Cell Surface Marker Expression Analysis to Track the Progression of Fibroblast Reprogramming.

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ABSTRACT

Pluripotent stem cells are appealing tools for drug discovery and in regenerative medicine. The process of somatic cell reprogramming is a three-week long process, and successful outcome is measured by the emergence of colonies positive for pluripotent markers. Methods to analyze reprogramming intermediates provide a powerful tool to track reprogramming and predict the potential successful outcome of the process at an early stage.

We had earlier reported the utilization of global gene expression analysis of fibroblasts, reprogramming intermediates, and fully reprogrammed cells to identify CD44 as a negative marker of pluripotent cells. Further, depletion of CD44 cells during the second week of reprogramming eliminated unreprogrammed and partially reprogrammed fibroblasts, thereby producing cleaner reprogramming cultures. In this study, CD44 was used in combination with the pluripotent marker SSEA4 to measure reprogramming kinetics. Fibroblasts from different reprogramming mediated transduced by either CytoTune® and or CytoTune™-iPS 2.0 Sendai Reprogramming kits showed distinct patterns of expression of the two markers with during reprogramming progression. The proportion of cells that were CD44 negative and SSEA4 positive at 7-8 days of reprogramming correlated with reprogramming efficiency. Analysis of CD44 and positive pluripotent markers thus enables the definitive identification, monitoring, and enrichment of pluripotent stem cells in reprogrammed cultures.

INTRODUCTION

As the field of reprogramming somatic cells into Induced Pluripotent Stem Cells (iPSC) evolves and becomes more accessible and more accepted in the various scientific fields, there still remains the need for cellular characterization tools to assist in identifying and qualifying the emerging iPSC. We previously identified novel pluripotency tools, like AP Live Stain[1] , which can be utilized in the early detection and selection process during reprogramming. During the subsequent usage and analyses of iPSC derived using AP Live stain we performed whole transcriptome analysis of parental somatic cells, established and early stage iPSC, and hESC and found various cell surface markers that showed differential expression in these cell types. One such marker , the cell adhesion protein CD44, was identified and subsequently qualified as a cell surface marker that is highly expressed in human fibroblasts and partially reprogrammed cells, and not in hESC and hiPSC[2]. This negative marker, in conjunction with other early pluripotency markers, such as SSEA4 can be used in identifying truly reprogrammed colonies through bioimaging. In this study we utilize this 2 differential marker approach , using CD44 and SSEA4, to elucidate the kinetic events during the somatic reprogramming process

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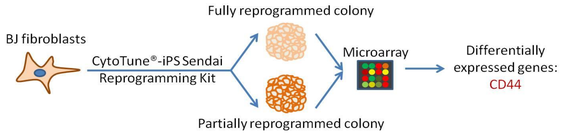
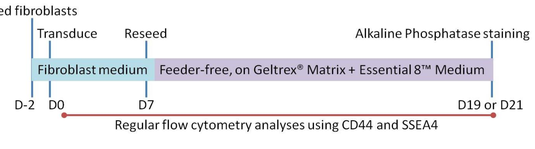
METHODS

Identification of differentially expressed biomarkers



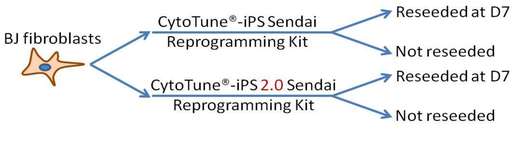
Fibroblasts were reprogrammed using CytoTune®-iPS Sendai Reprogramming Kits according to the recommended protocol. Clones were selected , characterized and assessed for biomarker expression via whole genome microarray analyses.

General reprogramming protocol



Reprogramming of fibroblasts was carried out using the CytoTune®-iPS Sendai Reprogramming Kits according to the recommended protocol for feeder-free conditions, then assessed using flow cytometry or Alkaline Phosphatase staining.

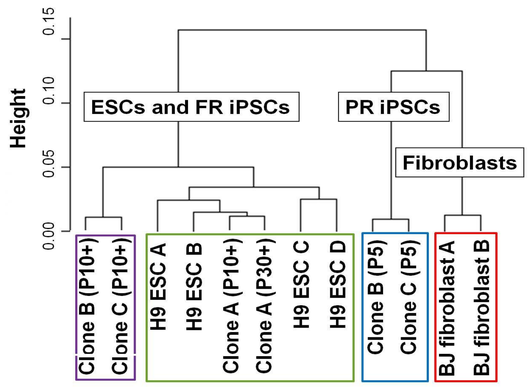
Using SSEA4 and CD44 to compare reprogramming kinetics



BJ and DF1 fibroblasts were reprogrammed using two versions of the CytoTune®-iPS Sendai Reprogramming Kit, with or without reseeding at Day 7. The impact of each parameter on the reprogramming kinetics was assessed by quantifying the SSEA4⁺ CD44⁻ population.

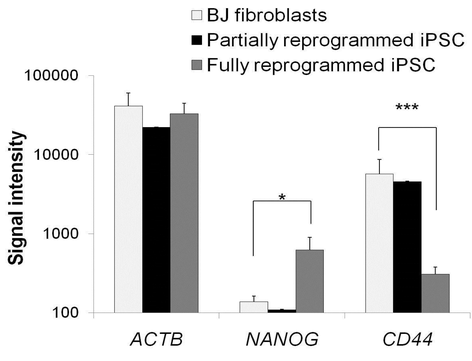
RESULTS

Figure 1. Microarray analysis distinguishes fully and partially reprogrammed clones



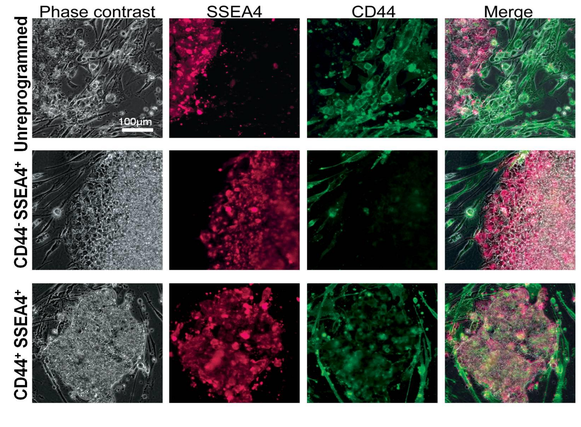
P5 clones (blue box) cluster away from embryonic stem cells (ESCs) (green box), and closer to BJ fibroblasts (red box). The same clones at passage 10+ (purple box) cluster with ESCs and away from BJ fibroblasts. Thus, the P5 clones were designated as partially reprogrammed (PR) while the P10+ clones were designated as fully reprogrammed (FR).

Figure 2. CD44 is differentially expressed during reprogramming



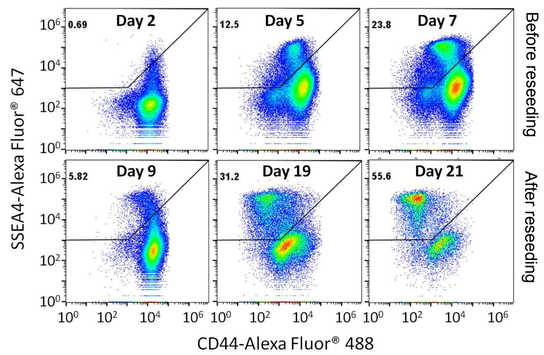
According to the microarray data, there were 135 potential negative PSC surface markers. CD44 has the second highest fold change within that list. The PSC marker *NANOG* is expressed in FR iPSCs, but not in BJ fibroblasts and PR clones. In contrast, *CD44* is expressed highly in BJ fibroblasts and PR clones, but not in FR iPSCs.

Figure 3. Double staining with CD44 and SSEA4 reveals three staining patterns



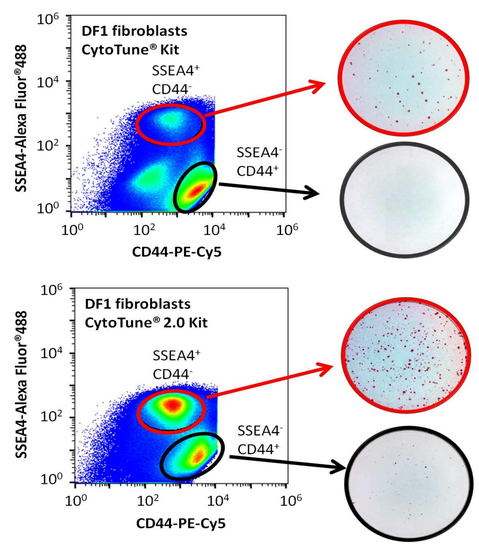
SSEA4 is a positive PSC marker which fails to differentiate between fully and partially reprogrammed cells. Double staining with CD44 and SSEA4 distinguishes SSEA4⁻ CD44⁺ ureprogrammed cells, SSEA4⁺ CD44⁻ reprogrammed colonies, and SSEA4⁺ CD44⁺ colonies. Therefore, CD44 enables the definite identification of fully reprogrammed iPSCs.

Figure 4. Flow cytometry with CD44 and SSEA4 tracks reprogramming kinetics



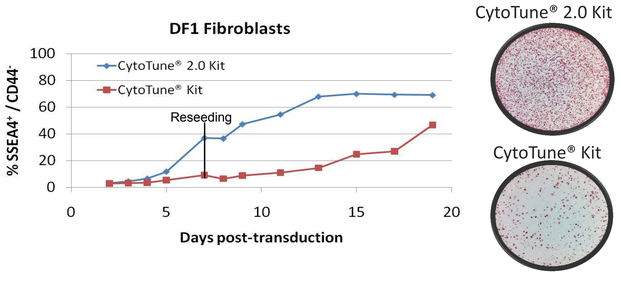
CD44 is gradually lost during reprogramming. At Day 2, there is a large population of SSEA4⁻ CD44⁺ cells and a small population of SSEA4⁺ CD44⁺ cells that becomes more distinct at Day 5. At Day 7, the double-positive population transitions towards the SSEA4⁺ CD44⁻ state. By Day 21, a large percentage of the cells in culture are already SSEA4⁺ CD44⁻. This time course shows that flow cytometry with CD44 and SSEA4 can be used to monitor reprogramming kinetics.

Figure 5. SSEA4⁺ CD44⁻ cells represent reprogrammed cells



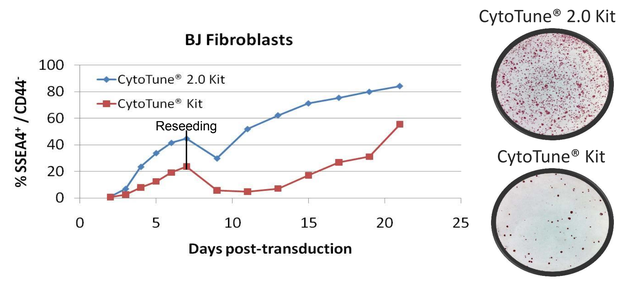
Cell populations present at Day 8 were sorted, plated and stained for Alkaline Phosphatase at Day 21. With both kits, the SSEA4⁺ CD44⁻ cells (red circle) form Alkaline Phosphatase positive iPSC colonies while the SSEA4⁻ CD44⁺ cells (black circle) do not, implying that they represent reprogrammed and unreprogrammed populations, respectively.

Figure 6. Percentage of SSEA4⁺ CD44⁻ cells predicts reprogramming efficiency



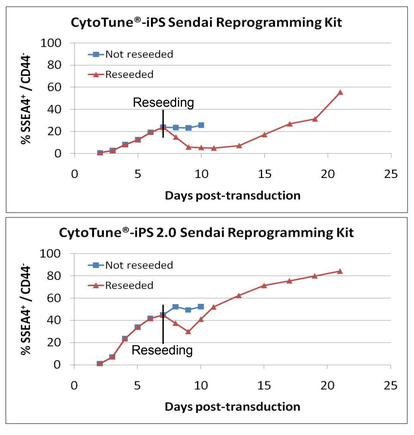
Reprogramming DF1 fibroblasts with the CytoTune® 2.0 Kit generates SSEA4⁺ CD44⁻ cells at a higher rate than its predecessor. Early quantification of this population can predict reprogramming efficiency, as shown by the higher number of Alkaline Phosphatase-expressing colonies produced by both kits by Day 19.

Figure 7. Analysis with CD44/SSEA4 confirms that the CytoTune® 2.0 Kit reprograms faster



Reprogramming of BJ fibroblasts confirms the observation with DF1 fibroblasts that the CytoTune® 2.0 Kit generates SSEA4⁺ CD44⁻ cells at a higher rate than its predecessor. Unlike with DF1 fibroblasts, reprogramming of BJ fibroblasts is clearly affected by reseeding at Day 7.

Figure 8. Analysis with CD44/SSEA4 elucidates the effect of reseeding at Day 7



When reprogramming BJ fibroblasts with the CytoTune® Kits, the SSEA4⁺ CD44⁻ population plateaus after Day 7 without reseeding. Reseeding at Day 7 temporarily sets back reprogramming but allows the SSEA4⁺ CD44⁻ population increase much further.

CONCLUSIONS

- CD44 is expressed in fibroblasts and partially reprogrammed clones, but not in fully reprogrammed iPSCs.
- Double staining with CD44 and SSEA4 differentiates between unreprogrammed, and partially and fully reprogrammed cells.
- The percentage of SSEA4⁺ CD44⁻ cells in a reprogramming culture can be used to predict the speed and efficiency of reprogramming.
- The gradual downregulation of CD44 as SSEA4 increases can allows us to monitor and compare the progression of the reprogramming process.
  - The CytoTune-®-iPS 2.0 Sendai Reprogramming Kit reprograms BJ and DF1 fibroblasts faster and more efficiently than its predecessor.
  - Reseeding has a different impact when reprogramming BJ fibroblasts and DF1 fibroblasts.
  - When using BJ fibroblasts, reprogramming plateaus around Day 7 unless cells are reseeded.

FUTURE PLANS

CD44 has been shown to be highly expressed in CD34⁺ lymphocytes, which are commonly used for reprogramming. Future experiments will explore CD44 as a negative iPSC marker when reprogramming from blood.

REFERENCES

1. Singh et al, Stem Cell Rev and Rep (2012) 8:1021–1029
2. Quintanilla, et al. (2014) **PLoS ONE** 9(1): e85419

TRADEMARKS/LICENSING