Epigenetic Changes Correlate with Loss of Pluripotency Marker Expression During Retinoic Acid-induced Differentiation of NTERA-2 Cells

Antony W. Wood , Susan Kane, Christopher J. Fry, Raffi Manoukian, and Curtis D. Desilets

Abstract

Identifying and investigating the molecular and biochemical events associated with changes in cell fate, such as those that occur during stem cell differentiation or somatic cell reprogramming, are fundamental goals in stem cell biology research. Achieving these objectives requires the development of highly specific and sensitive reagents that have been validated in a contextually appropriate manner. At Cell Signaling Technology, we are developing a portfolio of sensitive, rigorously validated antibody reagents designed to investigate the epigenetic, molecular, and biochemical events that underlie changes in cell fate. Here, we use retinoic acid (RA)-induced differentiation of NTERA-2 cells as a model to demonstrate the utility of our antibody reagents for interrogating the epigenetic and biochemical events associated with induced neuronal differentiation. Using chromatin immunoprecipitation, fluorescent immunocytochemistry and intracellular flow cytometry, we are able to demonstrate the predicted correlation between epigenetic changes at distinct genomic loci with changes in the expression of key pluripotency factors during directed differentiation. These data confirm the utility of these antibody reagents, employed across multiple technology platforms, for investigating the molecular mechanisms underlying cellular differentiation.

Materials and Methods

Cell Culture:

NTERA-2 embryonal carcinoma cells were treated with all-trans retinoic acid (RA) for 0–14 days to induce neuronal differentiation as previously described (1). Cells were collected for analysis at multiple time points representing different stages of neuronal differentiation.

Fluorescent Immunocytochemistry:

Cells were fixed for 15 min in 4% formaldehyde, permeabilized, blocked for 1 hr, then incubated overnight in primary antibody at 4°C. After washing, cells were incubated in secondary antibody for 1-2 hr at RT in the dark. Stained cells were imaged by laser scanning confocal microscopy.

Flow Cytometry:

Cells were harvested, fixed, permeabilized, and processed according to the standard CST flow cytometry protocol. In brief, cell pellets were resuspended in 4% formaldehyde (methanol-free) for 10 min at 37°C, followed by addition of ice cold 90% methanol for at least 30 min on ice. Cells were then immunostained for 1 hr at RT by suspension in an antibody cocktail containing Oct-4A Alexa Fluor® 488, Sox2 Alexa Fluor® 647, or Nanog Alexa Fluor® 594. After washing 2x with PBS/0.5% BSA, cells were analyzed on a 4 laser Galios™ flow cytometer (Beckman Coulter).

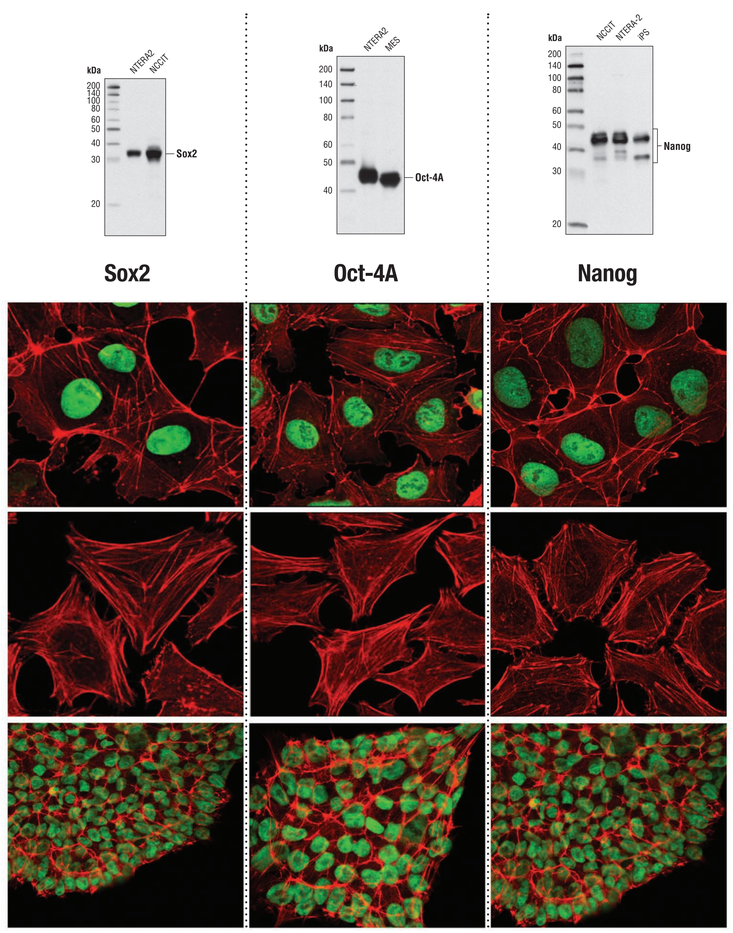
Chromatin Immunoprecipitation:

Chromatin Immunoprecipitations were performed with cross-linked chromatin from NTERA-2 cells using the indicated antibodies, or 2 µl of Normal Rabbit IgG #2729, using the SimpleChIP® Enzymatic Chromatin IP Kit (Magnetic Beads) #9003. The enriched DNA was quantified by real-time PCR using gene-specific primers. The amount of immunoprecipitated DNA in each sample is represented as a percentage of the total input chromatin.

Reference

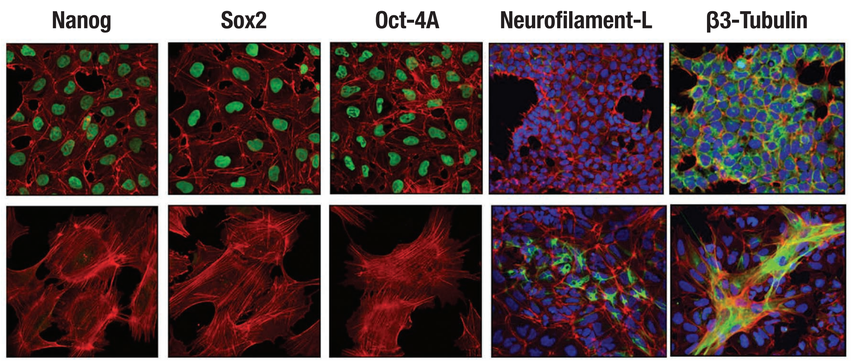
1. Lee, V.M. and Andrews, P.W. (1986) Differentiation of NTERA-2 clonal human embryonal carcinoma cells into neurons involves the induction of all three neurofilament proteins. *J. Neurosci.* 6, 514–521.

Antibodies validated for target specificity



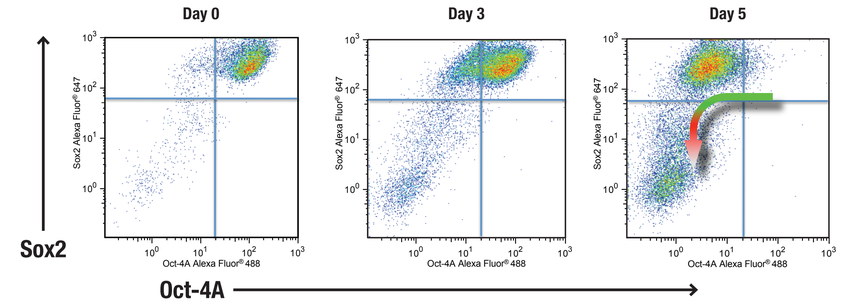
Confocal IF analysis of NTERA-2 cells (upper), HeLa cells (middle) and human iPS cells (lower) using antibodies against core pluripotency factors. Actin filaments have been labeled with DY-554 phalloidin (red). Blue pseudocolor = DRAQ5® #4084.

Immunocytochemical analysis shows reduction of pluripotency factor expression during differentiation

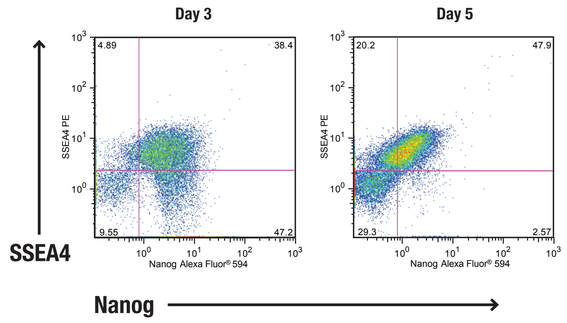


Confocal IF analysis of NTERA-2 cells, untreated (upper panels) or RA-treated (10 µM all-trans RA for 14 days, lower panel), using antibodies against core pluripotency factors, or neuronal markers. Actin filaments have been labeled with DY-554 phalloidin (red). Blue pseudocolor = DRAQ5® #4084 (fluorescent DNA dye). Note the loss of pluripotency transcription factor expression (green) as cells undergo neuronal differentiation.

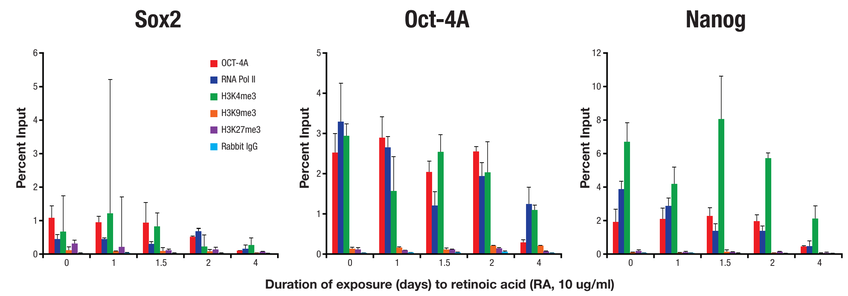
Flow Cytometric analysis shows reduction of pluripotency factor expression during differentiation



Flow cytometric analysis showing changes in pluripotency factor expression during RA-induced neuronal differentiation of NTERA-2 cells. **Upper panels:** Bivariate scatter plots showing expression of Sox2 (y-axis) and Oct-4A (x-axis) after 0, 3, and 5 days exposure to RA. **Lower panels:** Bivariate scatter plots showing expression of SSEA4 (y-axis) and Nanog (x-axis) after 3 and 5 days treatment with RA. Results demonstrate a time-dependent decline in the expression of pluripotency factors and an embryonic marker protein (SSEA4), detectable as early as 3 days after exposure to RA .



ChIP analyses show loss of epigenetic marks of active transcription at pluripotency loci during differentiation



Chromatin Immunoprecipitation analysis of selected epigenetic marks and transcription factors in NTERA-2 cells during RA-induced neuronal differentiation. Data indicate relative binding to indicated loci in untreated cells (Day 0) and after treatment with RA for the indicated durations. Data are normalized to total Histone H3 signal. Data are means +/- SD of 3 experiments.

Contact Information

Antony W. Wood, Ph.D.
Cell Signaling Technology | 3 Trask Lane, Danvers, MA 01923
email: antony.wood@cellsignal.com
www.cellsignal.com

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