

remain after (or have been selected by) treatment.

A prevailing view of carcinogenesis is of a stepwise accumulation of transforming genetic changes that arise in the progeny of an initially normal cell that has been exposed to some mutagenizing event. This “linear progression” of sequentially more mutated progeny is thought to culminate in a cell capable of sustaining the cancer (the CSC).

However, the variability in tumor-initiating capacities observed when the various cellular compartments of pretreatment cancer tissues are tested in different mouse recipients could imply that cells capable of sustaining cancer are heterogeneous and that multiple subclones capable of tumor initiation arise in parallel (as opposed to a strict linear evolution) and compete for dominance in a Darwinian fashion. Molecular genetic evidence for such heterogeneity has come from the study of lymphoid malignancies where global sequencing exposes molecular variability within individual tumors, suggesting that they are comprised of interrelated subclones derived from common ancestors (Campbell et al., 2008). Those clones that win the

initial battle of selection during carcinogenesis may not be the ones that survive/are selected by chemotherapy and which drive relapse.

Circumstantial evidence for the emergence of relapse propagating clones (RPCs) has come from single-nucleotide polymorphism studies of paired diagnostic and relapse samples from patients with childhood leukemia (Mullighan et al., 2008). Here, the genetic abnormalities that were dominant at the time of disease relapse often differed from those detected at presentation, when they represented a minor component of the disease.

The functional and molecular characterization of RPCs would be subject to the same limitations as for CSCs and would additionally require diligent archiving of matched diagnostic, remission, and relapse material.

This notion serves to underline the fact that patients represent the best “test tubes” for such work and may point the way forward for this fascinating field.

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Unraveling the Human Embryonic Stem Cell Phosphoproteome

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The human embryonic stem cell (hESC) transcriptome is well described, but minimal proteome characterization is available. In this issue of *Cell Stem Cell*, Brill et al. (2009) and Van Hoof et al. (2009) describe the hESC phosphoproteome and its changes upon differentiation.

Human embryonic stem cells (hESCs) are capable of differentiation into all lineages of the body, but directed differentiation to pure populations of cells has proven difficult to accomplish. Ideally, hESCs could be coerced to a particular lineage by making a series of changes to their

culture environment. These changes could be mediated via the application of growth factors, small molecules, and other effectors of cellular response, resulting ultimately in a signaling cascade(s) that directs differentiation along a particular path. Major transducers of these

environmental stimuli are the protein kinases, which transfer information by adding phosphate groups to Ser, Thr, and Tyr residues of proteins to create differences in the biochemical properties of their targets and thus their binding affinities (Pawson and Nash, 2003).

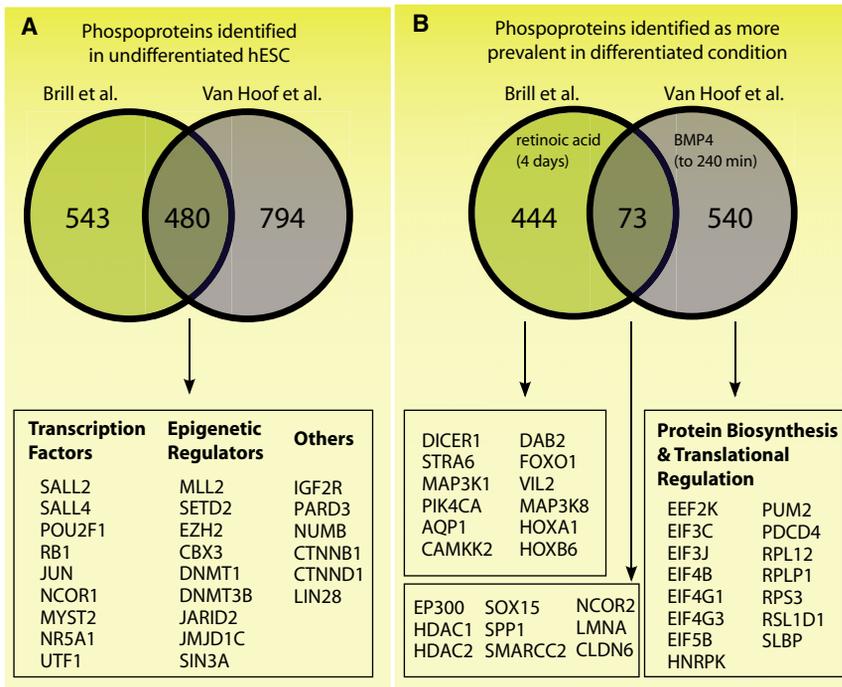


Figure 1. Comparison of the Two Phosphoproteomic Studies

A comparison between the phosphopeptides identified in Brill et al. (2009) and Van Hoof et al. (2009). Phosphopeptides identified in either study were overlapped via their gene symbol.

(A) Venn diagram showing the overlap of gene symbols between the two phosphoproteome studies and a selected list of phosphoproteins identified in both studies.

(B) A Venn diagram of the overlap between phosphoproteins identified as being upregulated in both studies when hESCs were differentiated with retinoic acid or BMP4, and a selected list of phosphoproteins identified in either one or both phosphoprotein list.

Identifying these information transfer points and how they change in response to stimuli is key to understanding and manipulating the biology of a cell. The interrogation of the phosphoproteome has been successful in helping unravel the role of cell signaling in controlling cell behavior in other cell types (Olsen et al., 2006). The characterization of the hESC phosphoproteome would provide an invaluable resource in designing rational approaches that bring about changes in cellular differentiation (or maintain pluripotency) and so allow precise control of hESC fate.

Comprehensive analyses of the hESC transcriptome and its regulation have yielded significant insight into the nature of pluripotency, but control at this level is only part of the story. Often it is assumed that if mRNA is present, so too is the protein. However, recent evidence suggests that substantial regulation of protein synthesis is mediated via translational control, at least during mouse ESC differentiation (Sampath et al., 2008).

Thus, it is essential to describe the proteome to uncover the functional units of the hESC protein-coding transcriptome. There has recently been some characterization of the hESC proteome (Swaney et al., 2008); however, to date, a systematic study of the hESC phosphoproteome has not been reported. In this issue, two independent research groups remedy this situation by using mass spectrometric approaches to analyze the phosphoproteome of hESCs, as well as tracking dynamic changes to these profiles upon exposure to differentiating stimuli (Brill et al., 2009; Van Hoof et al., 2009).

In one case, Ding and colleagues took advantage of multidimensional liquid chromatography-based mass spectrometry to identify phosphorylated peptides. The authors performed this analysis for two populations: undifferentiated hESCs (WiCell's H1 line) and hESCs differentiated for 4 days by the addition of retinoic acid. They identify 2546 phosphorylation sites on 1662 proteins (Brill et al., 2009). Krijgsveld and colleagues, in contrast,

used stable isotope labeling by amino acids in cell culture (SILAC) to measure relative quantitation of the phosphoproteins present in Harvard's HUES-7 hESC line and how the levels of these phosphoproteins change upon induction of differentiation with addition of BMP4 for 30, 60, and 240 min. The authors identify a total of 5222 proteins of which 1399 of these contained a total of 3067 phosphorylation sites (Van Hoof et al., 2009). A third phosphoproteome study was recently described providing a collective analysis of 10,844 unique phosphosites in undifferentiated hESC (Swaney et al., 2009), though specific phosphorylation sites were not disclosed and dynamic changes were not analyzed.

Kinase-mediated signaling events occur very rapidly (often on the order of seconds to minutes; Olsen et al., 2006), so neither group aimed to identify the primary events occurring upon the addition of their respective differentiation stimuli. The differentiated state was used rather as a reference to uncover phosphoproteins enriched or reduced in the undifferentiated state. The Van Hoof study, however, with its shorter time course of differentiation, was able to capture the cascade of phosphorylation events downstream of BMP/Smad signaling. Note, though, that both studies remove FGF2 from the differentiation media so that the interpretation of the data must take into account the loss of this receptor tyrosine kinase activator. Interestingly, both studies find significantly higher incidence of phosphorylation in the differentiating cells. This trend is perhaps not surprising, given that the comparison was between a cell population experiencing the dynamic changes that accompany differentiation (and presumably that require significant intracellular communication) and one in a relatively stable phenotype. In total, the Ding and Krijgsveld groups characterized 929 and 1091 phosphorylation sites, respectively, that exhibited differences between the undifferentiated and differentiated states.

Both groups probed the phosphoproteome of undifferentiated hESCs, so one might expect to detect some overlap between data sets. Indeed, an overlap between the two studies is observed, and the specific hits include transcription factors, epigenetic modifiers, as well as many other functional classes of

proteins (Figure 1A). Considering the significant differences between the methods used, and the analysis of distinct hESC lines between studies, the 35% overlap detected seems to be of some significance, and in line with previous comparisons (Olsen et al., 2006). At the same time, given the different methods of differentiation utilized by the two groups, the fact that BMP4 induction promotes extraembryonic lineages (Xu et al., 2002; Pera et al., 2004) whereas retinoic acid directs hESCs toward an ectoderm cell fate (Wichterle et al., 2002), and differing time points examined in each case, it should not be surprising that we see much less overlap (7%) between the two differentiated data sets (Figure 1B). However, it is interesting to note that a significant number of protein synthesis and translation regulators are found differentially phosphorylated within only 240 min of differentiation (a majority within as few as 30 min) (Figure 1B). This pattern implies that FGF removal and/or addition of BMP4 also regulate the cell at the translational level in addition to the transcriptional level.

Needless to say, both papers provide a substantial amount of data that stem cell scientists can mine for the purpose

of developing new hypotheses. These models can then be tested for their potential roles in the control of the undifferentiated state or in the initial steps of differentiation. Brill et al. (2009) undertake this approach by identifying additional receptor tyrosine kinases activated in hESCs and, in doing so, reveal an effect of PDGF in the maintenance of pluripotency. Meanwhile, Van Hoof et al. (2009) identify a phosphorylation site on Sox2 that mediates SUMOylation, potentially providing a mechanism to overcome the stem cell regulatory circuitry during the initial phase of differentiation.

Many of the phosphorylation sites identified in these studies remain uncharacterized, and their functions unknown, and yet describing these data sets is only an initial step in characterizing the hESC phosphoproteome, given that Swaney et al. (2009) have recently identified thousands more sites. Ultimately these types of studies will provide sufficient phosphoproteome resources to allow the stem cell community to integrate cellular regulation at all levels of control and achieve mastery over the hESC and its fate choices. It may be a daunting task, but it is exciting to see the progress made thus far.

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CD95/Fas in the Brain—Not Just a Killer

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Although CD95 (Fas/Apo-1) has long been known to be broadly expressed in the brain, its function has remained enigmatic. In this issue of *Cell Stem Cell*, Corsini et al. (2009) now show that CD95 serves as a potent activator of neurogenesis in both the healthy and injured brain.

CD95 is the best characterized and paradigmatic member of the TNF-receptor superfamily of “death receptors,” and the molecular mechanism of CD95-induced apoptosis is known specifically. After binding of CD95L, CD95 forms trimers and sequentially recruits the adaptor protein FADD, regulatory proteins (like DAXX or

FLIP), and procaspase 8, leading to the formation of a death-inducing complex (DISC). The oligomerization then results in the autoproteolytic cleavage of procaspase 8 and the initiation of the apoptotic cascade (Peter and Krammer, 2003). In the central nervous system, CD95 expression varies significantly during develop-

ment. In the adult brain, neurons in the hippocampus and cerebral cortex show the highest CD95 expression, although CD95 expression is also detectable on astrocytes and oligodendrocytes especially under pathological conditions. Conversely, the cognate ligand CD95L is constitutively coexpressed on neurons