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Phosphoproteomic Analysis of Human Embryonic Stem Cells

Laurence M. Brill[1,*,2,†], **Wen Xiong**[3,†], **Ki-Bum Lee**[3,4,†], **Scott B. Ficarro**[1,5], **Andrew Crain**[6,7], **Yue Xu**[3], **Alexey Terskikh**[6], **Evan Y. Snyder**[6,*], and **Sheng Ding**[3,*]

[1]Genomics Institute of the Novartis Research Foundation (GNF), 10675 John Jay Hopkins Drive, San Diego, CA 92109, USA

[3]The Scripps Research Institute, Department of Chemistry, 10550 North Torrey Pines Road, La Jolla, CA 92037, USA

[6]Burnham Institute for Medical Research, 10901 North Torrey Pines Road, La Jolla, CA 92037, USA

[7]University of California San Diego, Biomedical Sciences Graduate Program, 9500 Gilman Drive, La Jolla, CA 92093, USA

SUMMARY

Protein phosphorylation, while critical to cellular behavior, has been under-characterized in pluripotent cells. Therefore, we performed phosphoproteomic analyses of human embryonic stem cells (hESCs) and their differentiated derivatives. 2546 phosphorylation sites were identified on 1602 phosphoproteins; 389 proteins contained more phosphorylation site identifications in undifferentiated hESCs, whereas 540 contained more such identifications in differentiated derivatives. Phosphoproteins in receptor tyrosine kinase (RTK) signaling pathways were numerous in undifferentiated hESCs. Cellular assays corroborated this observation by showing that multiple RTKs cooperatively supported undifferentiated hESCs. In addition to bFGF, EGFR, VEGFR and PDGFR activation was critical to the undifferentiated state of hESCs. PDGF-AA complemented a sub-threshold bFGF concentration to maintain undifferentiated hESCs. Also consistent with phosphoproteomics, JNK activity participated in maintenance of undifferentiated hESCs. These results support the utility of phosphoproteomic data, provide guidance for investigating known and novel proteins in hESCs, and complement transcriptomics/epigenetics for broadening our understanding of hESC fate determination.

*To whom correspondence should be addressed: lbrill@burnham.org, (858) 646-3100 X 3815 (phone); esnyder@burnham.org, (858) 646-3158 (phone); and sding@scripps.edu, (858) 784-7376 (phone).
[2]Present address: Burnham Institute for Medical Research, NCI Cancer Center and Proteomics Facility, 10901 North Torrey Pines Road, La Jolla, CA 92037, USA
[4]Present address: Department of Chemistry and Chemical Biology, Institute for Advanced Materials, Devices and Nanotechnology, The Rutgers Stem Cell Research Center, Rutgers University, Piscataway, NJ, 08854, USA
[5]Present address: Dana-Farber Cancer Institute, 44 Binney St., Boston, MA 02115, USA
[†]These authors contributed equally to this work

ACCESSION NUMBERS OF THE SUPPLEMENTARY DATA

All supplementary data is in the PRIDE database: http://www.ebi.ac.uk/pride/; accession numbers are 9253–9257 and 9259–9264.

Details are in Supplementary Experimental Procedures.

INTRODUCTION

Human embryonic stem cells (hESCs) are a model developmental system that may have potential clinical value for mitigating diseases. Mechanisms of hESC fate determination are not well defined, although there has been progress in elucidating molecular circuitry of self-renewing ESCs. Transcriptional profiles of hESCs (Brandenberger et al., 2004; Sato et al., 2003; Sperger et al., 2003) and more limited ChIP-on-chip (Boyer et al., 2005) and proteomic (Bendall et al., 2007; Van Hoof et al., 2006) analyses suggest mechanisms underlying hESC self-renewal and differentiation. In addition to transcriptional and translational regulation, cell fate determination is controlled by protein phosphorylation, a critical determinant of cell signaling (Mann et al., 2002; Schlessinger, 2000). Recent phosphoproteomic analyses of human mesenchymal stem cells identified 716 and 703 protein phosphorylation sites (Thingholm et al., 2008a; Thingholm et al., 2008b). However, protein phosphorylation has not been well characterized in pluripotent cells. Therefore, we performed a large-scale multidimensional liquid chromatography- (MDLC) tandem mass spectrometry- (MS/MS) based phosphoproteomic analysis of undifferentiated hESCs and their differentiated derivatives for identification of protein phosphorylation sites in these cells.

Undifferentiated hESCs were cultured under feeder-free conditions with bFGF. Comparable differentiated derivatives were obtained by removal of bFGF and treatment with retinoic acid (RA), which induces nearly complete albeit non-specific differentiation to a heterogeneous population of cells. Removal of bFGF alone does not result in complete differentiation, whereas concurrent RA treatment causes virtually complete loss of the undifferentiated population in 4 days (required for this type of analysis). Our data provide a freely available resource of protein phosphorylation sites in hESCs and differentiated derivatives (http://www.ebi.ac.uk/pride/). These data have begun to prove informative and predictive. For example, as proof-of-concept, pathway analyses of the phosphoproteins suggested potential responses of hESCs to perturbations of receptor tyrosine kinase (RTK) signaling pathways. To test some RTK pathways for a role in the maintenance of undifferentiated hESCs, we treated hESC cultures with selected agonists or antagonists of these pathways. Their effects were consistent with predictions of the phosphoproteomic analyses. Furthermore, the data suggested a role of novel proteins in hESC self-renewal or differentiation, thus providing extensive guidance for future research.

RESULTS

Phosphoproteomic Analysis of hESCs

Because phosphoproteomic analysis is challenging (Mann et al., 2002) and has not been reported in hESCs, we chose to analyze the well-characterized hESC line H1 (WiCell; WA01) (Thomson et al., 1998), which has been used in molecular studies of hESCs e.g. (Bendall et al., 2007; Brandenberger et al., 2004; Wang et al., 2007). 59 hESC lines, including H1, showed remarkable conservation of hESC markers (Adewumi et al., 2007), which provided confidence that our findings would be representative. Before analyzing protein phosphorylation, the undifferentiated hESC markers OCT4 (Thomson et al., 1998) and SSEA-4 (Reubinoff et al., 2000) were examined to assess whether the hESCs were truly undifferentiated under our culture conditions, and whether differentiation was complete. Undifferentiated hESCs were cultured on matrigel-coated plates in feeder-free cultures using conditioned media (CM) that contained 8 ng/ml of added bFGF. A heterogeneous population of differentiated derivatives of the hESCs were obtained by removal of bFGF and treatment with 5 μM RA for 4 days. OCT4 was detected in ca. 97% of the hESCs under the feeder-free conditions, whereas it was nearly undetectable in differentiated derivatives (Figure 1). Similarly, SSEA-4 was positive in the undifferentiated hESCs and nearly absent in differentiated derivatives. Moreover, the nucleus-to-cytoplasm ratio, also monitored as an indicator of whether hESCs are undifferentiated or differentiated,

was consistent with OCT4 and SSEA-4 expression (Figure 1). These observations suggested that our cells represented two distinct populations -- "undifferentiated" or "differentiated" hESC derivatives -- that might then be reliably subjected to phosphoproteomic analysis, using MDLC-MS/MS technology, that can result in unbiased discovery of protein phosphorylation sites (Kruger et al., 2008).

Phosphoproteomic analyses of hESCs and their differentiated derivatives were performed using automated MDLC, a linear ion trap mass spectrometer and readily available bioinformatics algorithms. Phosphorylated peptides from total proteins from undifferentiated hESCs or their differentiated derivatives were separated, enriched, and analyzed using MDLC comprised of strong cation exchange chromatography (SCX), reversed-phase (RP) desalt-Fe^{3+}-immobilized metal affinity chromatography (desalt-IMAC) and RP HPLC coupled to nano-electrospray ionization-tandem mass spectrometry (ESI-MS/MS; Figure S1 **shows a schematic diagram**). IMAC, for phosphopeptide enrichment, coupled to RP HPLC-ESI-MS/MS is a robust technique for phosphoproteomic analyses (Bodenmiller et al., 2007; Brill et al., 2004; Gruhler et al., 2005) and automation improves reliability and reproducibility (Ficarro et al., 2005). Because phosphorylated proteins are frequently at low abundance, sub-stoichiometrically phosphorylated and difficult to identify (Mann et al., 2002), replicate analyses were performed to increase phosphoproteome coverage. Replicates increase proteome coverage, especially of lower abundance proteins (Liu et al., 2004), and the impact of experimental variation in LC-MS/MS can be minimized by replicates (Washburn et al., 2003). Phosphopeptides were identified with high confidence (see Supplementary Experimental Procedures). Examples of typical MS/MS spectra used to identify phosphopeptides are in Figure S2.

To complement identification, extracted ion chromatograms (XICs) were used to quantify the relative abundance of phosphopeptides. The normalized abundance of randomly selected phosphopeptides identified in all 4 phosphoproteomic analyses (2 biological replicates, *i.e.* phosphopeptides from two pairs of independent cultures of undifferentiated hESCs or their differentiated derivatives) demonstrated relatively low variability (Table S1). This degree of consistency agrees with previous findings in which proteomic data can be reliably compared among experiments (Washburn et al., 2003).

In contrast, differential phosphopeptide identification implies differential phosphopeptide abundance. We used data-dependent MS/MS, and peptide abundance and identification correlate in data-dependent MS/MS (Liu et al., 2004). Selected phosphopeptides identified in undifferentiated hESC or differentiated derivative cell populations were also quantified using XICs. Furthermore, signal from each of the selected phosphopeptides was manually sought in the MS/MS data from analyses in which it had not been identified by SEQUEST searches, in order to test whether the phosphopeptide was detectable, and if so, its relative abundance among the phosphoproteomic analyses. Only a fraction of the phosphopeptides not identified in SEQUEST searches was detectable (via a poor quality MS/MS spectrum) when searching the raw data (Table S2). However, every phosphopeptide that was examined demonstrated a higher normalized abundance in analyses in which it was identified than in analyses in which it was not identified by SEQUEST searches. Although lack of identification of a phosphopeptide is not evidence for its absence, identification vs. lack of identification implies that the phosphopeptide is likely to be more abundant in the cell population in which it was identified, consistent with our results (Table S2) and those of others (Liu et al., 2004).

Western blots were performed on proteins from undifferentiated hESCs and differentiated derivatives, using antibodies recognizing phosphorylation sites previously identified by MDLC-MS/MS. All 9 antibodies that were used recognized bands with the expected mobility on Western blots, providing confidence in phosphorylation site identifications.

Representative Western blots, including normalized integrated intensities of phosphoprotein bands, are shown in Figure S3. Phosphorylation of mTOR on Ser2448 was apparently more abundant in undifferentiated than differentiated cells (Figure S3A), and mTOR Ser2448 phosphorylation was identified in undifferentiated but not differentiated cells using MDLC-MS/MS (Table S3A). PAK1 phosphorylation on Ser144 was identified twice in undifferentiated cells and once in differentiated cells by MDLC-MS/MS (Table S5A) and Western blots suggested that PAK1 phosphoserine144 was more abundant in undifferentiated than differentiated cells (Figure S3B). Antibodies recognizing PTK2 phosphotyrosine576/577 suggested that phosphorylation of this site was more abundant in differentiated derivatives than undifferentiated hESCs (Figure S3C), consistent with identification of PTK2 phosphorylated on Tyr576, using MDLC-MS/MS, only in differentiated derivatives (Table S4A). Phosphorylation of CDK1/2/3/5 on Thr14 and Tyr15 (2 conserved residues in all 4 CDK proteins) was more abundant in undifferentiated cells (Figure S3D) and XIC peak areas suggested that phosphorylation of CDK1/2/3 on Thr14 and Tyr15 was more abundant in undifferentiated cells (Table S1). CDK1/2/3/5 phosphorylated on Thr14 and Tyr15 are recognized in Western blots (Supplementary Experimental Procedures), and the corresponding phosphopeptides identified by MDLC-MS/MS (IGEGT*YGVVY and IGEGTY*GVVY; for brevity, designated as originating from CDK2 in Table S1 and Table S5) are identical among CDK1/2/3, whereas the corresponding peptide from CDK5 differs at 2 amino acid residues (IGEGT*Y*G*TVF*), which is easily distinguishable by MS/MS. The relative abundance of JUN phosphorylated on Ser63, and HSP27 phosphorylated on Ser82 was similar in undifferentiated and differentiated cells on Western blots (not shown), and phosphorylated JUN Ser63 as well as phosphorylated HSP27 Ser82 were both identified the same number of times in undifferentiated and differentiated cells (Table S5A), demonstrating further agreement between Western blots and MDLC-MS/MS.

If subsequent studies focus on one or a few especially critical sites of protein phosphorylation, it is advisable to examine the phosphorylation site using an independent technique. However, MDLC-MS/MS is reliable for phosphoproteome analysis, and can yield unbiased, large-scale discovery of protein phosphorylation (Bodenmiller et al., 2007; Brill et al., 2004; Ficarro et al., 2005; Gruhler et al., 2005; Kruger et al., 2008; Thingholm et al., 2008a) and our findings support its accuracy. Together, these results suggest that application of MDLC-MS/MS for identification of phosphopeptides was suitable for phosphoproteomic analysis of undifferentiated hESCs and their differentiated derivatives.

Phosphopeptide identifications are in Table S3A– Table S5B. Each phosphoprotein, from which phosphopeptides were derived, was classified as either: **(1)** containing more phosphorylation site identifications in undifferentiated hESCs, **(2)** containing more phosphorylation site identifications in differentiated hESC derivatives, and **(3)** containing a similar number of phosphorylation site identifications in both cell populations. A protein is conservatively defined to contain more phosphorylation site identifications in a cell population if its phosphorylation was identified exclusively in this population or at least 3-fold more frequently than in the other population, otherwise the protein is considered to contain a similar number of phosphorylation site identifications in populations from both cell states. Although identification of protein phosphorylation sites was unlikely to be comprehensive, as implied by studies using different cell types (Bodenmiller et al., 2007; Mann et al., 2002), among the 2546 non-redundant phosphorylation sites, 472 were on proteins containing more phosphorylation site identifications in undifferentiated hESCs, whereas 726 were on proteins containing more phosphorylation site identifications in differentiated hESC-derivatives (Figure 2A). 94% of the peptides were singly phosphorylated, whereas the rest were doubly phosphorylated, similar to other studies using IMAC for phosphopeptide enrichment (Bodenmiller et al., 2007; Thingholm et al., 2008a). Serine, Thr and Tyr phosphorylation comprised ca. 82%, 14% and 4% of the sites, respectively (Table S3A–Table S5B), and Tyr

phosphorylation was relatively prominent in undifferentiated hESCs (Figure 2C). Among the 1602 proteins, 389 contained more phosphorylation site identifications in undifferentiated hESCs, whereas 540 contained more phosphorylation site identifications in differentiated hESC-derivatives (Figure 2B).

Transcription factors can reprogram differentiated cell types to ESC-like cells when ectopically expressed (Takahashi et al., 2007; Takahashi and Yamanaka, 2006; Yu et al., 2007), and were the most abundant known phosphoprotein category (Figures 2G–I). This observation, not typical of proteomic analyses, could reflect the growing consensus that many transcription regulators are important in control of ESC cell state. Among the 158 phosphorylated transcription regulators, 41 contained more phosphorylation site identifications in undifferentiated hESCs, 46 contained more phosphorylation site identifications in differentiated hESC-derivatives, and 71 contained a similar number of phosphorylation site identifications in both cell populations.

Most of the transmembrane receptors and predicted extracellular proteins contained more phosphorylation site identifications in either undifferentiated or differentiated hESCs, whereas fewer of these proteins contained a similar number of phosphorylation site identifications in both cell populations (Figures 2D–I, Table S6), implying that growth factors, cytokines, their receptors and corresponding signaling pathways could participate in controlling hESC fate. Furthermore, kinases, which are key players in cell signaling, represented the second largest category of known phosphoproteins (Figures 2G–I). Phosphorylation of cytoplasmic, cytoskeletal, and cell adhesion proteins was identified relatively frequently in differentiated derivatives (Figures 2D–I).

Phosphorylated Transcription Regulators in Undifferentiated hESCs

The transcription regulator ESG1 (official symbol TLE1; Table S7) is expressed only in pre-implantation embryos, ESCs, and primordial germ cells (Western et al., 2005). ESG1 is co-expressed with OCT4 and SOX2 in both mouse and human ESCs, suggesting it is a potential pluripotency marker (Western et al., 2005). In addition, SUPT16H and SSRP1 (Table S7,Table S8) were phosphorylated in undifferentiated hESCs, and are the two subunits of FACT (facilitates chromatin transcription). FACT destabilizes nucleosomes to allow transcription without disruption of the epigenetic state (Belotserkovskaya et al., 2003) and promotes initiation of DNA replication in the S-phase of the cell cycle (Tan et al., 2006). CREBBP (Table S7) has histone acetyltransferase activity. Its mRNA is enriched in undifferentiated hESCs (Brandenberger et al., 2004) (Table S8). AKT (Table S5A) phosphorylates CREBBP, increasing CREBBP acetyltransferase activity, promoting NF-κB-mediated transcription and enhanced cell survival (Liu et al., 2006). Furthermore, CREBBP increases ERK1 expression (Chu et al., 2005). ERK1 activity contributes to hESC self-renewal in the presence of bFGF (Li et al., 2007).

At least 18 phosphorylated transcription regulators identified in undifferentiated hESCs can modify chromatin structure via histone methylation or acetylation (Table S7) and may contribute to the epigenetic pattern that is likely to be important to hESCs (Bernstein et al., 2006;Lee et al., 2006;McCool et al., 2007). We identified phosphorylation of DNMT3B, MBD3 (Table S3A) and EZH2 (Table S5A) in undifferentiated hESCs. *DNMT3B* encodes a DNA-methyltransferase (Table S7), which was expressed in all 59 hESC lines tested (Adewumi et al., 2007), was enriched in undifferentiated hESCs (Brandenberger et al., 2004) and was phosphorylated in undifferentiated hESCs (Table S8). Differential phosphorylation could modulate EZH2 activity. Phosphorylation at S21 by AKT inhibits the histone H3 Lys27 methyltransferase activity of EZH2 (Cha et al., 2005), and we identified a novel phosphorylation site of EZH2 in undifferentiated hESCs (S371 or T372; Table S5A), a site whose phosphorylation was also identified in undifferentiated mouse ESCs (unpublished data).

Phosphorylated transcription regulators in undifferentiated hESCs can participate in transcriptional activation or repression, histone modification and more (Table S7). These and other functions may be integrated to favor the undifferentiated state of hESCs, as implied by the complexity of the phosphoproteome (Figure 2). Although some of these transcriptional and epigenetic regulators were previously reported to influence hESCs, the mechanisms are unclear. The identified phosphorylation sites provide focused information for future studies of the function of these factors in hESCs. Furthermore, we also identified hundreds of novel phosphoproteins whose presence in hESCs was unknown, providing a rich resource for further investigation. For instance, TNRC6A, a factor for gene silencing *via* RNA interference (Liu et al., 2005) was phosphorylated in undifferentiated hESCs (Table S3A).

Growth Factor-Mediated Signaling Pathways in Undifferentiated hESCs

Tyrosine phosphorylation, which plays a dominant role in growth factor/RTK signaling pathways (Schlessinger, 2000), was relatively prominent in undifferentiated hESCs (Figure 2C). Signaling pathways participating in self-renewal of hESCs include bFGF-, TGF-β/Activin-, Insulin/IGF-, EGFR family-, PDGF-, Wnt-, Neurotrophin-, Integrin- and Notch pathways (Beattie et al., 2005; Bendall et al., 2007; James et al., 2005; Pebay et al., 2005; Wang et al., 2007; Xu et al., 2005; Yao et al., 2006). However, detailed understanding of the action of these pathways is lacking. The phosphoproteins were grouped into signaling pathways, as described in Supplementary Experimental Procedures, to further explore their functional potential. 41 canonical and metabolic pathways were suggested using the phosphoproteins as input for pathway analysis (not shown). Proteins in RTK pathways were phosphorylated in undifferentiated hESCs, including the adaptors GAB1, SHC1 and NCK2, the kinases LCK, NEK4, MAPK6, MAPK7, mTOR, PIK3C3 and PIK3R4, phospholipases PLC-γ1 and PLC-γ2 and the phosphatase PPAP2B (Table 1). Some phosphoproteins are shared among pathways and some are more pathway-specific, such as APC in Wnt signaling and NUMB in Notch signaling. Table 1 and Figure 2C imply that a variety of signaling pathways are important in undifferentiated hESCs. For example, EGF pathway members ErbB2, AREG and EPS15L1 were phosphorylated in undifferentiated hESCs (Table 1, Table S5), complementing a report showing that the ErbB2/ErbB3 ligand heregulin-1β helps support undifferentiated hESCs (Wang et al., 2007). KDR (VEGFR2, FLK1) was phosphorylated in undifferentiated hESCs (Table 1), and stimulation of hESCs with CM elicits tyrosine phosphorylation (site(s) undefined) of PDGFRA (Wang et al., 2007). Components of the VEGF and PDGF pathways were phosphorylated in undifferentiated hESCs, including some proteins in Table 1. We also identified phosphoproteins from signaling pathways whose presence in hESCs have not been reported, and a large number of novel phosphoproteins were identified (Table S3A–Table S5B).

Molecular profiling studies typically lack biological follow-up, e.g. (Bodenmiller et al., 2007; Boyer et al., 2005; Brandenberger et al., 2004; Brill et al., 2004; Ficarro et al., 2005; Gruhler et al., 2005; Lee et al., 2006; McCool et al., 2007; Sperger et al., 2003; Thingholm et al., 2008a; Thingholm et al., 2008b; Van Hoof et al., 2006). However, a few, including transcriptomic (Armstrong et al., 2006) and proteomic (Bendall et al., 2007; Kratchmarova et al., 2005; Mukherji et al., 2006; Wang et al., 2006; Wang et al., 2007) studies demonstrated that cells responded to stimulation in manners consistent with molecular profiles. To test the cellular relevance of the phosphoproteomic and pathway analyses, we began by targeting EGF-, VEGF-and PDGF pathways in undifferentiated hESCs using inhibitors of their receptors. Although specificity of RTK inhibitors is imperfect, we used some of the widely accepted ones (see Supplementary Experimental procedures). Treatment of undifferentiated hESC cultures with an EGFR inhibitor at 10μM resulted in extensive apoptosis (not shown), similar to another report (Wang et al., 2007). The hESCs were also treated with 10μM KDR inhibitor II or 10μM Gleevec, a PDGFRA inhibitor (Zhang et al., 2003). Undifferentiated control colonies were

compact and expressed OCT4 and SSEA-4 (Figure 3B and not shown). In contrast, most cells differentiated in the presence of KDR or PDGFR inhibitor, shown by flattening of the colonies, altered cellular morphology and nearly undetectable OCT4 and SSEA-4 (Figure 3C and data not shown). Vehicle-only controls lacked any noticeable effect on the cells (Figure 3B). The results were similar under feeder-free conditions in CM and feeder-free conditions in chemically defined media (CDM; (Yao et al., 2006)). Furthermore, KDR or PDGFR inhibitor, at 10 μM, resulted in decreased expression of *NANOG* and *OCT4* (Figure 3A).

To further investigate the effect of RTK signaling pathways, we decreased bFGF to a sub-threshold 4 ng/ml (at least 20 ng/ml is required under feeder free conditions in CDM (Yao et al., 2006)) and systematically supplemented cultures with EGF, PDGF-AA or VEGF-AA at different concentrations to determine which trophic factor could complement bFGF deficiency. Although PDGF-AA without bFGF was unable to maintain long-term cultures of undifferentiated hESCs, PDGF-AA at 10 ng/ml and the sub-threshold concentration of 4 ng/ml of bFGF (subsequently abbreviated PDGF/bFGF) stably maintained undifferentiated hESCs under feeder-free conditions in CDM for >15 passages, and the hESCs remained undifferentiated throughout all 4 experiments (Figure 4D). The cells displayed undifferentiated morphology and robust expression of OCT4. In contrast, when undifferentiated hESCs, which had been stably maintained in CDM containing PDGF/bFGF for >15 passages were subsequently cultured for 4 days in CDM containing 4 ng/ml of bFGF but no PDGF, the cells differentiated (Figure 4B). FACS analyses demonstrated that ca. 89% of the hESCs in CDM containing PDGF/bFGF were positive for SSEA-4, comparable to cultures in CDM containing 20 ng/ml of bFGF (86%; Figure 4). Similar FACS results were obtained when cells were stained and sorted for the pluripotency marker Tra-1-60 (not shown). Moreover, PDGF/bFGF in CDM resulted in sustained expression of *NANOG* and *OCT4* transcripts, whereas their abundance declined within 4 d in the absence of PDGF-AA or the presence of the PDGFR inhibitor Gleevec (Figure 3A, Figure 4A), further supporting the proposal that PDGF-AA facilitates maintenance of undifferentiated hESCs. Together, phosphoproteomic and pathway analyses suggested that PDGF should favor maintenance of undifferentiated hESCs. PDGFR inhibitor, and separate use of PDGF-AA, provided clear evidence that PDGF, when bFGF is at a sub-threshold concentration, can promote the undifferentiated state of hESCs in CDM under feeder-free conditions, insights that derived directly from the phosphoproteomic analysis.

Our data further suggested that ErbB and VEGFR activation participate in maintenance of undifferentiated hESCs, because disruption of these pathways caused either apoptosis (not shown) and/or differentiation (Figure 3) (although EGF and VEGF-AA demonstrated limited efficacy at complementing the deficiencies of 4 ng/ml bFGF). The ErbB2/ErbB3 ligand heregulin-1β contributes to maintenance of undifferentiated hESCs (Wang et al., 2007). In addition, insulin/IGF pathway members (Bendall et al., 2007) were phosphorylated in hESCs (including proteins in the PI3K/AKT/mTOR pathway; Table 1).

Phosphoproteomics, cellular assays and other reports (Bendall et al., 2007; Wang et al., 2007; Yao et al., 2006), suggest that multiple RTK pathways are required, although none of them alone is sufficient to support self-renewal in the absence of bFGF. Also consistent with our results, although less clear due to the undefined media that was used, Sphingosine-1-phosphate plus PDGF contributes to maintenance of undifferentiated hESCs in the presence of mouse embryonic fibroblasts (MEFs) or MEF-conditioned media (Pebay et al., 2005). It previously appeared that bFGF alone might sustain self-renewal of hESCs. However, as predicted by our phosphoproteomic analysis, several other factors that exist in serum and/or are secreted by feeders, acting through autocrine or paracrine effects or as culture additives, are also important for hESC self-renewal (Bendall et al., 2007; Wang et al., 2007). Our phosphoproteomic and pathway analysis also imply that additional pathways could favor undifferentiated hESCs.

Phosphorylated Signal Transduction Proteins in Undifferentiated hESCs

PI3K signaling facilitates ESC self-renewal (Armstrong et al., 2006), and the PI3K pathway is activated by PDGF in mesenchymal stem cells (Kratchmarova et al., 2005), but the mechanism of action of the PI3K pathway has been unclear. PI3K/AKT/mTOR pathway members were phosphorylated in undifferentiated hESCs. For example, *PIK3C3* is enriched in undifferentiated hESCs (Brandenberger et al., 2004) and PIK3C3 was phosphorylated in undifferentiated hESCs (Table 1). mTOR (Table 1) plays a role in proliferation of undifferentiated hESCs (Wang et al., 2007) and is phosphorylated at Ser2448 during mitogenic stimulation (Chiang and Abraham, 2005). mTOR, phosphorylated at Ser2448 and Ser2454 in undifferentiated hESCs (Figure S3A, Table S3A) is a protein that enhances cell survival (Peponi et al., 2006). TSC1 was also phosphorylated in undifferentiated hESCs (Table 1). *TSC1* can limit cell size (Rosner et al., 2003) and its over-expression caused cells to form compact clusters with increased re-aggregation *in vitro* (Li et al., 2003), similar to the small size of undifferentiated hESCs and compact morphology of hESC colonies. Phosphorylated PI3K/AKT/mTOR pathway members in undifferentiated hESCs (Table 1) suggest which pathway members may regulate undifferentiated hESCs.

Phosphoproteins participating in MAPK signaling were identified (Table 1). The ERK pathway contributes to hESC self-renewal under conditions that include bFGF (Li et al., 2007), whereas JNK signaling in hESCs has not been reported. Some phosphoproteins downstream of RTK pathways are relatively specific to JNK signaling, such as TRAF4, MLK4, CRKL and MINK1 (Table 1). To test for JNK signaling in undifferentiated hESCs, we tested two JNK inhibitors in hESC cultures under feeder free conditions in CM. JNK inhibitor II, a small molecule (SP600125), widely used in JNK studies (Bennett et al., 2001;Han et al., 2001;Shin et al., 2002) and JNK inhibitor III, a polypeptide (Holzberg et al., 2003) were used. Each inhibitor, alone, resulted in cellular differentiation, demonstrated by colony morphology and decreased OCT4 expression (Figure S4). In contrast, controls lacking JNK inhibitors, including vehicle-only controls, remained undifferentiated (Figure S4 and not shown). Induction of differentiation by JNK inhibitors was similar under feeder-free conditions in CDM (not shown). Furthermore, *OCT4* and *NANOG* mRNA was depleted in the presence of JNK inhibitor II (Figure 3A). Thus, this phosphoproteomic analysis provides the first suggestion that JNK, an important signal transduction protein downstream of many RTKs, may facilitate maintenance of undifferentiated hESCs. Moreover, these experiments further demonstrate agreement between phosphoproteomic and cellular analyses in hESCs.

DISCUSSION

Analysis of molecular mechanisms underlying hESC properties is essential for optimal use of these cells. Complementing previous analyses of promoters, transcripts, and protein expression, our phosphoproteomic analysis suggests that multiple protein phosphorylation events participate in control of hESC fate. Application of MDLC-MS/MS-based phosphoproteomics to pluripotent cells may represent an important tool for stem cell biologists. While this study focused on its use for hESCs, one can envision its application to induced pluripotent somatic cells and other somatic stem cells.

Our phosphoproteomic analyses identified known and novel proteins potentially participating in self-renewal or differentiation of hESCs and focused attention on pathways heretofore under-appreciated and under-explored. Transcription regulators, including epigenetic and transcription factors, and kinases contained many phosphorylated members, suggesting that these proteins may be key determinants of hESC fate decisions. Although a variety of proteins have been implicated in hESC self-renewal, some of their functions have been unclear. The identified phosphorylation sites, some on central signaling proteins, expand the knowledge of protein phosphorylation in hESCs. We also identified many proteins whose potential functions

in hESCs is novel. In other words, phosphoproteomic analyses may provide guidance for a systematic, rather than solely serendipitous or overly broad-based approaches in future studies of self-renewal and differentiation of pluripotent cells.

Phosphoproteomic analyses identified proteins favoring an undifferentiated or differentiated state of hESCs. For example, phosphorylation of proteins in the JNK pathway was identified, and our cellular follow-up experiments, which are atypical of molecular profiling studies, suggested that inhibition of JNK leads to differentiation of hESCs. A role of JNK in undifferentiated hESCs has not been reported. The VEGF and PDGF pathways are candidates to favor maintenance of undifferentiated hESCs because inhibitors of their receptors resulted in hESC differentiation. However, the growth factors that were added singly could not replace bFGF. Together, these results suggested that activation of these pathways are necessary but not sufficient to sustain self-renewal of hESCs, consistent with increasing evidence that multiple growth-factor driven pathways act together to maintain undifferentiated hESCs. For example, PDGF-AA complemented a sub-threshold concentration of bFGF, shown by long-term maintenance of undifferentiated cultures under feeder-free conditions in CDM. Use of CDM allowed improved knowledge of the composition of the media, rather than use of undefined media in the presence of, or conditioned by, feeder fibroblasts (Yao et al., 2006), so the pathways that were targeted in our cellular assays were more clearly defined. Together, our results expanded the repertoire of pathways that facilitate hESC culture and support the suggestion that multiple signaling inputs are needed to maintain undifferentiated hESCs (Wang et al., 2007). Moreover, phosphoproteomic analyses complement epigenetics, gene expression profiles, and total protein MS to facilitate an improved understanding of hESC fate determination.

The functions of most phosphorylated proteins are unknown in pluripotent cells, including the plethora of novel ones, and should be evaluated for their influence on stem cell behavior. Application of further advances in proteomic and allied technologies should enhance future studies through improved analysis of protein phosphorylation. As phosphoproteins controlling pluripotent behavior are understood better, methods for developing model systems with stem cells, and potential therapeutic applications, may become increasingly clear.

EXPERIMENTAL PROCEDURES

Cell Culture, Phosphoproteomic Analysis

Feeder free cultures were in Matrigel coated plates in CM containing 8 ng/ml bFGF (Xu et al., 2001). Differentiation was with 5 μM RA and no added bFGF. In CDM, hESCs were cultured in Matrigel-coated plates in N2/B27-CDM (Yao et al., 2006). Phosphoproteomic analyses used cells from CM. Cells were rinsed with PBS, lysed, centrifuged, proteins precipitated with $(NH_4)_2SO_4$ and pelleted by centrifugation.

Proteins were re-suspended in 100 mM NH_4HCO_3, 8 M urea containing phosphatase inhibitors, reduced, alkylated, digested with trypsin, and peptides desalted. Peptides were separated by SCX, phosphopeptides enriched by desalt-IMAC (Brill et al., 2004; Ficarro et al., 2005), separated by nanoflow HPLC and analyzed by ESI-MS/MS. MS/MS spectra were matched to amino acid sequences using SEQUEST (Eng et al., 1994). All reported phosphopeptide identifications were manually verified (Bernstein et al., 2008; Brill et al., 2004; Ficarro et al., 2005).

Normalized XIC peak areas of some phosphopeptides were quantified. For analyses lacking the identification, MS/MS data was exhaustively searched for the phosphopeptide, which was rarely found *via* a poor quality MS/MS spectrum, and its XIC peak area was quantified.

Phosphoproteins were classified as containing more phosphorylation site identifications in undifferentiated hESCs or differentiated derivatives, or as containing a similar number of phosphorylation site identifications in the 2 cell populations, as described in the Results section.

Western Blot Analysis

Proteins were run on Bis-Tris gels, transferred to PVDF membranes, blocked, and incubated with antibodies recognizing phosphorylation sites identified by MDLC-MS/MS. Anti-GAPDH was the loading control. Membranes were washed, incubated with fluorophore-conjugated secondary antibodies, washed, imaged, and bands quantified according to the manufacturer (LiCor).

Phosphoprotein category, subcellular location and pathway Analysis

Ingenuity Pathway Analysis, Metacore, NCBI, Gene Ontology and peer-reviewed literature were used to identify phosphoprotein subcellular location, category, and signaling pathways.

Cellular Assays, RT-PCR

EGFR-, JNK- or PDGFR inhibitors were used. Untreated and vehicle-only controls were included for each experiment. PDGF-AA/bFGF were used in cultures for >15 passages.

For immunostaining and DAPI staining, monoclonal mouse anti-OCT4 and anti-SSEA-4 were used. Secondary antibodies were Cy2-conjugated rabbit anti-mouse IgM and Cy3-conjugated rabbit anti-mouse IgG. For RT-PCR, mRNA was isolated, cDNA was synthesized; *OCT4*, *NANOG* and *GAPDH* were amplified. For FACS, cells were incubated with mouse monoclonal anti-SSEA-4 or anti-TRA-1-60 antibodies, washed with PBS and incubated with Cy3-conjugated rabbit anti-mouse IgG.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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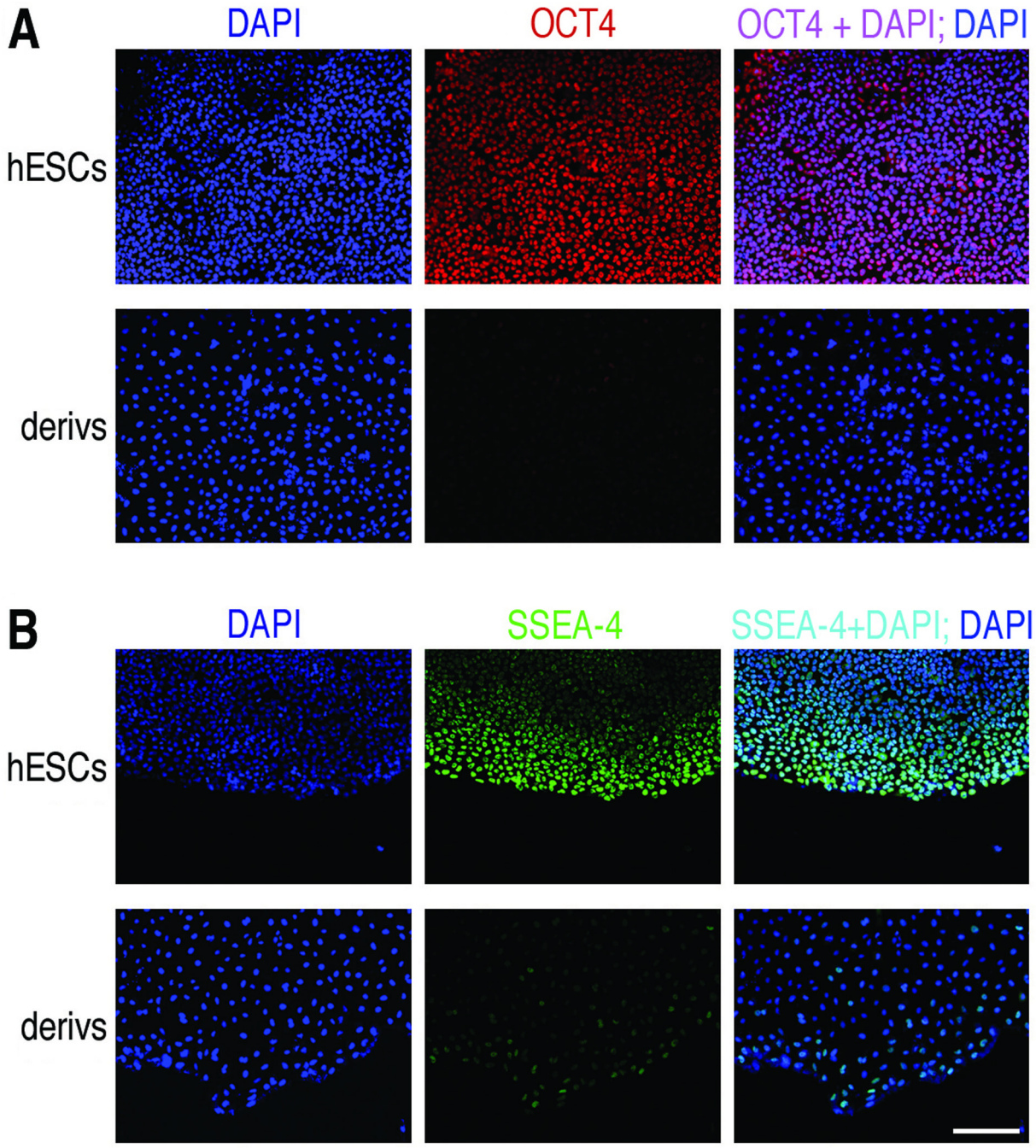

Figure 1. Undifferentiated hESCs Expressed Markers of Pluripotency, whereas the Markers were Down-Regulated upon Differentiation

Cells were cultured to yield undifferentiated hESCs ("hESCs"), or differentiated hESC-derivatives ("derivs") under feeder-free conditions by withdrawing bFGF and including 5 μM RA in the media for the final 4 days of culture. Nuclei were stained with DAPI **(A, B; left column)**. **(A)** Cells were stained with antibodies detecting OCT4 **(center column)**, and OCT4 and DAPI images were merged **(right column)**. **(B)** Cells were stained with antibodies detecting SSEA-4 (**center column**), and SSEA-4 and DAPI images were merged **(right column)**. All photomicrographs were at the same magnification. The scale bar represents 50 μM.

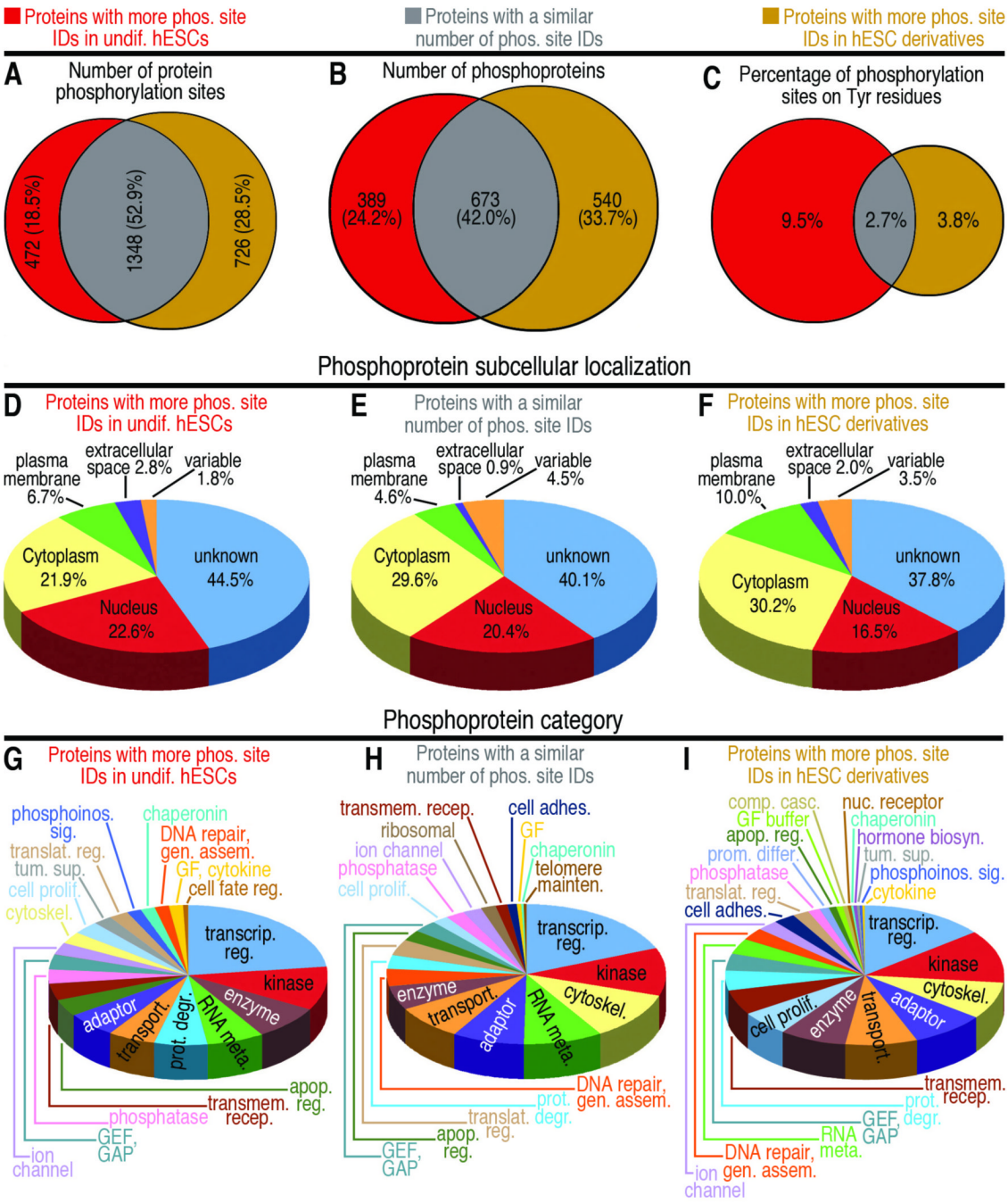

Figure 2. Number of Protein Phosphorylation Sites and Phosphoproteins Identified in hESCs and their Differentiated Derivatives, Prominence of Tyrosine Phosphorylation, Predicted Sub-Cellular Location of the Phosphoproteins and Phosphoprotein Categories

(A) Total number of non-redundant phosphorylation sites and **(B)** number of proteins with more phosphorylation site identifications in undifferentiated hESCs (line H1/WA01) (represented in red), RA-differentiated, H1-hESC-derivatives (represented in gold) or with a similar number of phosphorylation site identifications in the two cell populations (represented in gray). The percentage of the phosphorylation sites and phosphoproteins in each of the 3 groups of proteins is shown in parentheses. **(C)** Percentage of non-redundant Tyr phosphorylation sites, among the sites for which the phosphorylated residue could be defined

as Ser, Thr or Tyr (94% of all sites), that were on proteins containing more identified sites in undifferentiated hESCs, differentiated hESC derivatives or that were on proteins with a similar number of identified sites between undifferentiated and differentiated cells. **(D–F)** The sub-cellular localization of the phosphoproteins is shown; those widely associated with more than one sub-cellular location are designated as variable. **(G–I)** Phosphoprotein categories, among those whose functions are known, are shown. The percentage of proteins with known functions are 45.8, 55.7 and 57.2% for proteins with more phosphorylation site identifications in undifferentiated hESCs, differentiated hESC-derivatives or a similar number of phosphorylation site identifications between the 2 cell populations, respectively. Each chart progresses from the protein category containing the most to the fewest entries.
Abbreviations and definitions: transcript. reg. transcription regulator; enzyme, protein with enzymatic activity outside of the other categories; RNA meta., RNA-binding proteins and proteins participating in metabolic processes involving RNA; prot. degr., protein degradation; transport., transporter; apop. reg., apoptosis regulator; transmem. recep., transmembrane receptor; GEF, GAP, guanine nucleotide exchange factor, GTPase activating protein; cytoskel., proteins that are components of, closely associated with, or regulate cytoskeletal function; cell prolif., proteins participating in regulation of cellular proliferation and/or cell cycle progression; tum. sup., tumor suppressor; translat. reg., translation regulator; phosphoinos. sig., proteins participating in phosphoinositide signaling; gen. assem., genome assembly; GF, growth factor; cell adhes., proteins functioning in cell adhesion; telomere mainten., protein functioning in telomere maintenance; prom. differ., proteins promoting cellular differentiation; GF buffer, proteins regulating the availability of growth factors; comp. casc., complement cascade; nuc. receptor, ligand-dependent nuclear receptor; and hormone biosyn., hormone biosynthesis.

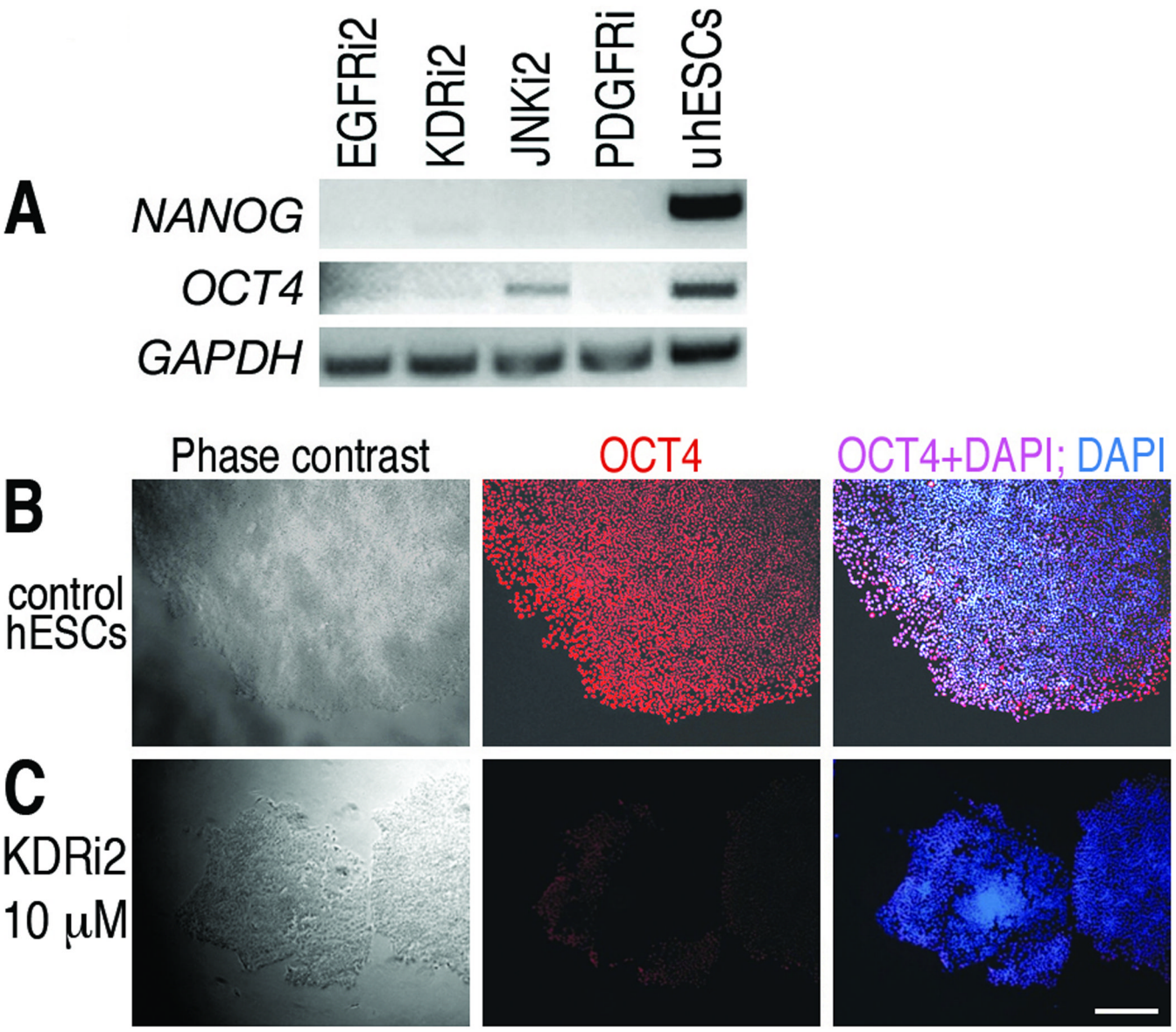

Figure 3. Protein Kinase Inhibitors Resulted in Differentiation of hESCs

(A) Expression of *NANOG* (Chambers et al., 2003) and *OCT4* mRNAs was assessed by RT-PCR, in the presence of protein kinase inhibitors that resulted in differentiation of hESCs. Cells were cultured with 20 ng/ml of bFGF, and inhibitors (10 μM) were included in the cultures for the final 4 days. Inhibitor identities are indicated in the figure. Slower decline of *OCT4* than *NANOG* was typically observed during hESC differentiation. *GAPDH* was an internal control. **(B, C)** Undifferentiated, vehicle-only control **(B)** and differentiated, KDR inhibitor-treated **(C)** cells are shown under imaging conditions indicated above the columns. All photomicrographs were at the same magnification, and the scale bar (bottom right) represents 50 μM **(B, C)**. **Abbreviations**: i, inhibitor; uhESCs, undifferentiated hESCs.

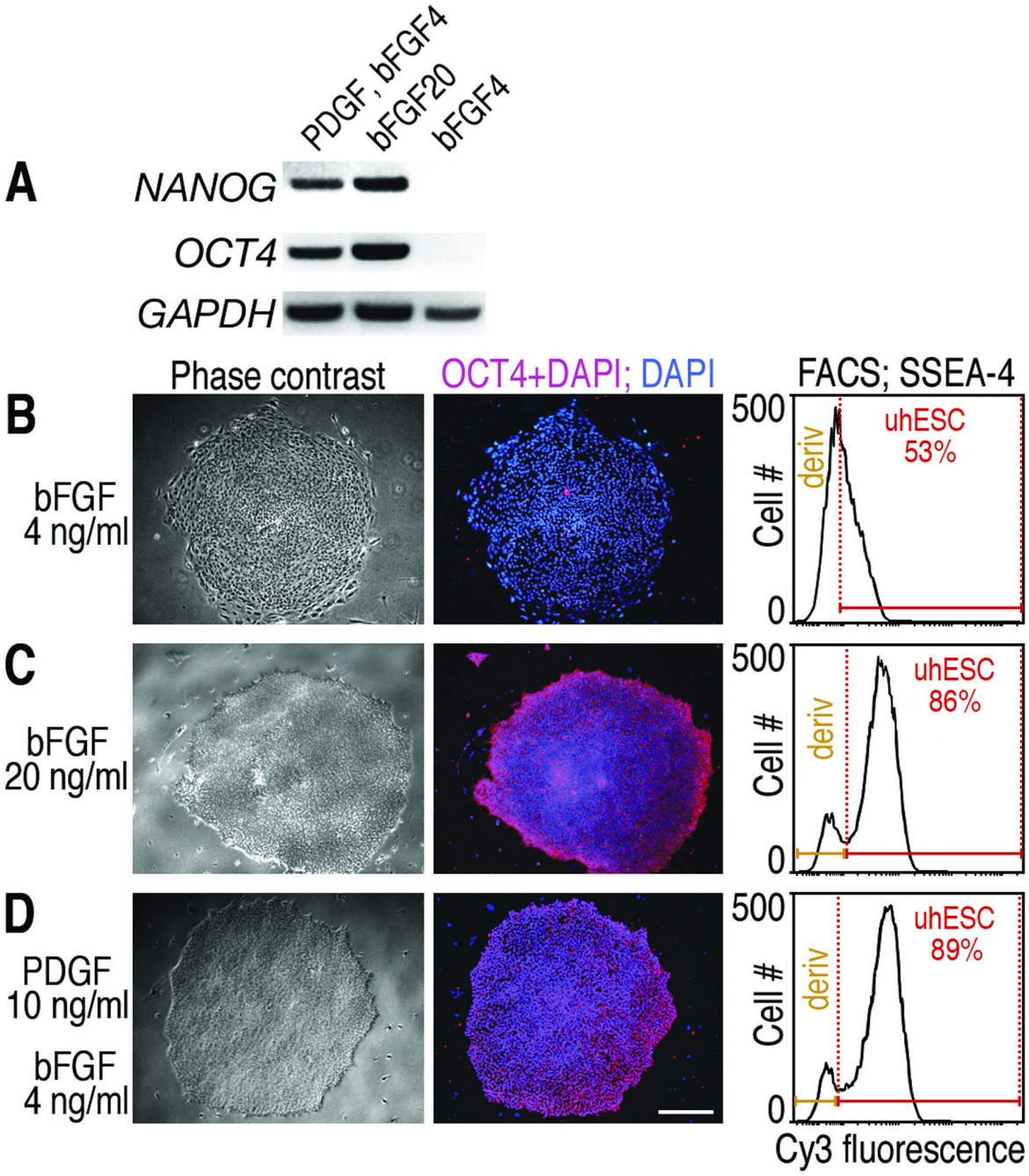

Figure 4. PDGF and a Sub-threshold Concentration of bFGF Sustained Long-Term Culture of hESCs

(A). RT-PCR to amplify *NANOG* and *OCT4* transcripts in long-term hESC cultures (>15 passages) in CDM containing 10 ng/ml of PDGF-AA and 4 ng/ml of bFGF (lane PDGF, bFGF4). Lanes bFGF20 or bFGF4 refer to 20 or 4 ng/ml of bFGF in the CDM for 4 days, respectively, in the absence of PDGF, following culture in 10 ng/ml of PDGF-AA and 4 ng/ml of bFGF for >15 passages. **(B–D)**: Colony morphology, OCT4-staining and fluorescence-activated cell sorting (FACS) demonstrated that PDGF/bFGF in CDM maintained undifferentiated hESCs passaged >15 times. Imaging conditions or FACS analyses of SSEA-4 expression, detected *via* Cy3 conjugated secondary antibodies, is indicated above the columns,

and the culture additives that were varied are indicated beside the rows. In FACS plots, dotted lines delineate boundaries of fluorescence intensity approximately indicative of cellular identity as undifferentiated hESCs ("uhESC"), and differentiated hESC-derivatives ("deriv"). Decline of SSEA-4 is incomplete in differentiated hESCs after 4 days (Figure 1). Following maintenance of the hESCs in CDM containing bFGF at 4 ng/ml and PDGF-AA at 10 ng/ml for >15 passages, cells were cultured for 4 d in CDM lacking PDGF and containing bFGF at 4 ng/ml **(B)** or 20 ng/ml **(C)**, or in the continued presence of bFGF at 4 ng/ml and PDGF-AA at 10 ng/ml **(D)**. All photomicrographs were at the same magnification, and the scale bar (bottom center panel) represents 100 μM **(B–D)**.

TABLE 1

Signaling proteins with more phosphorylation site identifications in undifferentiated hESCs than in hESC derivatives*

Proteins participating in Receptor Tyrosine Kinase signaling				
Receptors, Growth factors	**Kinases**	**Phospholipases**	**Adaptors**	**Other**
AREG, KDR, IGF2R, EPHA1	LCK, NEK4, MAPK6, MAPK7, FRAP1 (mTOR), PI3KC3, PI3KR4, DBF4, CDC42BPA, CRKL, MINK1, KIAA1804 (MLK4), CDKL5, EIF2AK1, CRKRS	PLCG1, PLCG2, PLCH1	SHC1, GAB1, NCK2, KIAA1303 (RAPTOR), CNKSR1, CNKSR2, ABI2, CDC37L1, PLEKHA1	PPAP2B, EPS15L1, TRAF4, APC, CDH17, IGFBP2, RAPGEF1, TRIP10, TSC1, WDR62, NUMB

Signal transduction pathways and member proteins	
MAPK: **JNK****, ERK	**CRKL, MINK1, KIAA1804 (MLK4), TRAF4**, TRIP10, WDR62, CNKSR1, DBF4, CDC42BPA, RAPGEF1, PLCG1, SHC1, PLCG2, GAB1, LCK, MAPK6, MAPK7, NEK4, NCK2
PI3K/AKT/mTOR	FRAP1 (mTOR), TSC1, GAB1, PIK3C3, PLCG2, PIK3R4, KIAA1303 (RAPTOR), ANRT (HIF-1β)

* Official symbols of the proteins, some of which are followed by synonyms in parenthesis, are used in this table.

** Symbols in bold text represent proteins that are relatively specific to JNK signaling.