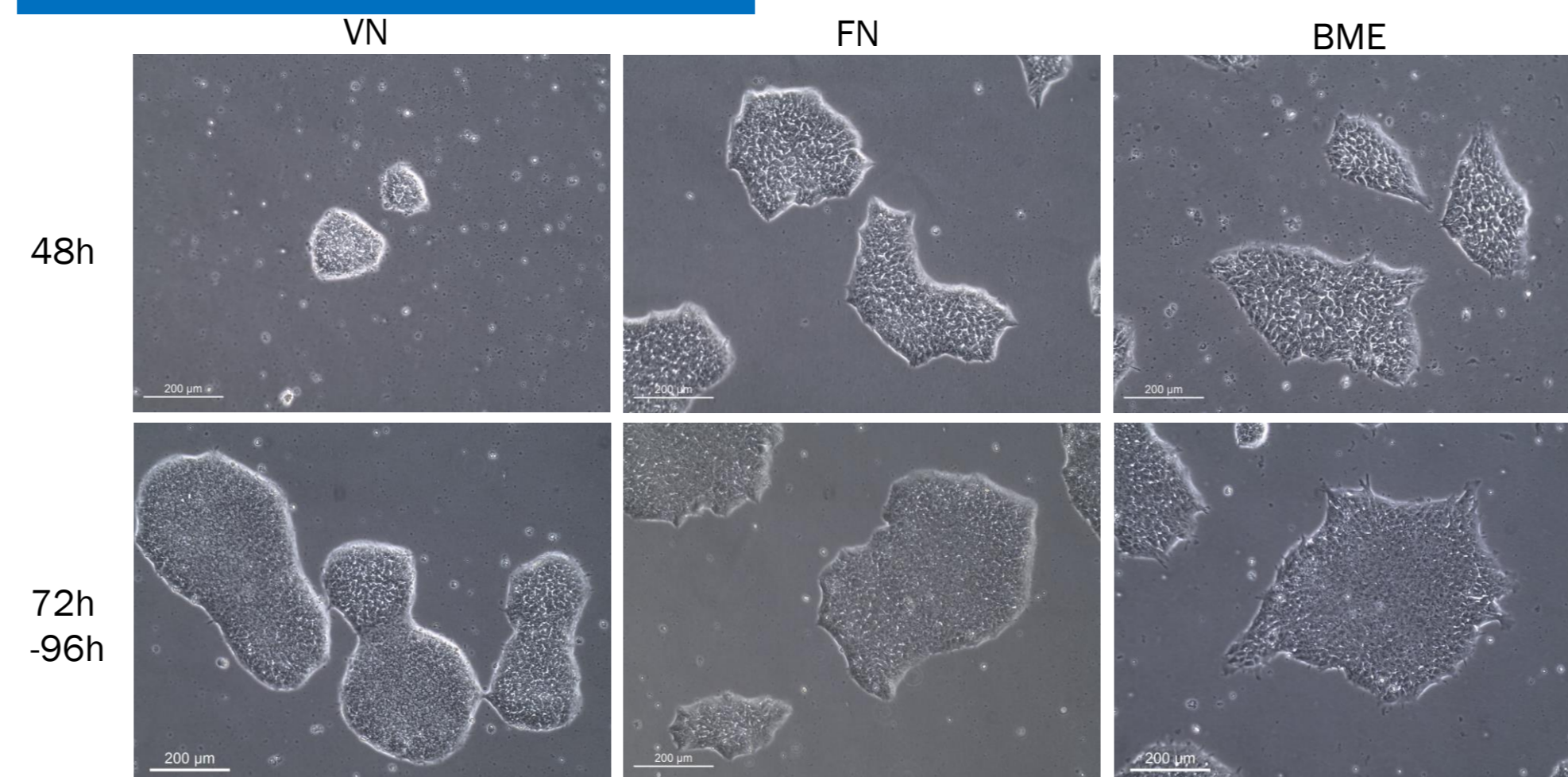
**Flow Cytometry:** iPSCs were dissociated using Accutase™ and stained for stemness markers such as Oct-3/4 and SSEA-1 using H/M Pluripotent Stem Cell Multi-Color Flow Cytometry Kit (R&D Systems, Catalog # FMC001). Cells were then processed with BD LSRFortessa and data analyzed in FlowJo.

**Cell Differentiation:** iPSC were differentiated to ectoderm or endoderm lineages using StemXVivo® Ectoderm (R&D Systems, Catalog# SC031B) or Endoderm Differentiation Kit (R&D Systems, Catalog # SC019B). SOX17+ (endoderm) and Otx2+ (ectoderm) cells were quantified using Operetta.

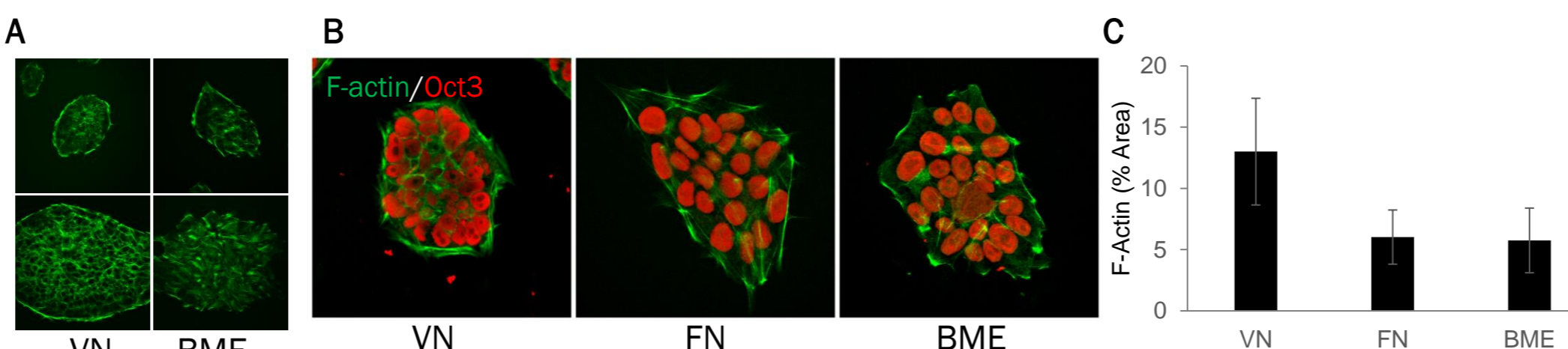
**Immunocytochemistry:** Cells were fixed with 4% PFA and permeabilized with 0.3% Triton-X-100. Primary antibodies include Oct-3/4 (R&D Systems, Catalog # AF1759), Paxillin (R&D Systems, Catalog # AF4259), β1 (R&D Systems, Catalog # MAB17781), and F-actin. Differentiated cells were stained with primary antibodies against SOX17 (R&D Systems, Catalog # AF1924) or Otx2 (R&D Systems, Catalog # AF1979). Cells were then washed with PBS and stained with appropriate NorthernLights™ secondary antibodies (R&D Systems, Catalog # NL001, NL007) and DAPI.

**Simple Western:** Colony lysates were harvested using cold RIPA buffer and then analyzed using Protein Simple's Wes/Jess. Antibodies include FAK (R&D Systems, Catalog# AF4467), pFAK (R&D Systems, Catalog# MAB4528), SRC (R&D Systems, Catalog# AF3389), pSRC (R&D Systems, Catalog# AF2685), Integrin αV (R&D Systems, Catalog# AF1219), or Integrin β5 (R&D Systems, Catalog# AF3824) and appropriate HRP secondary antibodies (R&D Systems, Catalog# HAF005, HAF016, 042-205, 043-522, 042-206)

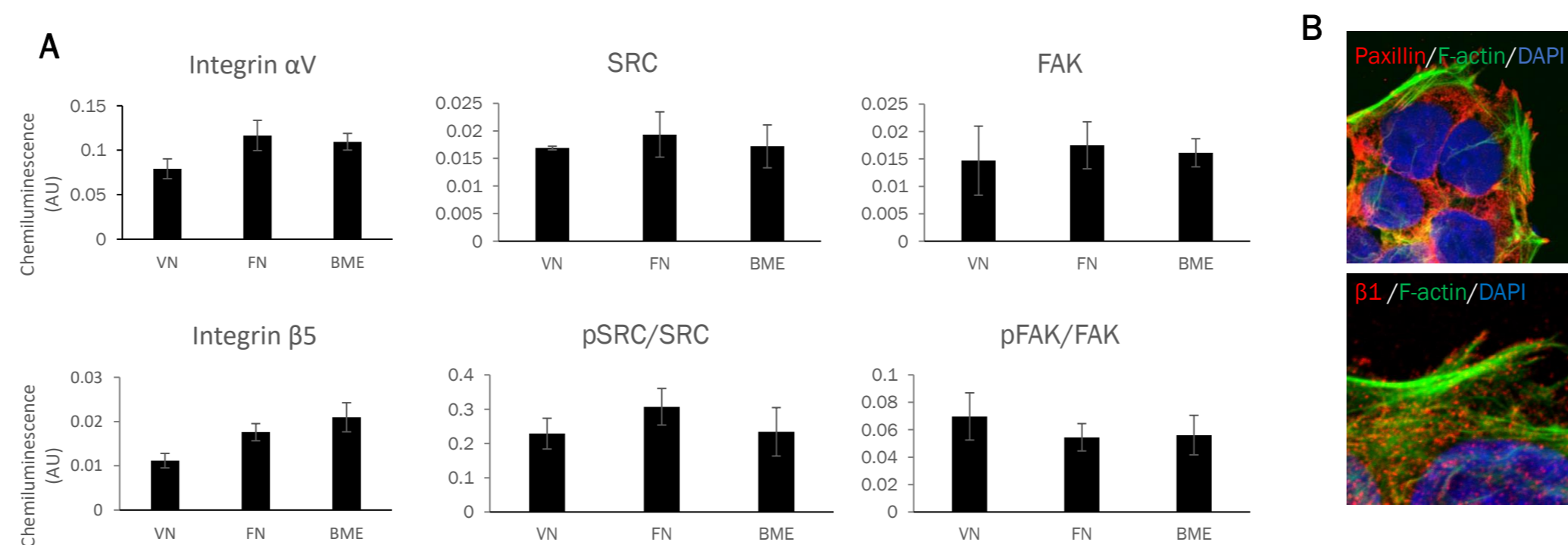
## RESULTS



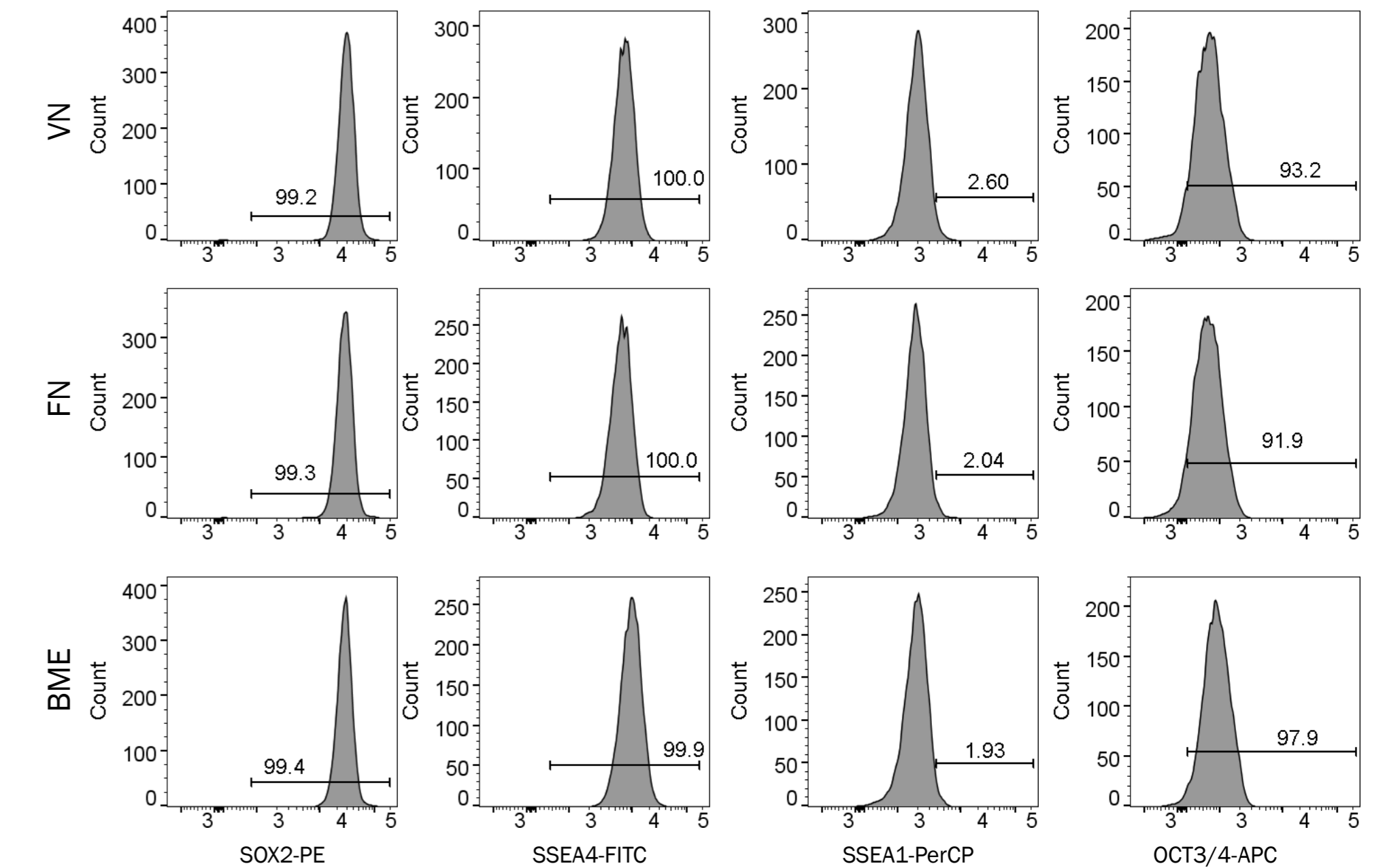
**Figure 1. Colony Morphology is Influenced by Adherence Substrate.** Brightfield images of small and large (long axis > 400 µm) iPSC colonies cultured on VN, FN and BME at 48 h and 72-96 h. More symmetrical, "rounder" and seemingly denser packed colonies with VN compared to other surfaces.



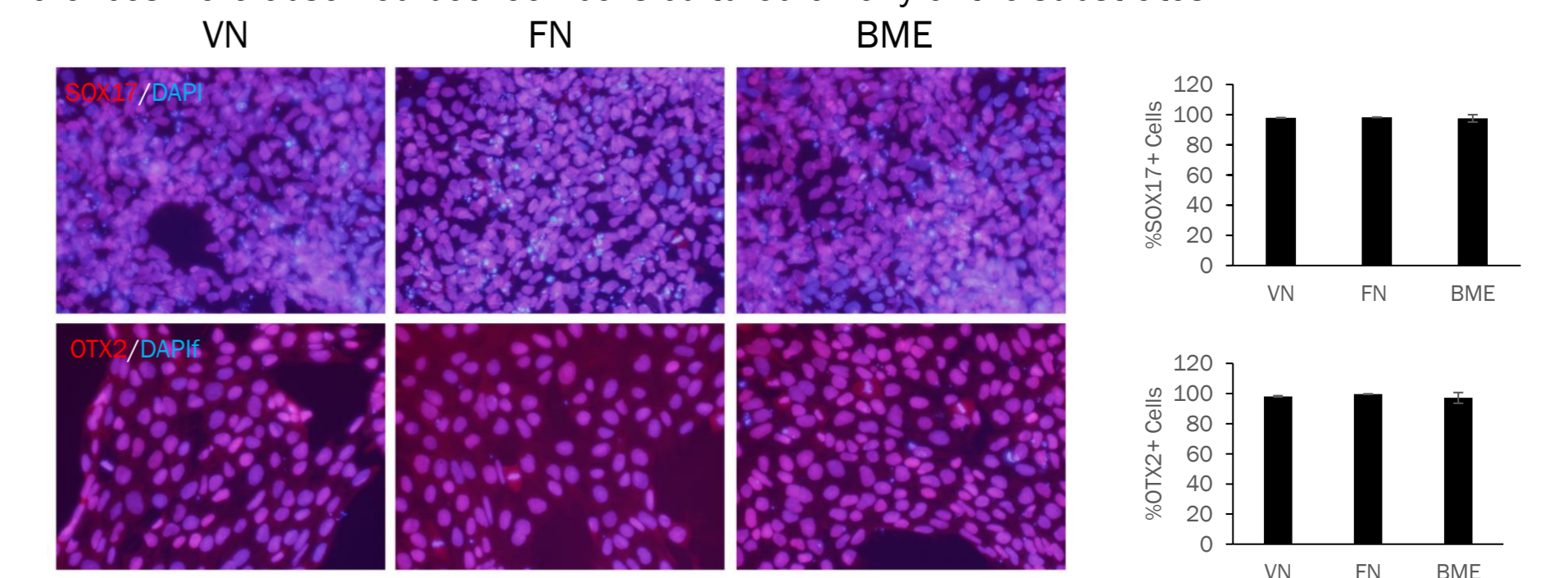
**Figure 2. Increased F-Actin Localization to Colony Perimeter with VN.** A. F-actin "fence" at edge of colony edge formed in small (top) and large (bottom row) colonies on B. all substrates. C. Quantification of % F-actin fence area of iPSC colonies.



**Figure 3. iPSC Colonies on Different Substrate Show Similar FAK/SRC Profiles.** A. Simple Western analysis of focal adhesion and integrin proteins. B. Representative images of focal adhesion (paxillin) formation (top) and β1 integrins (bot) for colonies on all substrates.



**Figure 4. Pluripotent Stemness Marker Expression is Maintained and Similar Across ECM Substrates.** Stemness markers of iPSC on VN, FN, and BME characterized by flow cytometry. Cells expressed positively for Oct-3/4, Sox2, and SSEA-4 and negatively for SSEA-1. No significant differences were observed between cells cultured on any of the substrates.



**Figure 5. Functional Pluripotency Maintained Across ECM Substrates.** iPSC grown on VN, FN, or BME expressed positive markers of definitive endoderm (SOX17, top row) or ectoderm (Otx2, bottom row) lineages after differentiation. Quantification of showed no significant differences in percentage of differentiated cells between substrates.

## CONCLUSION

Despite differences in morphology and the F-actin fence on the edges of iPSC colonies, defined (VN, FN) and undefined ECM (BME) all supported growth and propagation of iPSC colonies for multiple passages without adversely affecting their differentiation potential or stemness markers.