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Utilizing FUCCI reporters to understand pluripotent stem cell biology

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ABSTRACT

The fluorescence ubiquitination cell cycle indicator (FUCCI) system provides a powerful method to evaluate cell cycle mechanisms associated with stem cell self-renewal and cell fate specification. By integrating the FUCCI system into human pluripotent stem cells (hPSCs) it is possible to isolate homogeneous fractions of viable cells representative of all cell cycle phases. This method avoids problems associated with traditional tools used for cell cycle analysis such as synchronizing drugs, elutriation and temperature sensitive mutants. Importantly, FUCCI reporters allow cell cycle events in dynamic systems, such as differentiation, to be evaluated. Initial reports on the FUCCI system focused on its strengths in reporting spatio-temporal aspects of cell cycle events in living cells and developmental models. In this report, we describe approaches that broaden the application of FUCCI reporters in PSCs through incorporation of FACS. This approach allows molecular analysis of the cell cycle in stem cell systems that were not previously possible.

1. Introduction

Human and mouse pluripotent stem cells (hPSCs and mPSCs, respectively) have an unusual cell cycle structure comprised of short G1 and G2 gap-phases (Fig. 1). At any point in time >50% of PSCs are in S-phase, primarily because of the short intervening time between replicative and mitotic phases [1]. This structure is considerably different from somatic cells that devote more time to G1-phase and show characteristically slower division rates. During differentiation of PSCs, the cell cycle remodels such that the time taken to transition through G1- and G2-phase increases and the rate of cell division decelerates. This general pattern of cell cycle regulation is also exhibited by peri-implantation stage PSCs *in vivo* [1].

The atypical cell cycle structure of PSCs has attracted intense interest and has led to various models proposing links between cell cycle regulation and maintenance of the pluripotent state. A long-standing idea has been that PSCs retain a short G1-phase that somehow favors self-renewal over exit from pluripotency and, that initiation of PSC differentiation occurs during G1-phase [1,2]. The latter concept is intriguing because it provides a potential link between the cell cycle and fate decisions and would imply that transition through G1-phase is mechanistically linked to initiation of differentiation [1]. Bringing these ideas to a conclusion has been difficult however, due to technical limitations associated with tools previously used for cell cycle analysis (Fig. 2). For example, in our experience conventional approaches such as the use of synchronizing drugs are problematic due to their inherent cytotoxicity and perturbation of cell cycle events. Centrifugal elutriation has also been used to a lesser extent in PSCs [3] and although useful, is hampered by limitations in the resolution of cell cycle phases and because specialist equipment is required. Finally, factor withdrawal to impose a G_0 arrest is ineffective and leads to differentiation of PSCs [4] and, other tools such as temperature-sensitive mutants are not generally available.

In 2008, Miyawaki and colleagues described the first fluorescent-protein based approach to monitor and isolate living cells in different phases of the cell cycle [4]. This system, termed FUCCI for Fluorescent Ubiquitinated-Cell Cycle Indicator, exploits cell cycle-regulated oscillations in the stability of two cell cycle regulated proteins. The first, CDT1, accumulates in G1 phase due to enhanced stability and then as cells enter S-phase it is degraded by ubiquitin-mediated proteolysis. The second protein, GEMININ, accumulates from early S-phase through to M-phase. Periodicity of protein stability is important for the respective functions of CDT1 and GEMININ and in each case, destruction is regulated by a small peptide motif known as a degron that directs cell cycle-dependent proteolysis. The FUCCI system exploits cell cycle regulated degron-sequences by fusing them to one of two fluorescent proteins with non-overlapping excitation and emission wavelengths; Kusabira Orange-2 (KO2) and Azami Green (Az1).

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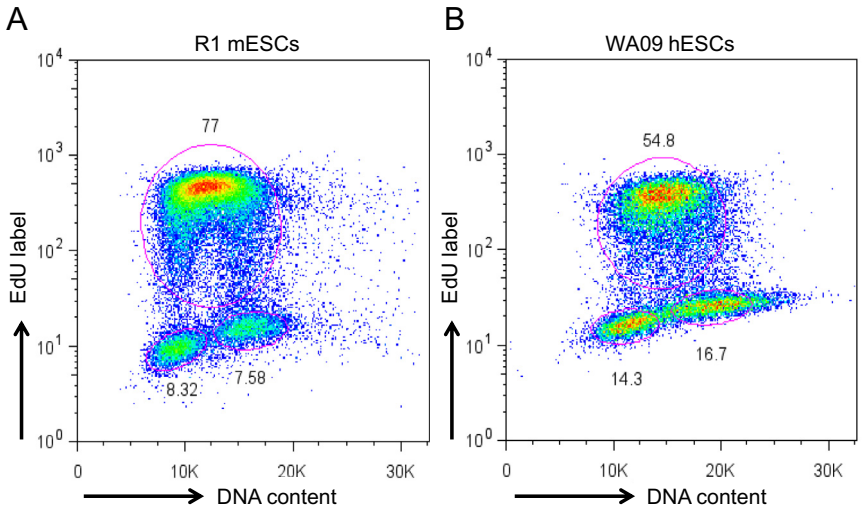

Fig. 1. Cell cycle structure of murine and human ESCs. Two-dimensional cell cycle analysis of (A) murine and (B) human embryonic stem cells (ESCs) where the X-axis indicates DNA content of the cell through propidium iodide staining and the Y-axis indicates cells in S-phase by incorporation of EdU, over a 15 min brief pulse-label period. PSCs typically have short gap phases and proportionally, are heavily enriched in S-phase cells.

The resulting fusion proteins turnover in a cell cycle-dependent manner that mirrors the accumulation of native CDT1 and GEMININ, allowing them to be used as accurate reporters of cell cycle position at the single cell level in living cells (Fig. 3).

The FUCCI system exploits transition of cells through different color states, indicative of cell cycle position. Directly following mitosis (early G1), cells become double negative (DN) for both fluorescent reporters but as they transition through G1, orange fluorescence ($KO2^+$) progressively increases. The transition from DN to $KO2^+$ allows early and late G1 cells to be readily separated. Previously, isolation of cells at different stages of G1 was possible by elutriation, which sorts on the basis of cell volume or, by drug synchronization that separates cells based on post-release time (Fig. 2). Both of these approaches don't specifically result in isolation of single cells at a defined stage of G1 because of the isolation criteria used (e.g. volume, time). The FUCCI system offers a solution to these limitations because it precisely identifies cells functionally, based on the biochemical state of the cell. Upon entry into S phase, KO2 begins to be degraded and Az1 is stabilized, resulting in a brief period where cells are double positive for green and orange fluorescence. As KO2 is degraded, cells become increasingly green as they progress through S-phase (AzL). Finally, as cells enter G2, Az1 accumulates to higher levels reaching a maximum at the end of each cell cycle (Az-high, AzH). These fluorescent reporters allow live cells to be tracked as they progress through the cell cycle *in vitro* and *in vivo*, allowing temporal and spatial aspects of cell cycle regulation to be characterized in ways that were not previously possible [4].

Another advantageous feature of the FUCCI system is that cells from each stage of the cell cycle can be isolated by fluorescence activated cell sorting (FACS) and used for additional single cell or population-based experiments; including studies of differentiation, trans-differentiation and reprogramming. While some viable DNA-binding dyes such as Hoechst 33342 and Vybrant Dye Cycle (Life Technologies) can also be used for FACS isolation of cells, this approach is somewhat limiting because cell cycle fractionation is based on cellular DNA content and therefore, it has limitations with regards to early and late G1-phase separation. Overall, the establishment of the FUCCI system provides tremendous benefits to researchers interested in the role of the cell cycle in development and disease and has resulted in many publications since first being reported seven years ago in models ranging from flies to human stem cells.

Several studies have now described the utility of FUCCI reporters in mouse and human PSCs and established new aspects of pluripotent cell biology [5–10]. One important finding has been confirmation that PSCs initiate the differentiation program from the G1-phase and that this represents a "window of opportunity" where cells make fate decisions [7,8,11–13]. As one example, we have recently used FUCCI indicators to identify genes that are cell cycle-regulated in PSCs by performing FACS of cell cycle fractions followed by RNA-sequencing. Surprisingly, transcription of developmental genes increases during late-G1 thereby contributing to stem cell heterogeneity but more importantly, indicating that PSCs are poised for differentiation at this time. Activation of developmental genes in late-G1 requires signals generated by retinoid, WNT, FGF, BMP and TGFβ family-members indicating that cell cycle and cell signaling pathways converge to control fate decisions. Epigenetic marks such as 5-hydroxymethylcytosine (5hmC) also appear to oscillate during the cell cycle, indicating a deeper layer of regulation in response to G1 transition. Detailed information on 5hmC assays is already available [14–16]. Overall, these findings illustrate the utility of FUCCI in characterization of PSCs and how it can be used to address questions that were previously intractable. In the following sections we will provide a framework for how the FUCCI system can be established and utilized in hPSCs.

2. Materials and methods

2.1. Construct design considerations

Obtaining stable, ectopic expression of transgenes in hPSCs can sometimes be problematic and promoters used to drive transcription are an important variable. The cytomegalovirus (CMV) promoter for example is unreliable due to silencing. Instead, *EF1α*, *PGK1* and *UBC* promoters are better options and provide different levels of expression. The most reliable, constitutive promoter used in PSCs however, is the *CAG* promoter that consists of chicken beta-actin 5′ regulatory sequences flanked by the CMV enhancer [17]. We therefore expressed *CDT1-KO2 and GEMININ-AZ1* fusion genes from the *CAG* promoter because robust expression is required in this system. We also strongly recommend coupling the expression of genes to an internal ribosome entry site (IRES)-linked drug-resistance marker such as puromycin^r, neomycin^r, blastacidin^r, or zeocin^r. This approach significantly increases the yield of desired cell lines by reducing the frequency of 'false positive' clones. As