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Review Article

Expanding applications of protein analysis using proximity ligation and qPCR ☆

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ABSTRACT

The correlation of gene and protein expression changes in biological systems has been hampered by the need for separate sample handling and analysis platforms for nucleic acids and proteins. In contrast to the simple, rapid, and flexible workflow of quantitative PCR (qPCR) methods, which enable characterization of several classes of nucleic acid biomarkers (i.e. DNA, mRNA, and microRNAs), protein analysis methods such as Western blotting are cumbersome, laborious, and much less quantitative. However, TaqMan® Protein Assays, which use the proximity ligation assay (PLA™) technology, now expand the range of qPCR applications to include the direct detection of proteins through the amplification of a surrogate DNA template after antibody binding. Here we describe an integrated qPCR approach for measuring relative changes in gene and protein expression from the same starting sample and on a single analytical platform that pairs TaqMan® Gene Expression (GEx) Assays with TaqMan® Protein Assays. We have monitored the changes in mRNA, microRNA, and protein expression of relevant biomarkers in the pluripotent human embryonal carcinoma cell line, NTERA2, upon differentiation to neuronal cells. In addition, TaqMan® Protein Assays have been used to monitor protein expression in induced pluripotent stem cells (iPSC) that have been reprogrammed from human somatic cells. The data presented establishes a general paradigm utilizing real-time PCR instruments and reagents for studying the relationship between the stem cell transcriptome and proteome.

1. Introduction

Human embryonic stem (ES) cells can be propagated indefinitely in culture, but will differentiate into major cell lineages under specific culture conditions. NTERA2, a pluripotent human embryonal carcinoma cell line, can be induced to differentiate along neuroectodermal lineages upon exposure to retinoic acid (RA) [1]. Pluripotent cells can also be derived from somatic cells that have been reprogrammed. These induced pluripotent stem (iPS) cells have many of the same characteristics as ES cells, including the ability to differentiate into various cell lineages [2–4]. Understanding maintenance of pluripotency and commitment to cell differentiation on a molecular level is crucial to understanding developmental processes of pluripotent cells. The OCT4, NANOG, SOX2, and LIN28 proteins play critical roles in establishing or maintaining pluripotency in human ES cells. OCT4, NANOG, SOX2, and LIN28 are highly expressed in undifferentiated ES cells and can be used as markers for pluripotency [5].

mRNA transcripts often serve as the biomarker type of choice for assessing, monitoring, and/or determining the state of a cell. This is largely due to the relative ease with which such markers can be analyzed. However, it still remains to be proven that transcript profiles faithfully reflect the cellular proteome and the presence and relative abundance of their encoded proteins. TaqMan® Protein Assays enable an integrated qPCR approach for measuring relative changes in gene and protein expression from the same starting sample and on a single analytical platform that pairs TaqMan® Gene Expression Assays with TaqMan® Protein Assays. To validate this approach, we used NTERA2 cells as a model system and determined both mRNA and protein expression profiles and fold changes throughout the time course of induction for four stem cell pluripotency markers and two differentiation markers, NCAM1 and ALCAM. In addition, we used TaqMan® Protein Assays to monitor gene transfer success in somatic cells that have been transduced with several of the reprogramming genes (OCT4, SOX2, and KLF4) that are required to generate induced pluripotent stem cells (iPSC) [2–4].

TaqMan® Protein Assays are an adapted form of PLA™, a proximity ligation assay technology invented by Ulf Landegren, Simon Fredriksson, and colleagues, that combines antibody–protein binding with real-time PCR-based detection of the reporter nucleic acid sequence [6,7]. The three basic steps of the assay are shown in Fig. 1. The first step involves binding of a protein target by paired

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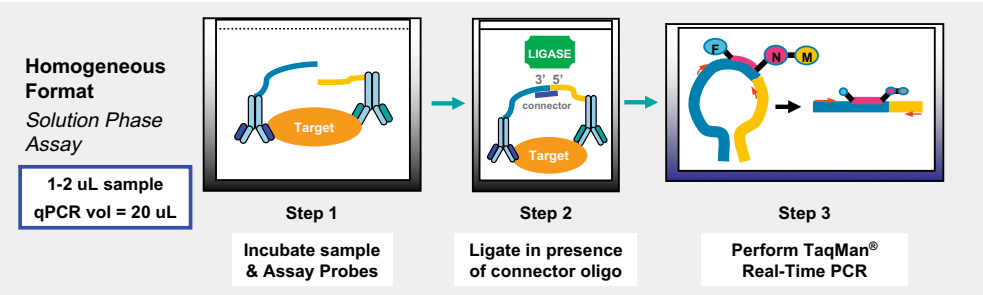

Fig. 1. The TaqMan® Protein Assay is a 3-step homogeneous process that involves (1) binding of paired antibody–oligonucleotide probes to a protein target in whole cell lysates, (2) templated ligation of the oligonucleotides in proximity, and (3) qPCR detection. No wash step are required and results are obtained within 3.5 h.

assay probes. The assay probes are target-specific antibodies conjugated to oligonucleotides through a biotin–streptavidin linkage. Each oligonucleotide in the probe pair presents a 5′ or 3′ end; these ends are brought into proximity when the antibody components of the assay probe pair bind to two different epitopes on the target protein. The second step involves ligation of the oligonucleotides by DNA ligase. The substrate for the ligase is a bridge structure formed by hybridization of a third oligonucleotide complementary to the oligonucleotide ends of the assay probe pair. This structure forms preferentially when the assay probes are in proximity to each other. Subsequent protease treatment inactivates the ligase. The final step involves amplification and detection by real-time PCR with a TaqMan® probe. The ligation product serves as a template in the TaqMan® real-time PCR assay.

2. Materials and methods

2.1. NTERA2 retinoic acid induction

NTERA2 cells (4×10^6) were cultured in T75 flasks in the presence or absence (untreated control) of 10 μM trans-retinoic acid (RA) for 28 days. Cells were harvested by scraping at multiple time points and analyzed for protein and gene expression. After 28 days, the cells were harvested and replated on PDL/laminin coated plates (BD Biosciences) in the presence of 1 μM cytosine D-arabinofuranoside and 10 μM uridine (both from Sigma–Aldrich) for 21 days at which time the RA-treated cells were harvested for protein and gene expression analysis. Only the treated cells survived the replating procedure and showed morphological changes indicative of neuronal differentiation.

2.2. NTERA2 sample preparation

NTERA2 cell lysates were prepared with Protein Expression Sample Prep Kit (Applied Biosystems) and assayed directly for protein. In parallel, crude cell lysates were processed for RNA with the PARIS™ kit (Applied Biosystems) and subsequently treated with TURBO DNA-*free*™ DNAse (Applied Biosystems). The PARIS™ mirVana kit (Applied Biosystems) was used for mircoRNA purification.

2.3. Fibroblast transduction and sample preparation

Baculovirus particles (1×10^7) containing a polycistronic vector expressing OCT4, SOX2, KLF4, and tagRFP (Invitrogen) was transduced onto 1×10^6 human dermal fibroblasts. Cells were harvested 24 h and 4 days post-transduction and cell lysates were prepared using the Protein Expression Sample Prep Kit (Applied Biosystems).

2.4. TaqMan® Protein Assays and reagent kits

All TaqMan® Protein Assays and associated reagents kits were obtained from Applied Biosystems. TaqMan® Protein Assays with NTERA2 cell lysates were carried out using cell lysate dilutions of 500, 125, 32, and 0 cells per reaction. TaqMan® Protein Assays with transduced fibroblast cell lysates were carried out using 500 and 0 cells per reaction.

2.5. TaqMan® Gene Expression Assays and reagent kits

mRNA assays and One-Step RT–qPCR reagents were obtained from Applied Biosystems. Gene expression Assays were performed according to the manufacturer's instructions. No reverse transcription (RT) controls verified the removal of genomic DNA after DNAse treatment. microRNA assays and Multiplex RT and qPCR reagents were obtained from Applied Biosystems.

2.6. Real-time PCR System and data analysis

qPCR assays were performed on a StepOnePlus™ Real-Time PCR System (Applied Biosystems). C_T values were determined from ampli-

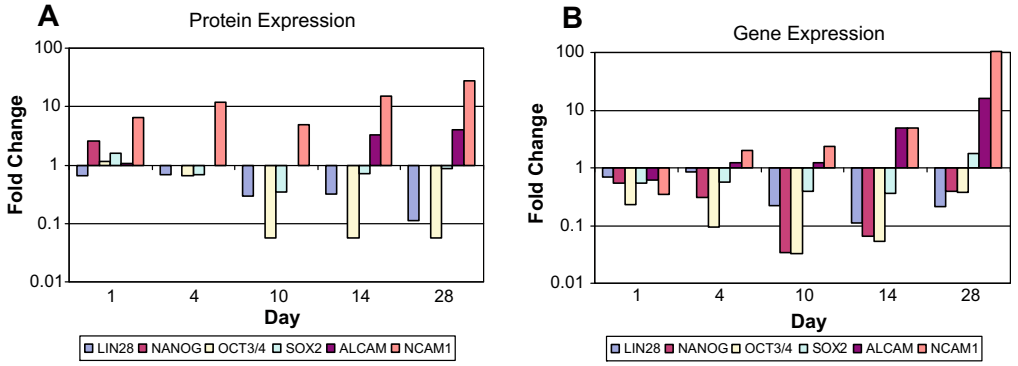

Fig. 2. Relative quantification was monitored for 6 proteins (A) and their corresponding mRNA (B) (4 pluripotency and 2 differentiation markers [NCAM1, ALCAM]) during RA induction of NTERA2 cells. The relative abundance of proteins and mRNA was measured with TaqMan® Protein Assays and TaqMan® Gene Expression Assays, respectively. Expression changes are relative to day 0 (untreated).

fication plots using a threshold of 0.2. Protein expression fold changes between untreated and treated samples were determined by first calculating ΔC_Ts (C_T for cell input minus C_T for no cell input) for each lysate dilution point and for each protein target. ΔC_Ts were then plotted vs. cell input per assay reaction. The slopes from the resulting plots were used to determine fold changes. All fold changes (up and down) are relative to day 0 (untreated cells). Gene expression fold changes between untreated and treated samples were determined by first calculating ΔC_Ts (C_T for each target minus C_T for ACTB [mRNA], RNU48 [miRNA]) for each sample. Gene expression changes were calculated relative to day 0 using the $\Delta\Delta C_T$ method [8].

3. Results

3.1. Monitoring protein, mRNA and microRNA expression levels during stem cell differentiation using TaqMan based Protein Assays, Gene Expression Assays, MicroRNA Assays and qPCR

TaqMan® Protein Assays were used to monitor protein levels during the course of RA treatment of NTERA2 cells while the mRNA and microRNA levels were monitored using TaqMan Gene Expression Assays and MicroRNA Assays, respectively. During the course of RA treatment, the stem cell protein markers NANOG, OCT4, LIN28 and SOX2 all show decreased expression in NTERA2 (Fig. 2A). NANOG is no longer detectable by day 4. LIN28 and OCT4 continue to be expressed, but at progressively lower levels. SOX2 protein levels decrease somewhat during the course of treatment, but recover to near normal levels by day 28. The differentiation markers NCAM1 and ALCAM both exhibit increased expression during the time course, with NCAM1 exhibiting a greater and earlier increased expression than ALCAM.

For the mRNA assays (Fig. 2B) the relative levels of NANOG, OCT4, and LIN28 mRNA are all decreased by day 10 of the RA induction time course. Levels of NANOG and OCT4 mRNA are noticeably reduced by day 4, while SOX2 mRNA levels are somewhat reduced during the course of treatment but recover by day 28. In contrast, levels of NCAM1 and ALCAM increase throughout the induction period, with maximal expression at day 28.

Relative expression changes ($\Delta\Delta C_T$s) for a panel of microRNAs (treated cells minus untreated, day 0) are shown for NTERA2 cells after 5 days of treatment (treated cells, day 5 minus untreated cells, day 0) (Fig. 3). Negative values denote a decrease in expression, while positive values denote an increase in expression. The temporal changes in expression of specific mature microRNAs such as the Let-7 series during RA induction correlate well with temporal changes in LIN28 expression, supporting the link between LIN28 and microRNA-mediated cellular differentiation.

3.1. Monitoring protein expression after viral transduction

Human dermal fibroblasts were transduced with a vector expressing OCT4, SOX2, KLF4, and RFP (red fluorescent protein) (Fig. 4). RFP expression can be observed 24 h post-transduction, but cannot be observed 4 days post-transduction. In contrast, OCT4, SOX2, and KLF4 protein expression was observed 24 h and 4 days post-transduction using TaqMan® Protein Assays.

4. Summary

TaqMan® Protein Assays were used to determine the relative expression of six target proteins in untreated and RA-treated NTERA2 cells. As expected, the levels of OCT4, NANOG, LIN28, and to a lesser extent, SOX2, declined or became undetectable in RA-induced cells as they became differentiated. In contrast, the differentiation markers NCAM1 and ALCAM were detected at much lower levels in untreated NTERA2 cells compared to RA-induced cells. TaqMan® Gene Expression Assays were used to determine the relative expression of the corresponding six target transcripts in untreated and RA-treated NTERA2 cells. As expected, OCT4, NANOG, SOX2, and LIN28 transcripts were detected at high levels in untreated cells and decreased in RA-induced cells. In contrast, transcripts for NCAM1 and ALCAM increased with RA-treatment. Overall, the protein expression profiles correlate with gene expression profiles in terms of timing and direction of changes over the course of RA induction. However, the protein and mRNA expression profiles are not identical, and the differences observed in the protein and mRNA expression profiles demonstrated here could be significant with respect to our understanding of fundamental biological processes.

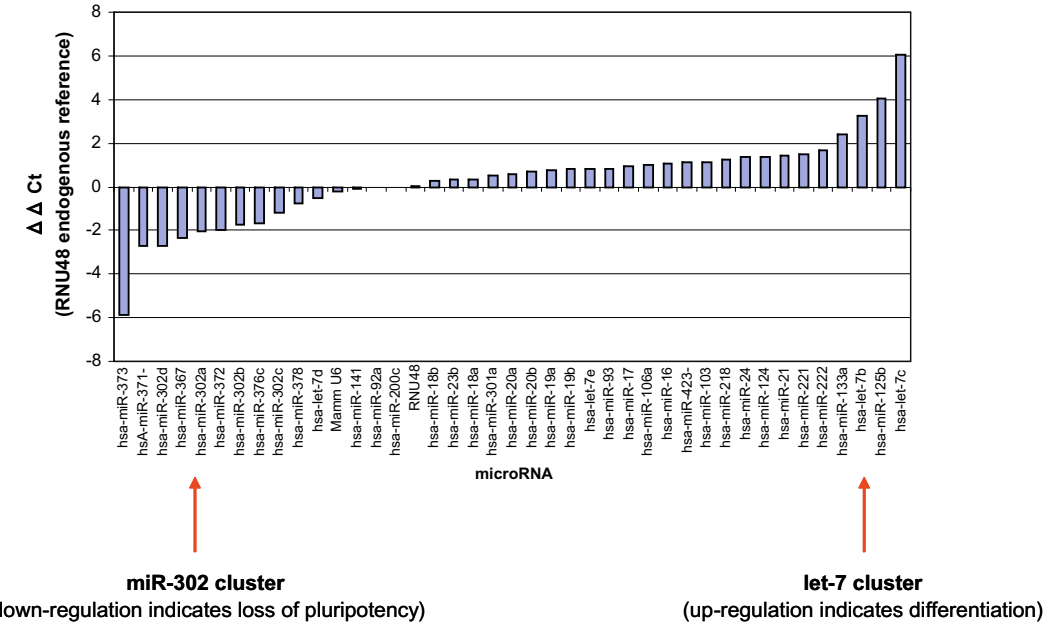

Fig. 3. Relative quantification for a panel of microRNAs (treated cells, day 5 minus untreated, day 0). Negative values denote a decrease in expression in cells at day 5 of treatment relative to untreated cells, while positive values denote an increase in expression.

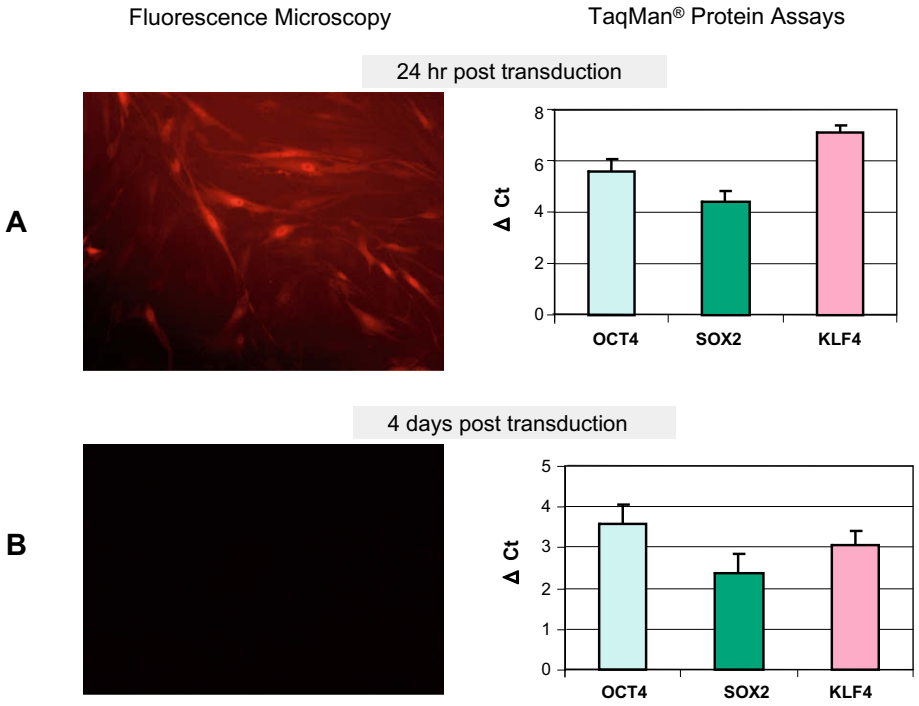

Fig. 4. Baculovirus particles (1×10^7) containing a polycistronic vector expressing OCT4, SOX2, KLF4, and RFP was transduced onto 1×10^6 human dermal fibroblasts. Protein expression was monitored by either fluorescence microscopy (RFP) or TaqMan® Protein Assays (OCT4, SOX2, and KLF4) at either 24 h post-transduction (A) or 4 days post-transduction (B). RFP expression can be visualized after 24 h, but not 4 days post-transduction, whereas OCT4, SOX2, and KLF4 expression can be observed after 24 h and 4 days post-transduction.

TaqMan® Protein Assays were also used to monitor the expression of proteins in cells transduced with a vector expressing OCT4, SOX2, KLF4, and RFP. The TaqMan® Protein Assays proved to be more sensitive and more quantitative than fluorescence microscopy in monitoring protein expression. The TaqMan® Protein Assays could be used to in lieu of more cumbersome and time-consuming techniques such as immunohistochemistry or Western blotting to screen for potential iPS colonies.

TaqMan® Protein Assays provide a simple, rapid, and sensitive method for protein relative quantification in cell lysates. Proteins can typically be detected in reactions containing 500 or less cell equivalents. Compared to typical protein detection methods such as Western blotting, the TaqMan® Protein Assay requires less sample, is more sensitive, and has a simpler and faster workflow. Moreover, the TaqMan® Protein Assay allows users to determine relative protein quantification and enables correlation to mRNA and/or miRNA expression results using the same analytical platform and provides a general paradigm for studying the relationship between the stem cell transcriptome and proteome.

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