New monoclonal antibodies to explore human pluripotency

Introduction

Monoclonal antibodies (mAbs) are effective tools for objective characterization of various cell types. More specifically, monoclonal antibodies that detect cell-surface proteins are useful because they can be utilized for positive and negative selection of cell populations by using techniques such as fluorescence-activated cell sorting (FACS) or magnetic-activated cell sorting (MACS).

Historically, there has been a select panel of monoclonal antibodies used to characterize human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs), which are collectively termed as human pluripotent stem cells (hPSCs) [1]. This select panel includes monoclonal antibodies that detect the stage-specific embryonic antigens 3 and 4 (SSEA-3 and SSEA-4). SSEA-3 and SSEA-4 antibodies detect globo-series glycolipids, and were raised against mouse embryos and embryonic carcinoma cells.

Another series of antibodies raised to embryonic carcinoma cells (TRA-1-60, TRA-1-81, and GCTM-2) are associated with a pericellular matrix keratin sulfate/chondroitin sulfate proteoglycan. Interestingly, the protein epitopes that these antibodies bind to are not fully defined, and the identity of the genes encoding the proteins detected are either unknown or ambiguous. The majority of these antibodies are of the IgM class and are composed of a pentameric polypeptide. This structure, when compared to other immunoglobulin classes, is larger, more difficult to handle and label, and is subject to higher levels of background staining. Hence, the availability of new, well-characterized monoclonal antibodies detecting cell-surface epitopes on hPSCs would provide useful research tools to investigate the cellular mechanisms underlying human pluripotency and states of cellular reprogramming.

Prediction of new cell-surface markers for hPSCs

A research team recently described the generation of new monoclonal antibodies that detect cell-surface proteins present on primed and naive human hESCs and hiPSCs [2]. This confirms our previous prediction that these proteins were present on the cell surface of hPSCs [3]. Our predictions were based on a microarray-based gene signature of spontaneously differentiating hPSCs. These hPSCs were rapidly losing cell-surface immunoreactivity to 2 cell-surface markers (GCTM-2 and CD-9) previously shown to be associated with human pluripotency. Bioinformatic analysis of the gene signature allowed us to identify 88 known cell-surface proteins, most of which had not been previously associated with undifferentiated hPSCs.

Production of new cell-surface markers for hPSCs

Based on these predictions, we aimed to produce new monoclonal antibodies to protein targets that did not have available antibodies capable of detecting protein epitopes on live hPSCs. To achieve this, we first produced recombinant proteins corresponding to the extracellular portions of our target proteins. These recombinant proteins were then used to immunize mice for the production of hybridomas at the Monash Antibody Technologies Facility (**platforms.monash.edu/matf/**). All hybridomas that were demonstrated to bind to the target proteins by antigen microarray were then screened by high-throughput flow cytometry to identify the ones that could bind to the target proteins on hPSCs. Hybridomas that passed this screening were then subcloned and subjected to extensive characterization.

Characterization of new cell-surface markers for hPSCs

Monoclonal antibodies to four predicted proteins have now been produced and characterized. These antibodies detect CUB domain–containing protein 1 (anti-hCDCP1; CSTEM26), platelet F11 receptor (anti-hF11R; CSTEM27), desmoglein-2 (anti-hDSG2; CSTEM28), and cadherin-3 (anti-hCDH3; CSTEM29). Analysis by ELISA demonstrated that the monoclonal antibodies detected only the target cell-surface proteins and did not detect other proteins [2]. Indirect immunofluorescence staining of fixed hPSCs clearly shows that each of these monoclonal antibodies detects cell-surface protein (Figure 1).

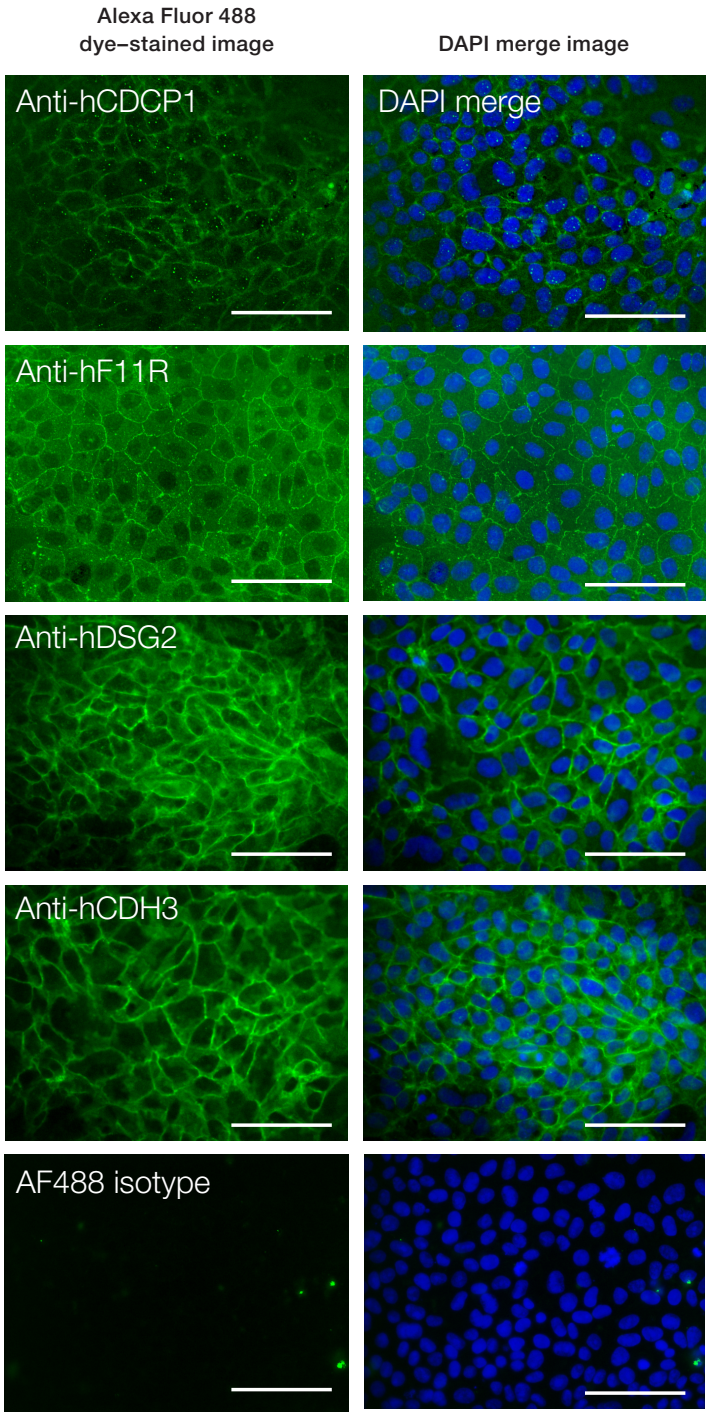

Figure 1. Representative images of undifferentiated MEL1 hESCs. The cells, cultured on a feeder layer of mouse embryonic fibroblasts in hPSC medium for 4–7 days, show surface immunostaining with Invitrogen™ Alexa Fluor™ secondary antibodies) of anti-hCDCP1, anti-hF11R, anti-hDSG2, and anti-hCDH3 antibodies (green) merged with 4′,6-diamidino-2-phenylindole (DAPI)–counterstained nuclei (blue). The images at the bottom show isotype controls. Scale bars = 100 µm.

Each of the four monoclonal antibodies also detects target protein on live hPSCs by flow cytometry (Figure 2). The data show a high correlation by intracellular flow cytometry for the transcription factor Oct-4 (Figure 3) and a high correlation by live-cell flow cytometry for TRA-1-60 and SSEA-4—the hESC markers (Figure 4). This property of the antibodies can be utilized to sort live cells that are highly expressing each of the target proteins using FACS, and replate them to form self-renewing cell colonies that contain high percentages of Oct-4–positive hPSCs [2]. Moreover, using FACS or MACS with these monoclonal antibodies enables the enrichment for hPSCs suitable for transcriptome or differentiation studies.

The published research by O'Brien et al. also showed that the anti-hCDCP1, anti-hF11R, and anti-hDSG2 monoclonal antibodies detected greater than 80% of human naive PSCs (produced using three different methodologies), demonstrating high immunoreactivity; whereas, anti-hCDH3 antibody only detected 50–70% of naive cells depending on how the naive cells were produced [2]. This raises an interesting question of whether there are distinct subpopulations within human naive PSCs that may have different potential.

The anti-hDSG2 (CSTEM28) and anti-hF11R (CSTEM27) monoclonal antibodies were also demonstrated to be useful for detecting rare Oct-4–positive cells in embryonic cell cultures at 7, 14, and 28 days of differentiation [2].

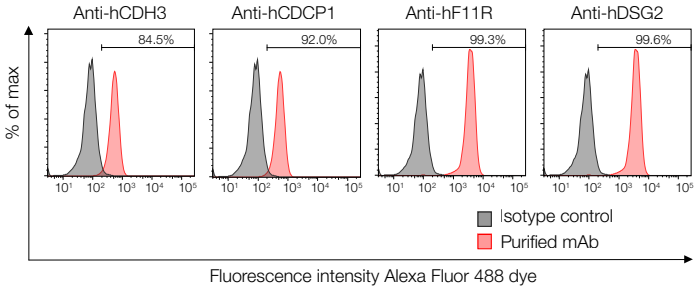

Figure 2. Undifferentiated hPSC cultures immunolabeled with purified mAbs such as anti-hCDCP1, anti-hF11R, anti-hDSG2, and anti-hCDH3. Representative flow cytometry histogram plots show fluorescence detection of protein epitopes corresponding to all mAbs on live cells stained with Alexa Fluor 488 antibody conjugates. A high percent of total hESCs from the WA09 cell line was analyzed.

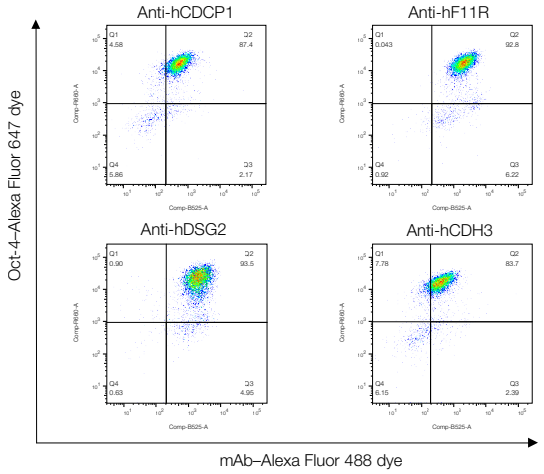

Figure 3. Representative flow cytometry dot plots showing high coexpression of target proteins. The mAbs detected hPSC-surface proteins (Alexa Fluor 488 dye) and Oct-4 (Invitrogen™ Alexa Fluor™ 647 dye) against isotype controls following sequential live- and fixed-cell immunolabeling of WA09 hESCs.

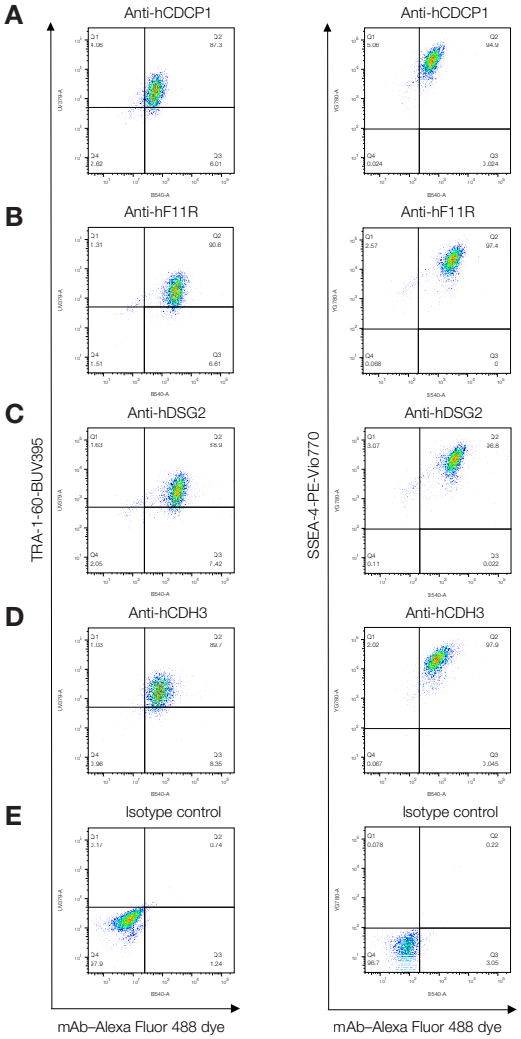

Figure 4. Representative multicolor immunostaining and flow cytometry analysis of live cells co-detected by mAbs. Live WA09 hESCs were co-detected by **(A)** anti-hCDCP1, **(B)** anti-hF11R, **(C)** anti-hDSG2, and **(D)** anti-hCDH3 mAbs with pluripotency-associated antibodies such as TRA-1-60 (left panel) or SSEA-4 (right panel), compared with **(E)** isotype controls.

Direct coupling of anti-CDCP1, anti-F11R, or anti-CDH3 monoclonal antibodies to the Alexa Fluor 488 fluorophore or phycoerythrin (PE) allowed for the detection of the majority of TRA-1-60–positive cells from a mixture of human iPSCs and the murine C2C12 cell line (Figure 5). This experiment clearly demonstrates the ability of these 3 antibodies to detect hPSCs in mixed cell populations.

Non-pluripotency–associated uses of the new monoclonal antibodies

Three of the pluripotency-associated antibodies were also able to detect antigen expression on human mammary epithelial and stromal subpopulations of cells by flow cytometry. Specifically, immunoreactivity of anti-hCDCP1, anti-hF11R, and anti-hDSG2 monoclonal antibodies was seen on human mammary epithelial luminal progenitor cells and on mature luminal cells [2]. This finding suggests that these antigens may be used as possible biomarkers for breast cancer.

Finally, downregulation of DSG2 protein was demonstrated in cancerous intestinal epithelial cell populations from three donors when compared to the corresponding normal tissues from the same donors—raising the intriguing possibility of use of this antibody as a cancer-associated biomarker [2].

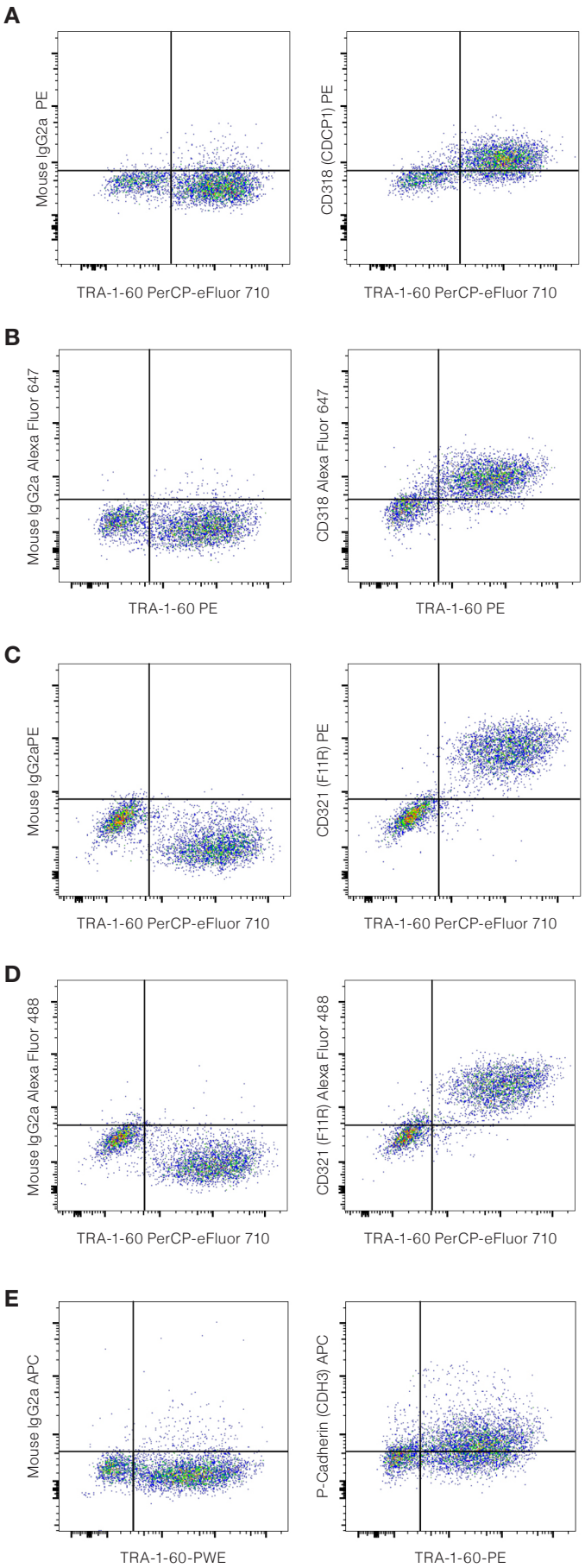

Figure 5. Detection of TRA-1-60–positive cells from a mixture of human iPSCs and murine C2C12 cells. The cell mixture was stained with **(A)** Invitrogen™ eBioscience™ Anti-Human TRA-1-60 (Podocalyxin) PerCP-eFluor™ 710 antibody, and 0.25 µg Invitrogen™ eBioscience™ Mouse IgG2a K Isotype Control PE (left) or Anti-Human CD318 (CDCP1) PE antibody (right); **(B)** Anti-Human TRA-1-60 (Podocalyxin) PE, and 0.125 µg Invitrogen™ eBioscience™ Mouse IgG2a K Isotype Control Alexa Fluor™ 647 (left) or Anti-Human CD318 (CDCP1) Alexa Fluor 647 antibody (right); **(C)** Anti-Human TRA-1-60 (Podocalyxin) PerCP-eFluor 710 antibody, and 0.125 µg Mouse IgG2a K Isotype Control PE (left) or Invitrogen™ eBioscience™ Anti-Human CD321 (F11R) PE antibody (right); **(D)** Anti-Human TRA-1-60 (Podocalyxin) PerCP-eFluor 710 antibody, and 0.25 µg Invitrogen™ eBioscience™ Mouse IgG2a K Isotype Control Alexa Fluor™ 488 (left) or Invitrogen™ eBioscience™ Anti-Human CD321 (F11R) Alexa Fluor 488 antibody (right); and **(E)** Anti-Human TRA-1-60 (Podocalyxin) PE, and 0.25 µg Invitrogen™ eBioscience™ Mouse IgG2a K Isotype Control APC (left) or Anti-Human P-Cadherin (CDH3) APC antibody (right).

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Conclusion

In summary, we have described the prediction, production, characterization, and use of four new monoclonal antibodies. These antibodies are composed of unique clones exclusively available through Thermo Fisher Scientific and have recently been published as pluripotent stem cell markers by an international collaborative team [2]. The antibodies are validated* on live hPSCs by flow cytometry and are monoclonal IgGs detecting known cell-surface proteins on live cells. The genes encoding the proteins detected by the antibodies are known, and the monoclonal antibodies detect both primed and naive hPSCs. These antibodies can serve as effective tools for purifying and selecting subsets of stem cell populations for enriching hPSCs before they differentiate, and help enable quantitative comparison of various hPSC lines. Notably, these antibodies also detect subsets of breast and colon tissues and may be applicable as potential cancer biomarkers.

References

1. Laslett AL, Filipczyk A, and Pera MF (2003) Characterization and culture of human embryonic stem cells. *Trends in Cardiovas Med* 13:295–301.
2. O'Brien CM, Chy H, Zhou Q et al. (2017) New monoclonal antibodies to defined cell surface proteins on human pluripotent stem cells. *Stem Cells* 35:626–640.
3. Kolle G, Ho M, Zhou Q et al. (2009) Identification of human embryonic stem cell surface markers by combined membrane-polysome translation state array analysis and immunotranscriptional profiling. *Stem Cells* 27:2446–2456.

Ordering information

Clone	Target	Format	Quantity	Cat. No.
CSTEM26	CD318 (CDCP1)	Unconjugated	25 µg	14-3189-80
			100 µg	14-3189-82
		PE-conjugated	25 tests	12-3189-41
			100 tests	12-3189-42
		Alexa Fluor 647 dye–conjugated	25 tests	51-3189-41
			100 tests	51-3189-42
CSTEM27	CD321 (F11R)	Unconjugated	25 µg	14-3219-80
			100 µg	14-3219-82
		PE-conjugated	25 tests	12-3219-41
			100 tests	12-3219-42
		Alexa Fluor 488 dye–conjugated	25 tests	53-3219-41
			100 tests	53-3219-42
CSTEM29	P-cadherin	Unconjugated	25 µg	14-2237-80
			100 µg	14-2237-82
		APC-conjugated	25 tests	17-2237-41
			100 tests	17-2237-42
CSTEM28	Desmoglein-2	Unconjugated	25 µg	14-9159-80
			100 µg	14-9159-82
		PE-conjugated	25 tests	12-9159-41
			100 tests	12-9159-42
		Alexa Fluor 488 dye–conjugated	25 µg	53-9159-80
			100 µg	53-9159-82

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* The use or any variation of the word "validation" refers only to research use antibodies that were subject to functional testing to confirm that the antibody can be used with the research techniques indicated. It does not ensure that the product(s) was validated for clinical or diagnostic uses.