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Modeling Human Neural Development Using Pluripotent Stem Cells



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# Modeling Human Neural Development Using Pluripotent Stem Cells

A dissertation submitted in partial satisfaction of the
requirements for the degree Doctor of Philosophy
in Molecular, Cell and Developmental Biology

by

Michaela Cyr Patterson

2012

## ABSTRACT OF THE DISSERTATION

Modeling Human Neural Development Using Pluripotent Stem Cells

by

Michaela Cyr Patterson
Doctor of Philosophy in Molecular, Cell and Developmental Biology
University of California, Los Angeles, 2012
Professor William Lowry, Chair

Human pluripotent stem cells (hPSCs) are derived from the developing blastocyst or through transcription factor based reprogramming. hPSCs have the capacity to self-renew and give rise to all cell types of the human body. These two defining characteristics make them attractive for a variety of uses including regenerative medicine and modeling human development and disease. While it is clear that hPSCs can differentiate into all cell types, it is unknown how similar hPSC derivatives are to cells that have undergone development *in vivo*. Using previously established differentiation protocols we demonstrated that both types of hPSCs could generate functional motor neurons that displayed repetitive action potentials characteristic of mature motor neurons. Furthermore, these motor neurons differentiated through a progressive and predictable induction of developmental stages, suggesting hPSC differentiation appears to obey normal developmental progression. In a comprehensive comparison of hPSC derivatives

and tissue-derived counterparts, we determined that the progeny of hPSCs are most similar to a cell type found prior to 6 weeks of gestation. These hPSC derivatives continued to express genes normally found at 3-5 weeks of gestation, including *LIN28*; while tissue derived counterparts had already silenced these genes. *In vitro* function demonstrated that hPSC derivatives also acted like developmentally primitive cells. These findings suggest that developmental timing is conserved *in vitro* and perhaps even innate to the differentiating cell. Finally, we explored the role of the *LIN28*/let-7 pathway on the developmental maturity of hPSC-derived neural progenitor cells (hPSC-NPCs) as measured by their ability to generate neurons or glia. We determined that addition of let-7s into hPSC-NPCs can correct a significant number of the genes differentially expressed between hPSC derivatives and tissue-derived counterparts. Furthermore, the manipulation has a modest, but significant effect on the functional maturity of PSC-NPCs. Taken together, these data demonstrate that hPSCs can serve as an excellent and tractable model of human development.

The dissertation of Michaela Cyr Patterson is approved.

Alvaro Sagasti

Karen Lyons

Harley Kornblum

William Lowry, Chair

University of California, Los Angeles
2012

I dedicate this dissertation to my grandfather, John Stanley Patterson.

He has forever instilled in me the importance of love and family.

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## POSTERS AND PRESENTATIONS

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# CHAPTER 1:

# Introduction

**Preface**

Human pluripotent stem cells (hPSCs) have two defining characteristics: 1) the ability to self-renew; and 2) the capacity to give rise to all cell types of the human body. These attributes make them an attractive biological tool to clinicians and research scientists alike.

One proposed use of hPSCs is for regenerative medicine or cell transplantation, whereby disease relevant cell types can be generated from hPSCs and then transplanted into a patient who lost those cells to disease or injury. Parkinson's disease and type-I diabetes are prime examples of diseases that may benefit from this proposed technique. In each case, a specific cell type, dopamine neurons or pancreatic β-cells respectively, is lost to disease. There are no known cures for either disease, and alternative sources of cells for transplantation are scarce or non-existent. hPSC technology could provide an unlimited source of these cells for the purpose of transplantation into patients.

Another use would be for drug discovery and toxicity testing. Currently, the field of drug discovery is hampered by inadequate tools and cell sources for testing efficacy and possible toxicity of drugs prior to advancing to clinical trials. Cellular based screening is typically performed on accessible primary cells or, more commonly, on established cell lines. More relevant cell types, those affected by the disease itself, are rarely accessible or used. Furthermore, unless drugs are tested on a variety of cell types it will remain unknown if they have toxic side effects on other tissues. Animal models can be a good tool for understanding how a drug might affect an entire organism, however they are expensive and the results do not always mirror what happens in humans. hPSCs could serve as a cost-effective tool for testing the efficacy and toxicity on a variety of human cell types, so we can better anticipate how a drug will act once it advances to the clinic.

Finally, hPSCs can be exploited to better understand human development and disease. As a scientific community, we rely heavily on animal models to understand how an organism or even a tissue is generated, however developmental and disease mechanisms do not always translate from one organism to another. Furthermore, capturing specific transient events can be challenging when using a whole animal. hPSCs are an *in vitro* tool thought to reflect the embryonic epiblast state, which can give rise to all the cells of an entire organism. Theoretically, we can follow the differentiation of hPSCs to a specific cell lineage to better understand the molecular mechanisms required to generate that cell type. The fact that these events occur in a petri dish could make them a tractable model of human development.

These three proposed uses of hPSCs make them an exciting biological tool and it is no wonder that the number of PSC-related publications and funding opportunities have steadily grown over the last decade. However, there is still much to understand about these potent cells and the derivatives they make. In this chapter, I will provide background information on hPSCs and their progeny. I will discuss the advances in the field and identify the areas where more focus is warranted. Finally, I will introduce the aims of this dissertation and our contributions to understanding the types of cells we can derive from hPSCs.

**Pluripotent stem cell derivation and characterization**

There are two main sources of hPSCs. Embryonic stem cells (ESCs), isolated from a pre-implantation embryo, represent the gold-standard PSC. Induced pluripotent stem cells (iPSC), generated through the reprogramming of a somatic cell to a pluripotent state, represent an *in vitro*, artificially generated PSC. The isolation and characterization of both sources will be discussed further here.

*Human Embryonic Stem Cells (hESCs)*

ESCs are isolated from the inner cell mass (ICM) of a pre-implantation blastocyst. They were first isolated from mouse embryos in 1981[1-2], followed by non-human primates in 1995[3] and finally from human blastocysts in 1998[4]. Most hESC lines are generated from discarded embryos produced by in vitro fertilization (IVF) for clinical purposes.

Remarkably, cells of the ICM only have a transient capacity to self-renew as they begin to differentiate within a day; however when placed into culture, these cells can be passaged indefinitely and have high telomerase activity indicative of their immortality and self-renewing capacity[4]. Typically they grow in flat, tightly compact colonies with well-defined borders. hESCs can spontaneously differentiate into all three embryonic germ layers. This pluripotency is typically measured two ways: 1) *in vitro* embryoid body formation, where undifferentiated colonies are removed from their feeder layer and maintenance growth factors thus allowing them to form a differentiating sphere of cells containing all three germ layers; and 2) *in vivo* teratoma formation, where undifferentiated cells are injected into an immune compromised mouse resulting in large benign tumors containing cell types representing all three germ layers. A third method, accessible only in animal models, entails the generation of a chimeric offspring or a complete animal through tetraploid complementation; however for ethical reasons this cannot be repeated for hPSCs.

Since the isolation of the first hESC lines, we have learned a great deal about the mechanisms that control their pluripotency and self-renewal. Originally, three key transcription factors, *OCT3/4*, *SOX2*, and *NANOG*, were individually identified for their involvement in the maintenance of the undifferentiated state[5-8]. It is now believed that these factors work in unison

as regulatory factors that positively regulate each other's expression and that of the other genes required for self-renewal[9].

While hESCs have great promise as a source of hPSCs and they remain the gold standard in the field, there are many technical limitations associated with their clinical use. First, as previously discussed, they are isolated from discarded embryos derived for IVF purposes. These embryos are sometimes damaged or considered less than ideal for implantation, which could suggest that they are less than ideal for generation of hESCs as well. Second, it takes many passages to establish a new line and genetic abnormalities increase with continued passaging (discussed further in a later section). Currently, there is little to no access to low passages of available hESC lines. Finally, should hESCs be used for tissue regeneration they would not be a genetic match to the recipient and therefore patients would require immunosuppressive drugs to avoid graft rejection.

*Human Induced Pluripotent Stem Cells (hiPSC)*

Decades before ESCs were isolated, Gurdon and colleagues demonstrated in xenopus that an enucleated oocyte had the capacity to reprogram a somatic cell nucleus to the undifferentiated state in a process now called somatic cell nuclear transfer (SCNT)[10]. In 2006, Takahashi and Yamanaka set out to identify factors that could be responsible for this reprograming event. They started with twenty-four candidate genes specifically expressed in ESCs and asked if any individual or combination of those genes could reprogram somatic cells into PSCs. Remarkably, they determined that just four transcription factors, *OCT3/4*, *SOX2, KLF4,* and *C-MYC*, when virally transduced into mouse fibroblasts could reprogram them into a pluripotent, embryonic-like state[11].

Within a year, four groups repeated the experiment on human fibroblasts[12-15]. The resulting induced pluripotent stem cells (hiPSC) had seemingly similar characteristics to hESCs: they grew as tightly compact colonies with well-defined borders; they could be passaged indefinitely and had high telomerase activity; and they expressed markers of hESCs and even turned on the endogenous locus for the virally transduced genes[12]. hiPSC could form both embryoid bodies and teratomas, thereby meeting the gold standard for human pluripotency.

**hESCs versus hiPSC as a biological tool**

hiPSC technology may have advantages over hESCs because of several defining features. The first benefit is that hiPSC can be derived from patients harboring a genetic disease. Disease-specific hiPSCs could allow us to model diseases in a petri dish to better understand the mechanisms that cause the disease. Furthermore, the disease-specific hiPSCs could be used for drug screens. Not only can these cells generate disease-relevant cell types, as previously discussed, but they would also contain the specific genetic mutation that causes the disease. Therefore drug efficacy could easily be tested on disease-relevant cells harboring the disease causing lesion (Figure 1)[16]. hiPSC lines have now been generated from patients with ALS[17], SMA[18], Rett syndrome[19] and many others.

Another benefit is that hiPSC lines can be patient specific. Theoretically, they can be generated from each individual, such that if a patient requires transplantation, hiPSCs can be derived from that patient, forced to become the desired cell type and transplanted back into the same patient with lower risk of immune rejection. Furthermore, as the technology improves, the patient's genetic mutation, which caused their disease in the first place, can be corrected prior to

transplantation (Figure 1). This theoretical process has already been achieved in mouse iPSCs (miPSC) harboring the mutation for sickle cell disease[20].

Despite significant progress in reprogramming methodology, hiPSC technology does carry substantial risks to clinical application. hiPSC were originally derived through retroviral integration of the four transcription factors previous described. The use of retroviruses creates random integrations in the host genome, which could result in unwanted and potentially harmful mutations. Furthermore, some of the reprogramming genes themselves are known oncogenes. The exogenous transgenes should theoretically be silenced upon differentiation just as the endogenous pluripotency genes are; however a big concern is that the exogenous transgenes will not be under the same control and therefore may be aberrantly reactivated even after differentiation. Aberrant expression of the oncogenic transgenes in the wrong cell type could lead to tumorigenesis. Indeed, when mice were generated from miPSC to test for germline contribution, 20% of the offspring generated tumors attributable to the reactivation of the C-MYC transgene[21].

Many groups have been working on alternative methods for deriving hiPSC that would avoid these risky side effects. Some proposed alternatives include: using constructs that can be removed after successful reprogramming, so that genes cannot be reactivated later[22]; and using non-integrating methods to express the reprogramming factors such as episomal vectors[23], miRNAs[24], proteins[25], or even through small molecules[26-27].

**Differences between hESCs and hiPSCs - the undifferentiated state**

Some of the first papers stemming from Yamanka and Takahashi's finding concentrated on the similarity of hiPSC to hESCs. On a superficial level, hiPSC seemed identical to their

embryonic counterparts; they had the right morphology, expressed the right markers and could generate the same types of cells. However, their drastically different methods of derivation begged the question of how truly similar were they?

When researchers took a more global approach to identify differences between hiPSC and their embryonic counterparts, they observed subtle variances that distinguished the two sources of PSCs. One of the first studies described a transcriptional variance between hESCs and hiPSC regardless of which lab had generated the hiPSC clones[28]. Chin et al. pointed out that while hiPSC were more similar to hESC than to their parental fibroblasts, they still retained a transcriptional signature akin to the parental cell type. Interestingly, early passage hiPSCs (<p10) had greater retention of this parental fate, while later passage hiPSCs (>p30) had corrected some, but not all, of the discrepancies. Furthermore, similar results were found when comparing miPSC to mESCs[28]. This suggests that there is an incomplete resetting of the parental fate that is universal to the reprogramming process. Many other groups have confirmed these observations even when alternative donor cell types and alternative reprogramming methods are used (29-31) and some have offered possible mechanisms underlying these observed transcriptional differences.

*Genomic aberrations*

One explanation as to why hiPSC are transcriptionally different from hESCs is that the reprogramming process introduces genetic mutations into the clones. Chin et al. reported that there were no conserved genomic abnormalities when comparing hESCs to hiPSC by gross karyotyping[28]. However, when more sensitive, genomic sequencing of protein coding regions was instead performed, it was found that hiPSC had a higher level of mutational load compared

to their parental fibroblasts[32]. In this study, Gore et al. did not sequence hESC lines so it is unclear if the additional mutations were the result of the reprogramming event itself or simply due to continued passaging.

Another group, using high resolution genotyping, reported that hiPSCs had greater copy number variation (CNV) on average than somatic cells. In contrast, while hESCs had greater variation in CNV than somatic cells, the average CNV was not significantly higher[33]. In addition, Laurent et al. determined that the CNV changes were a dynamic process during reprogramming. Low passage hiPSC had high percentage of deletions in tumor suppressor genes, and some of these aberrations were even necessary for efficient reprogramming. On the other hand, late passage hiPSC had corrected some of the observed tumor suppressor deletions and instead displayed an increase in duplications of proliferation genes. These duplications were also observed in the variable hESC lines. These findings together suggest that while reprogramming is a likely cause of some of the observed genomic aberrations, continued time in culture is also a probable source.

*Epigenetic Memory*

While genetic mutations could theoretically affect the transcriptional output of cells and therefore could explain some of the gene expression differences between hESCs and hiPSCs, they do not fully account for the observation that a significant portion of the differences resulted from failure to silence the parental cell's transcriptome. An alternative mechanism that has been proposed is that the epigenetic marks known to control gene expression are incompletely rewritten during the reprogramming process and as a result hiPSC retain an epigenetic memory of their parental cell. Several groups have provided evidence supporting this hypothesis.

Histone modifications are one such example of an epigenetic mark that can contribute either positively or negatively to gene expression. For example, Histone3 lysine27 tri-methylation (H3K27me3) correlates with repressed genes. Chin et al. and Hawkins et al. both determined that this mark was almost completely reset to an hESC state during the reprogramming process[28, 34], suggesting it cannot account for the transcriptional differences observed between the two sources of hPSCs. On the other hand, H3K9me3, another repressive mark, tagged a significant number of promoters in hiPSC, 68% of which were not tagged in hESCs[34]. Furthermore, the genes corresponding to these aberrantly marked promoters significantly overlapped with the original list of genes that Chin et al. identified in their comparison of hESC and hiPSC. This finding suggests that aberrant H3K9me3 repressive marks contribute to a greater portion of the gene expression differences than H3K27me3. On a more global level, it also suggests that specific histone modifications may be more relevant than others.

Another type of epigenetic modification is DNA methylation. Several groups have reported the existence of differentially methylated regions (DMRs) when comparing hESCs, hiPSC, and parental somatic cells[30, 35-37]. In a thorough investigation of epigenetic memory in miPSC, one group demonstrated that miPSC derived from either fibroblasts or blood cells had a strong propensity to differentiate back into their cell of origin and were inefficient at contributing to a new lineage[38]. On the other hand, mESCs and mPSCs derived by SCNT (ntPSC) were capable of generating both cell types with good efficiency. Kim et al. later linked this observation to residual DNA methylation signatures found on miPSC that linked them back to their somatic cell of origin, suggesting the DNA methylation signature was incompletely rewritten during transcription factor induced reprogramming. Furthermore, Kim and colleagues

demonstrated that they could correct this epigenetic memory either by serial reprogramming, where cells were reprogrammed, forced to differentiate into a new lineage, and reprogrammed again; or by administering chromatin-modifying drugs during the reprogramming process[38]. They found similar results, when comparing hiPSC derived from human cord blood or human fetal keratinocytes[37]. These studies demonstrate that not only do iPSC have an epigenetic memory of their parental cell type, but that this memory also affects their capacity to differentiate into new lineages. Furthermore, their findings in mPSCs suggest that transcription factor based reprogramming may not be as faithful as nuclear transfer reprogramming.

As more papers are published and more methods are used to compare hiPSC to hESCs it has become clear that the original assessment that hiPSC are seemingly identical to their embryo-derived counterpart was naïve. hiPSC are similar, but not perfect. One hypothesis that has been offered to explain the discrepancies found between hiPSC and hESCs, is that if one compares any number of hESC lines to each other based on any criteria (i.e. epigenome, transcriptome, proteome, differentiation efficiency, etc.) there will be a level of variance between the group; no two cell lines will be perfectly identical. If the analysis is repeated for hiPSC, it appears that the variance between lines is increased, however there exist subsets of hiPSC clones that fall into the cloud of variance encompassed by hESC lines (Figure 2)[39]. This increased variance observed amongst hiPSC lines is perhaps not surprising as the number of methods for deriving the lines is vast. Furthermore, other contributing factors (i.e. sites of integration, stochastic control of transgene expression, etc.) are hard to hold constant between derivations.

The effect these discrepancies observed in the undifferentiated state have on the progeny of hiPSC following differentiation is still not well understood. As was previously described, it

appears that the differential epigenetic signatures may contribute to the efficiency of iPSC to generate specific cell lineages[37-38], but in the event that a cell of interest is generated from hiPSC, regardless of efficiency, does the resulting progeny function to the same capacity as a counterpart derived from hESCs? I will address this question further in chapters 2 and 3 of this dissertation.

**Deriving clinically relevant cell types – hPSC progeny**

Ultimately, when thinking about the proposed utility of hPSCs, it is not the undifferentiated cells that we are interested in exploiting. Instead, it is their progeny or the cells we generate from them that are clinically relevant. It is well established that hPSCs, when removed from their normal culturing conditions, can randomly differentiate into many cell lineages; however, in most cases, scientists and clinicians are instead interested in generating one pure cell type without contamination from other lineages. To achieve this, directed differentiation and purification from the mixed culture are required.

*Current methodology*

Many have applied the knowledge of mammalian development to generate sequential protocols for the accurate induction of specific cell types of interest. These protocols usually include: the addition of growth factors or small molecules to activate only those signaling pathways important for that lineage, while blocking aberrant signaling pathways (Figure 3); co-culture with a supporting cell to recapitulate the niche; and/or use of extracellular matrices and engineered substrates to produce 3-dimentional structures akin to a whole organ (reviewed in [40]).

Once a putative cell type of interest has been generated, it is important to demonstrate that it displays the unique characteristics of that lineage. Currently, several methods are used to demonstrate the fate of a cell. These methods include: morphology, expression of specific cell fate markers, *in vitro* function, and *in vivo* transplantation or rescue of an animal model of disease. Our own group demonstrated that by using differentiation protocols, established by the hESC field, we could generate motor neurons from both hESCs and hiPSCs. The resulting motor neurons had undergone a predictable induction of motor neuron lineage markers and after eight weeks could produce repetitive action potentials characteristic of mature motor neurons[41]. This work is discussed further in Chapter 2.

*Advancing the methodology*

As we observed when comparing hESCs and hiPSC, it may be dangerous to characterize a cell only by superficial examination of its traits. We may not fully appreciate the nature of a cell unless more comprehensive analyses are used. In addition, it is also important to compare the *in vitro* generated cell to an appropriate positive control, such that if it displays an identifying characteristic (i.e. the expression of a specific gene) it does so at a level comparable to the positive control. In the case of hPSC derivatives, the most appropriate control would be an equivalent cell isolated from a human tissue source. This "natural counterpart" has undergone development normally, a process which scientists are attempting to recapitulate when they differentiate hPSCs. Furthermore, these tissue-derived cells are precisely the cells that are meant to be replaced if hPSC technology advances to the clinic, so it will be important to determine if hPSC derivatives will behave like cells found in the human body. Unfortunately, this control is rarely used and so it remains unknown how similar *in vitro* differentiation of an hPSC mirrors

normal, *in vivo* development. If differences are observed between hPSC progeny and their tissue-derived counterpart it will be important to determine what consequence if any they may have. I will begin to discuss this issue in Chapters 3 and 4.

Our own work comparing three hPSC derivatives, representing each of the embryonic germ layers, to their respective tissue-derived equivalent uncovered a list of genes that were differentially expressed between the two sources regardless of cell type. The differential expression of these genes, along with functional data and microarray analysis from 3-19 week human embryos suggested that derivatives of hPSCs are most similar to a tissue derived counterpart found before 6 weeks of gestation[42]. I will address this issue further in Chapter 3 of this dissertation.

**Significance and specific aims of this dissertation**

The work presented in the next three chapters represents my own contributions to our understanding of the types of cells we are generating from hPSCs and how similar they are to a counterpart found in the human body.

*Aim I: Determine if hiPSC and hESCs, generate functional and clinically relevant cell types.*

In chapter 2 of this dissertation, I present our work demonstrating that hiPSC and hESCs can generate functional motor neurons and that they differentiate down this lineage via a progressive induction of predictable developmental markers. These findings suggest that *in vitro* differentiation appears to obey normal development. This chapter was previously published in *Stem Cells*[41] and is reprinted in its entirety here with permission from John Wiley & Sons, Inc and all co-authors.

*Aim II: Determine if hiPSC and hESC derivatives are "identical" to one another and if they represent a cell type found in the human body.*

In chapter 3, I will present our work where we globally compare hESC-, hiPSC- and tissue-derived cells. We demonstrate that derivatives of hiPSC and hESCs are nearly indistinguishable, suggesting that the differences observed in the undifferentiated state dissipate upon differentiation. Furthermore, we determine that, based on transcriptional and functional data, derivatives of either hPSC source are perhaps most similar to a cell type found prior to six weeks of gestation. These finding suggest that developmental timing during *in vitro* differentiation is faithful to *in vivo* development. This chapter was previously published in *Cell Research*[42] and is reprinted in its entirety with permission from Nature Publishing Group and all co-authors.

*Aim III: Establish a functional role for LIN28/let-7 in the developmental maturity of hPSC-derived neural progenitor cells (PSC-NPCs).*

In this final chapter, we explore the functional relevance of one of the gene pathways identified in our *Cell Research* paper as being differentially expressed between hPSC-derivatives and their respective natural counterpart. We determined that the *LIN28*/let-7 pathway can correct a significant portion of the genes differentially expressed between PSC-NPCs and fetal tissue-derived NPCs and can functionally mature PSC-NPCs, suggesting that it may be one pathway involved in the developmental timing of cells. This work is published here with permission from all co-authors.

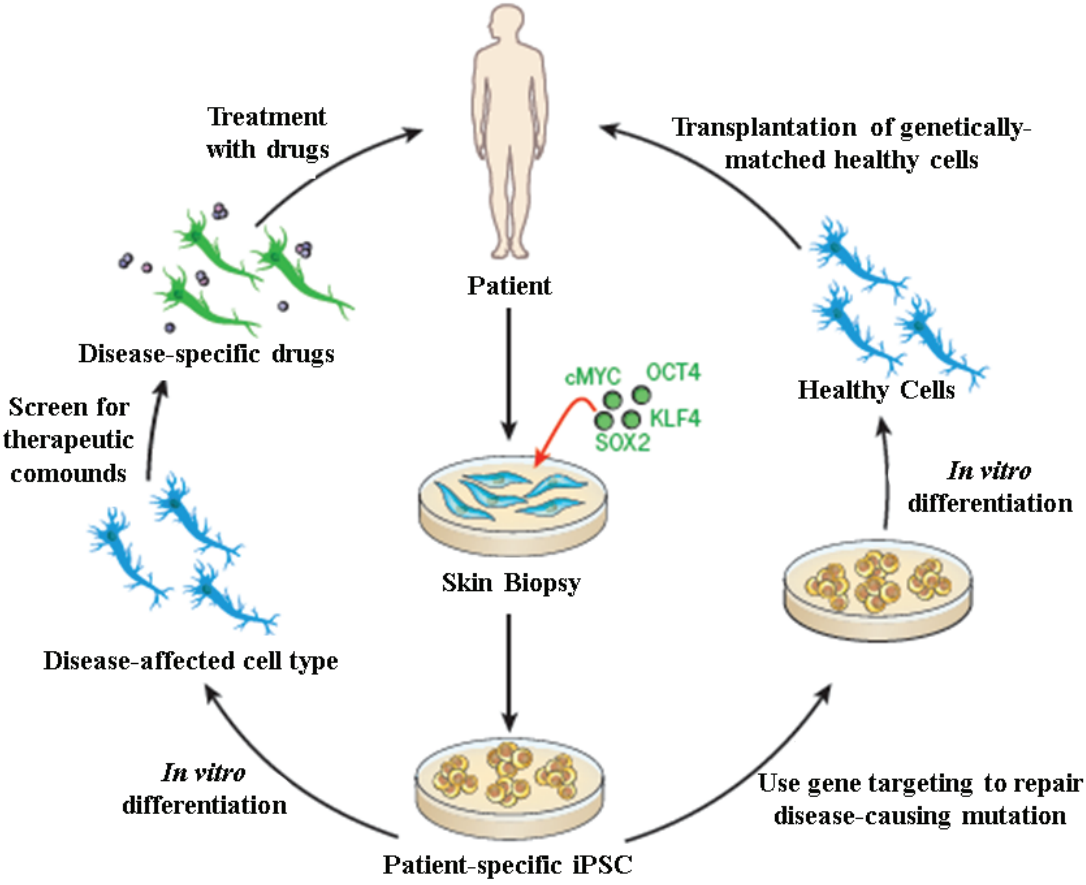


**Figure 1.1 Patient-specific treatments in the advent of iPSC technology.**
With the ability to reprogram somatic cells to the pluripotent state, a source of hPSC can now be generated from patient biopsies, thereby harboring all patient-specific genes. These iPSCs can either be used to screen for new drugs in a patient specific manner (left), or be used for tissue transplantation following correction of the disease-causing mutation (right). Adapted from Robinton and Daley[16] with permission from Nature Publishing Group.

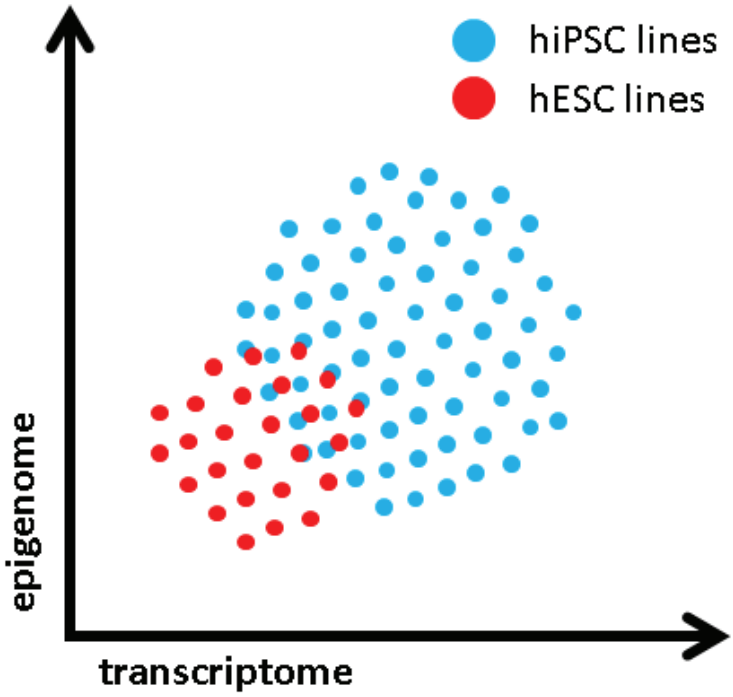


**Figure 1.2 Epigenetic and transcriptional variation between hESCs and hiPSCs.**
If hESC lines (red) are compared by any characteristics (represented here by epigenetic and transcriptional signatures) then they will display a degree of variance between lines. If the same analysis is done on hiPSC lines (blue) then the variation will be greater, however a subset of lines will fall within the normal variation of hESCs. Adapted from Bock et al.[39] with permission from Elsevier.

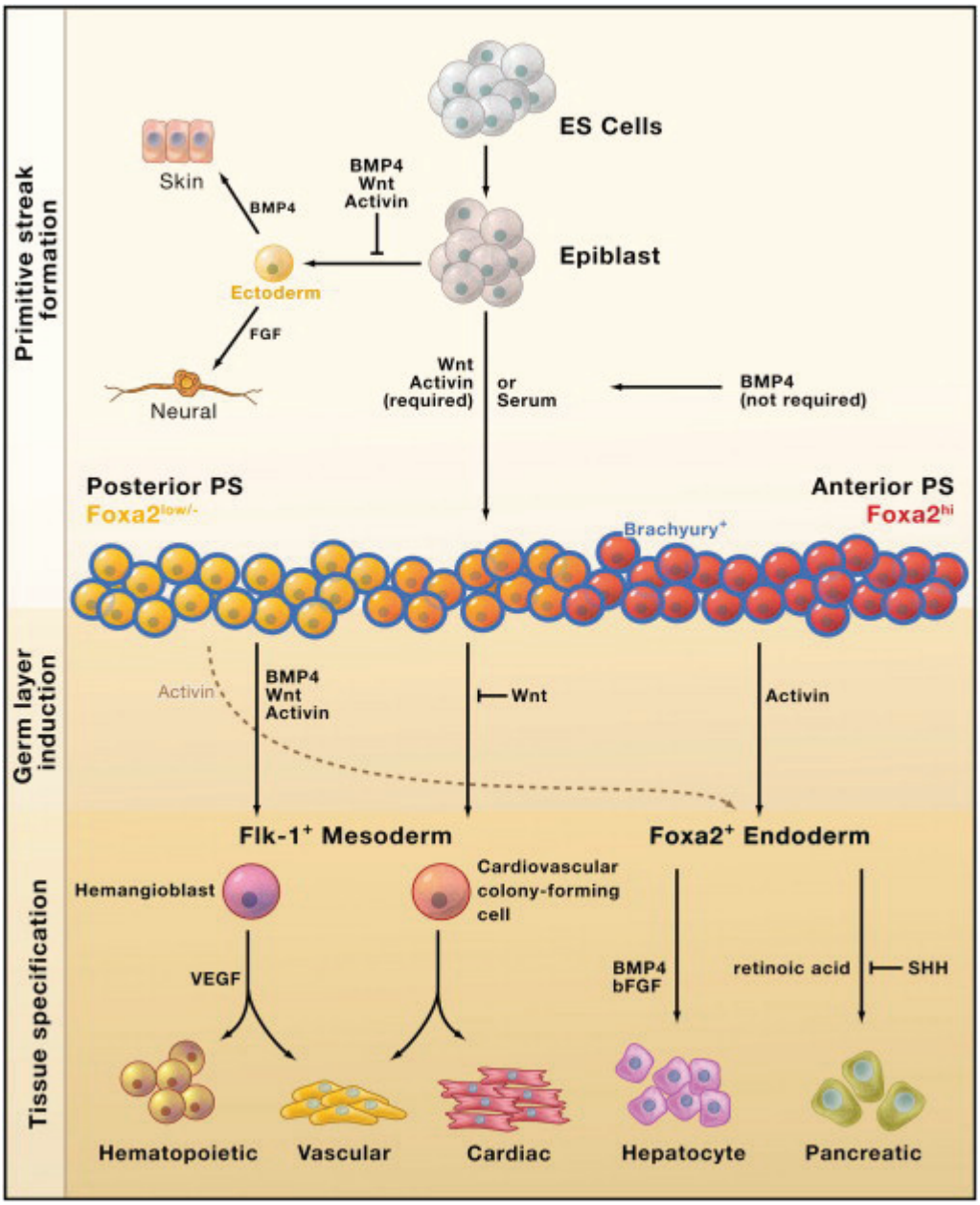


**Figure 1.3 Directed differentiation of PSCs to clinically relevant cell types.**
hESCs and hiPSC can be directed to differentiate into various cell types through the sequential addition or repression of growth factors as established by our understanding of mammalian development. Reprinted from Murray and Keller[40] with permission from Elsevier.

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# CHAPTER 2:

## Directed differentiation of human induced pluripotent stem cells generates active motor neurons

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# Directed Differentiation of Human-Induced Pluripotent Stem Cells Generates Active Motor Neurons

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**Key Words.** Embryonic stem cells • Neural differentiation • Neuron • Reprogramming • Pluripotent stem cell

ABSTRACT

**The potential for directed differentiation of human-induced pluripotent stem (iPS) cells to functional postmitotic neuronal phenotypes is unknown. Following methods shown to be effective at generating motor neurons from human embryonic stem cells (hESCs), we found that once specified to a neural lineage, human iPS cells could be differentiated to form motor neurons with a similar efficiency as hESCs. Human iPS-derived cells appeared to follow a normal developmental progression associated with motor neuron formation and possessed prototypical electrophysiological properties. This is the first demonstration that human iPS-derived cells are able to generate electrically active motor neurons. These findings demonstrate the feasibility of using iPS-derived motor neuron progenitors and motor neurons in regenerative medicine applications and in vitro modeling of motor neuron diseases.** STEM CELLS *2009;27:806–811*

Disclosure of potential conflicts of interest is found at the end of this article.

## INTRODUCTION

Several groups have demonstrated the feasibility of reprogramming various types of human somatic cells to an embryonic state upon the introduction of pluripotency factors that yield induced pluripotent stem cells (iPS cells) [1–4]. The excitement surrounding iPS technology is predicated upon the potential to treat disease or injury with derivatives of patient-specific stem cells. In the more immediate term, their value may lie in the opportunity to model human diseases in vitro for which the etiology is unknown. Motor neurons are lost in many conditions, including spinal cord injury, amyotrophic lateral sclerosis (ALS), and spinal muscular atrophy (SMA). A major therapeutic goal is to develop the means to functionally replace these cells and to model their states of disease in vitro. Previous studies have outlined methods to derive functional motor neurons from human embryonic stem cells (hESCs) [5–7], and two recent studies applied similar methods to human iPS cell lines derived from patients with ALS and SMA [8, 9]. Before human iPS cells are used for regenerative medicine or to model motor neuron diseases, it is imperative to demonstrate that these cells can generate electrically active motor neurons with the characteristics of their natural counterparts.

Human iPS cells were capable of generating derivatives representing the three embryonic germ layers both in vitro in embryoid bodies (EBs) and in vivo teratoma assays ([1] and supporting information Fig. S1). Here, we show that two established methods to derive motor neurons from murine cells and hESCs can also be used to produce functionally mature motor neurons from human iPS cells [1]. First, an EB differentiation protocol was used to enrich for motor neuron differentiation [10, 11]. Second, an adherent approach was used to enable the characterization of the differentiated cells for their gene

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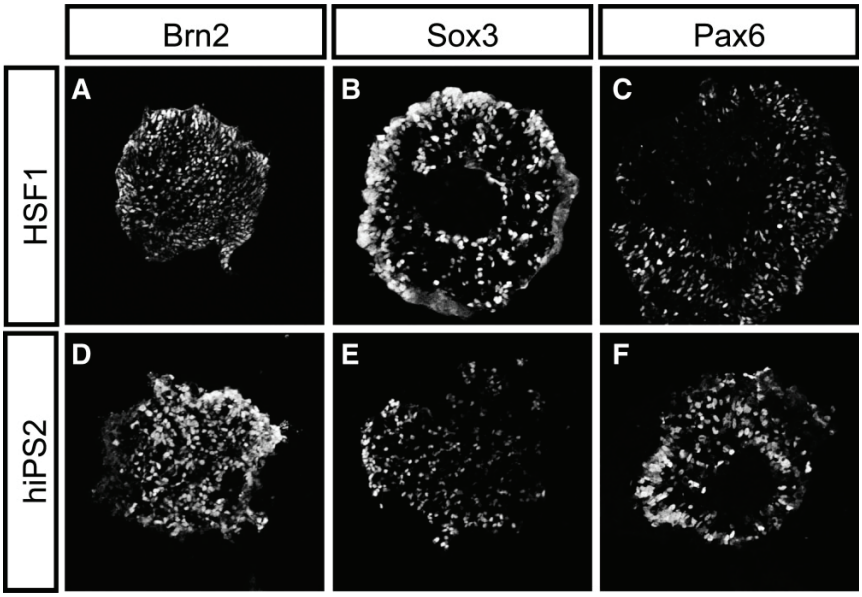


**Figure 1.** Human embryonic stem cells and human iPS-derived cells can both generate neural progenitors. Both HSF1 and hiPS2-derived embryoid bodies (EBs) were grown for 5-7 days in the presence of retinoic acid (1 $\mu$M, Sigma) and the Shh pathway agonist purmorphamine (1.5 $\mu$M, Calbiochem, San Diego, http://www.emdbiosciences.com) and generated EBs full of neural progenitors as judged by immunostaining for Brn2, Sox3, and Pax6 (**A–F**). Similar results were obtained using a different Shh pathway agonist (HhAg1.3, 500 nM, Curis, data not shown). Abbreviations: hiPS2, human-induced pluripotent stem cells; HSF1, human embryonic stem cell line.

expression and electrophysiology [6, 7]. For both approaches, we directly compared the ability of different human iPS cell lines and the well characterized hESC line HSF1 to generate both motor neuron progenitors and differentiated motor neurons. The results of this study should provide a platform for future studies of human motor neuron diseases in vitro.

## Materials and Methods

### Immunostaining

Antibody staining was performed on 4% parformaldehyde fixed, cryosectioned EBs and cultured cells as previously described [11]. Antibodies used include the following: goat anti-Brn2 (sc-6029; Santa Cruz Biotechnology Inc., Santa Cruz, CA, http://www.scbt.com); goat anti-choline acetyl transferase (ChAT; Millipore, Billerica, MA, http://www.millipore.com); rabbit anti-Hoxa3 and guinea pig anti-Hoxa5 (generously provided by J. Dasen and T. Jessell) [13]); goat anti-Hoxc6 (SC-46135; Santa Cruz Biotechnology); mouse anti-Hoxc8 clone C952-7E (MMS-266R; Covance, Princeton, NJ, http://www.covance.com); goat anti-Isl1 (AF1837; R&D Systems Inc., Minneapolis, http://www.rndsystems.com); mouse anti-Isl1 clone 39.4D5 (Developmental Studies Hybridoma Bank, Iowa City, IA, http://www.uiowa.edu/dshbwww); mouse anti-Lim3 clone 4E12 (Developmental Studies Hybridoma Bank); mouse anti-Nkx6.1 clone F55A10 (Developmental Studies Hybridoma Bank); rabbit anti-Nkx6.1 (generously provided by S. Morton and T. Jessell) [14]; guinea pig anti-Olig2 [11]; mouse anti-Pax6 (Developmental Studies Hybridoma Bank); mouse anti-Pax7 (Developmental Studies Hybridoma Bank); mouse anti-Nestin (Neuromics); rabbit anti-Sox1, anti-Sox2, and anti-Sox3 (all generously provided by T. Edlund and J. Muhr) [15]; and rabbit anti-$\beta$-III tubulin (MRB-435P, Covance). The monoclonal antibodies obtained from the Developmental Studies Hybridoma Bank were developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA. Alexa488-, FITC-, Cy3-, and Cy5-conjugated secondary antibodies were obtained from either Invitrogen (Carlsbad, CA, http://www.invitrogen.com) or Jackson Immunoresearch Laboratories (West Grove, PA, http://www.jacksonimmuno.com). Fluorescence and DIC images were collected using a Zeiss Axioobserver microscope equipped with the Apotome optical imaging system, or a Zeiss LSM5 Exciter confocal imaging system. Images were processed using the Zeiss Axiovision and LSM Exciter software suites (Carl Zeiss, Oberkochen, Germany, http://www.zeiss.com), and Adobe Photoshop CS2 (Adobe Systems, San Jose, CA, http://www.adobe.com).

### Electrophysiology

Electrophysiology was performed at 20-23°C using standard whole-cell, current-clamp techniques. Patch pipettes [3-5 $\mu$M] were filled with the following: 140 mM potassium gluconate, 10 mM Na HEPES, 1 mM EGTA, 4 mM ATP-Mg, 0.3 mM GTP, pH 7.2 [adjusted with KOH]. Cells were bathed in 120 mM NaCl, 1.2 mM $KH_2PO_4$, 1.9 mM KCl, 26 mM $NaHCO_3$, 2.2 mM $CaCl_2$, 1.4 mM $MgSO_4$, 10 mM D-Glucose, 7.5 mM Na HEPES [pH with NaOH to 7.2] equilibrated with 95% $O_2$ and 5% $CO_2$ and resting potentials were maintained at about −70mV. Graded current injections used durations of 0.5 milliseconds (in steps of 100 pA) or 250 milliseconds (in steps of 20 pA). Signals were sampled at 10 kHz using a Digidata 1322A analog to digital converter and acquired and stored on a computer hard drive using pClamp six software. Data were analyzed offline using pClamp six (Clampfit; Molecular Devices, Sunnyvale, CA, http://www.moleculardevices.com/home).

## Results

To demonstrate whether human iPS are able to differentiate down neural lineages to form motor neurons, we generated

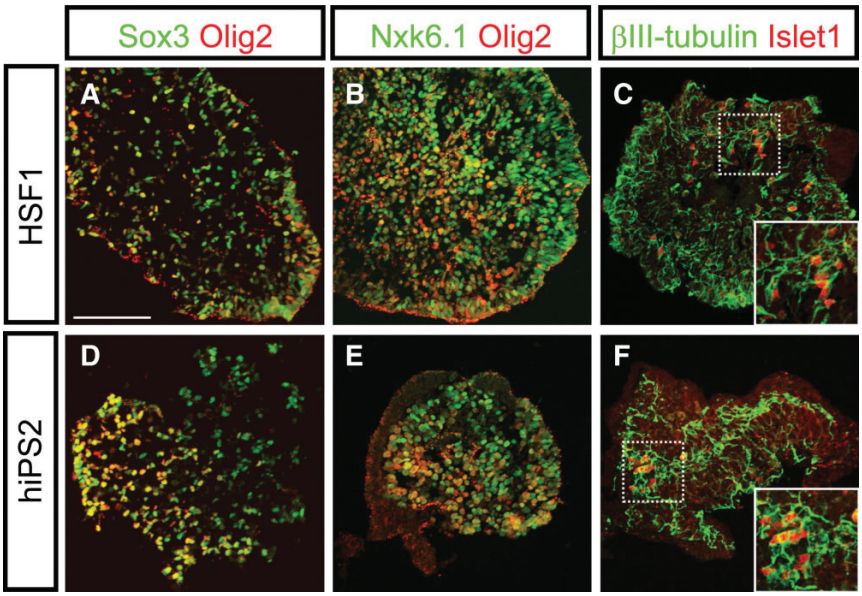


**Figure 2.** The directed differentiation of human embryonic stem cell and human iPS-derived cells recapitulates the stereotypical progression associated with motor neuron formation. After 8-10 days in the presence of retinoic acid, Shh pathway agonists and neurotrophic factors, both HSF1 and human iPS-derived embryoid bodies (EBs) contained $Sox3^+$, $Nkx6.1^+$, and $Olig2^+$ motor neuron progenitors (**A, B, D, E**). Both HSF1 and human iPS cells were further able to produce differentiated Islet1 and $\beta$III-tubulin-positive motor neurons within these EBs (**C, F**). Scale bar = 100 $\mu$m (**A–F**). Abbreviations: hiPS2, human-induced pluripotent stem cells; HSF1, human embryonic stem cells.

EBs from human iPS cells (hiPS2) and hESCs (HSF1), as previously described [1]. The EBs were cultured for 1 week in hESC media lacking FGF2 and then treated for an additional week with retinoic acid (RA; 1 $\mu$M) and a Sonic Hedgehog pathway agonist (purmorphamine, 1.5 $\mu$M) [16]. This method is known to both neuralize and ventralize EBs, as defined by the expression of ventral neural progenitor markers [11]. Both HSF1 and human iPS cells followed a standard course of development, serially differentiating from pluripotent cells to neural progenitors to fully differentiated motor neurons. As the EB protocol initiates specification in a somewhat stochastic manner, only a proportion of EBs from either HSF1 or iPS cells were specified to be neural, as demonstrated by immunostaining for the neural progenitor markers Brn2, Sox3, and Pax6 (Fig. 1A–1F and supporting information Fig. S2).

We did observe marked differences in the efficiencies by which HSF1 and hiPS cells underwent specification down the neural lineage (supporting information Fig. S2). Given the well-described variation of differentiation potentials among pluripotent stem cell lines [17], it is unclear whether this finding reflects an inherent difference between embryonic and induced pluripotent stem cells. However, within those EBs that were specified as neural, the percentage of cells expressing neural progenitor markers Brn2, Sox3, and Pax6 was similar whether the EBs were derived from HSF1 or human iPS cells (Fig. 1A–1F and data not shown). These findings suggest that both HSF1 and human iPS cells can be directed to form comparable neural progenitors.

After another week in the presence of RA and Shh pathway agonists, along with neurotrophic factors known to promote motor neuron survival [ciliary neurotrophic factor (CNTF) 20 ng/ml, brain-derived neurotrophic factor (BDNF) and glial-derived neurotrophic factor (GDNF), 10 ng/ml each], the EBs were fixed, cryosectioned, and immunostained for Sox3 and the motor neuron progenitor markers Nkx6.1 and Olig2. In the EBs that expressed markers of neural progenitors, the extent of labeling with Nkx6.1 and Olig2 antibodies was similar between HSF1 and human iPS-derived cells (Fig. 2B, 2E), and the percentage of $Sox3^+$ cells that expressed Olig2 was comparable (59.1% $\pm$ 7.07% for HSF1 and 57.6% $\pm$ 9.88% for human iPS-derived cells). Further analysis was conducted with a combination of markers known to be specific to differentiated motor neurons. Within EBs that were specified towards a neural fate and expressed markers of motor neuron progenitors (Nkx6.1 and Olig2), a small but significant number of Islet1 and $\beta$III-tubulin double-positive neurons were observed (Fig. 2C, 2F). The physical limitations of the EB differentiation method precluded detailed functional analysis of these cells, but these results together provide evidence that both HSF1 and human iPS cells can be induced to generate differentiated motor neurons.

To enable a physiological characterization of these iPS-derived motor neurons, we used another method of directed differentiation using previously described adherent conditions [6, 7]. Neural rosettes generated from HSF1 and human iPS1, iPS2, and iPS18 cells were mechanically isolated and then replated onto laminin-coated dishes in medium containing RA (1 $\mu$M) and Shh (200 ng/ml). After a week, neurotrophic factors were added (BDNF, CNTF, and GDNF; 10 ng/ml each), the Shh concentration was lowered (50 ng/ml), and the cells were allowed to differentiate for 3-5 weeks. Both HSF1 and human iPS-derived cells followed the expected course of differentiation, from Nestin-positive neuronal progenitors (Fig. 3B, 3F) to mature motor neurons ($\beta$III-tubulin, ChAT, and Islet1-positive; Fig. 3C–3H). In both HSF1 and human iPS derived $\beta$III-tubulin-positive cells, a similar percentage of Islet1-positive cells was detected (28.2% $\pm$ 5.7% for HSF1, 33.6% $\pm$ 12% for human iPS2) (Fig. 3C, 3G), suggesting again that once specified to a neuronal fate, human iPS-derived cells and HSF1-derived cells are equally efficient at generating motor neurons in these conditions.

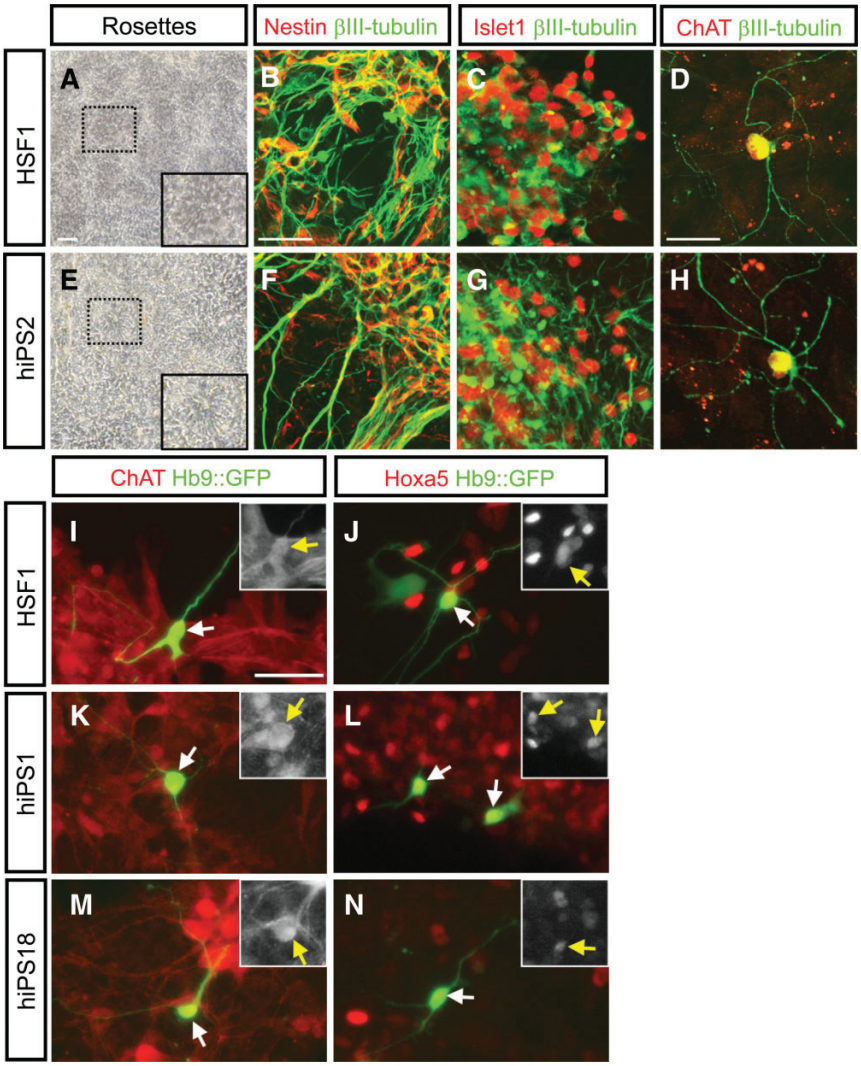


**Figure 3.** Neurons derived from human iPS cells and human embryonic stem cells express several motor neuron markers. Neural rosettes formed after 8-10 days in adherent culture (**A, E**). After mechanical dissection of rosettes from both HSF1 and hiPS2, Nestin-positive neural progenitors remain while differentiated neurons expressing $\beta$III-tubulin are formed (**B, F**). Confocal imaging demonstrates the generation of cells double stained for definitive markers of motor neurons including $\beta$III-tubulin and Islet1 (**C, G**) or $\beta$III-tubulin and ChAT (**D, H**). Late-stage differentiated neurons from were transiently transfected with a reporter indicative of Hb9 expression (Hb9:: green fluorescent protein [GFP]; **I–N**). Staining with an antibody recognizing ChAT demonstrates the specificity of the reporter for mature motor neurons (**I, K, M**). Costaining with antibody against Hoxa5 demonstrates a rostral cervical character of both HSF1 and hiPS-derived motor neurons (**J, L, N**). Insets in panels (**I–N**) show the single channel stains for either ChAT or Hoxa5 in the Hb9::GFP-positive cells indicated by the arrows. Scale bars = 200 $\mu$m (**A, E**); 70 $\mu$m (**B, C, F, G**); 50 $\mu$m (**D, H**); 50 $\mu$m (**I–N**). Abbreviations: ChAT, choline acetyl transferase; hiPS2, human-induced pluripotent stem cells; HSF1, human embryonic stem cells.

To further demonstrate that this differentiation protocol generates spinal cord neurons, cells from both HSF1 and human iPS cells were also stained with markers of various regions of the spinal cord and a reporter specific for activity of *Hb9* (*Mnx1* or *Hlxb9*), which encodes for a transcription factor specifically expressed by mature motor neurons [6]. This Hb9-driven green fluorescent protein (GFP) reporter was transfected into HSF1 and human iPS derived cells to enable the identification and targeting of motor neurons in which *Hb9* was transcriptionally active [6]. Activity of this reporter tightly correlated with markers characteristic of rostral cervical motor neurons such as Hoxa5 and ChAT in both HSF1 and hiPS-derived cells (Fig. 3) [11, 12].

Finally, to establish the phenotypic maturation of the human iPS-derived neurons, we studied their electrophysiological properties. It is well established that the firing of repetitive action potentials in response to current injection is typical of the behavior of adult vertebrate motor neurons [10] and that this repetitive firing develops as function of their maturation [18]. The excitability of HSF1 and human iPS-derived motor neurons was assayed by whole cell patch clamping in the current clamp mode. Action potentials were

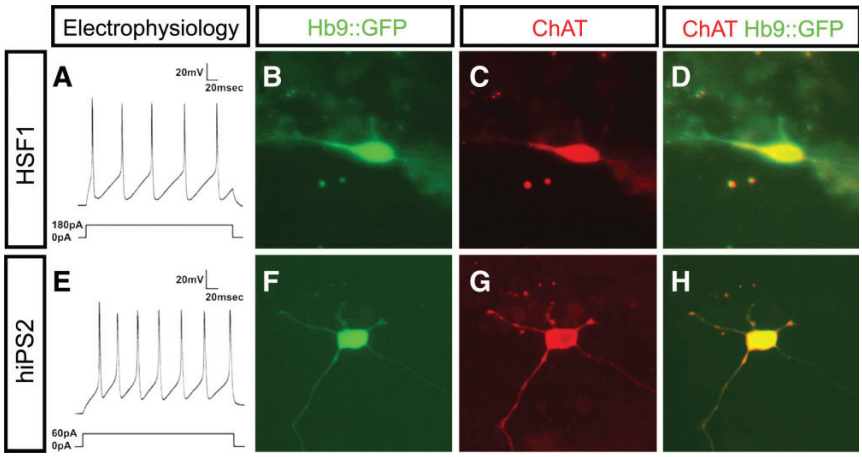


**Figure 4.** Neurons derived from human iPS cells and human embryonic stem cells (hESCs) display mature motor neuron characteristics. Whole cell patch clamp recordings from Hb9:: green fluorescent protein (GFP) expressing hESC and human iPS-derived neurons show repetitive firing after stimulation (**A, E**). Results shown are representative of recordings made from at least 20 cells derived from both hESCs and human iPS cells. Imaging of cells fixed after electrophysiological recordings show that these cells expressing the Hb9::GFP reporter also contained ChAT (**B–D** and **F–H**). Abbreviations: HSF1, human embryonic stem cells; hiPS2, human-induced pluripotent stem cells; ChAT, choline acetyl transferase.

recorded 48-62 days after plating. Upon application of current to either hESC or human iPS-derived neurons with Hb9::GFP activity, roughly half responded with single action potentials, whereas the other half responded with repetitive action potentials (Fig. 4A, 4E). After recordings were made, the neurons were fixed and analyzed for Hb9::GFP expression and ChAT staining to confirm that those cells that generated a typical motor neuron response to electrical stimulation also possessed cholinergic properties (Fig. 4B–4H). Identical results were achieved with motor neurons derived from three independent human iPS cell lines and were indistinguishable from motor neurons derived from HSF1 (supporting information Fig. S3). Together, these results demonstrate the general feasibility of the generating electrophysiologically active motor neurons from human iPS cells.

## Discussion

A primary objective of hESC and human iPS cell technology is to be able to generate relevant cell types to enable the repair of tissue damage and in vitro modeling of human disease processes. Here, we demonstrate the successful generation of electrically active motor neurons from multiple human iPS cell lines and provide evidence that these neurons are molecularly and physiologically indistinguishable from motor neurons derived from hESCs. The generation of motor neurons from human iPS cells isolated from patients harboring ALS and SMA mutations has recently been described, even though the electrophysiological activity of these motor neurons was not assessed [8, 9]. Demonstrating that human iPS cells can adopt this key hallmark of functional maturation is essential for any future application of human iPS cells in the study or treatment of motor neuron diseases. To our knowledge, our results present the first demonstration of the electrical activity of human iPS-derived neurons and further suggest the feasibility of using these cells to explore how changes in motor neuron activity contributes to the degeneration of these cells underlying that underlies motor neuron disorders [19, 20].

It remains unclear why the potential for the human iPS cell lines described here to appeared to undergo neural specification with a lower efficiency than HSF1 in these experiments. Marked variability in neuralization competence has been described among different lines of hESCs [21], and thus it seems likely that such differences may be noted also in human iPS cells. More importantly, the data presented here demonstrate that, of the cells that were specified to become neural, human iPS-derived neural progenitors proved as competent at generating motor neurons as their HSF1-derived counterparts. These findings support the possibility that reprogrammed somatic cells might prove to be a viable alternative to embryo-derived cells in regenerative medicine. Finally, as the human iPS cells appeared to obey a normal developmental progression to mature, electrically active neurons, it seems possible that disease-specific somatic cells may be reprogrammed and used to model and ultimately to treat a variety of human neurological disorders.

## Acknowledgments

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## Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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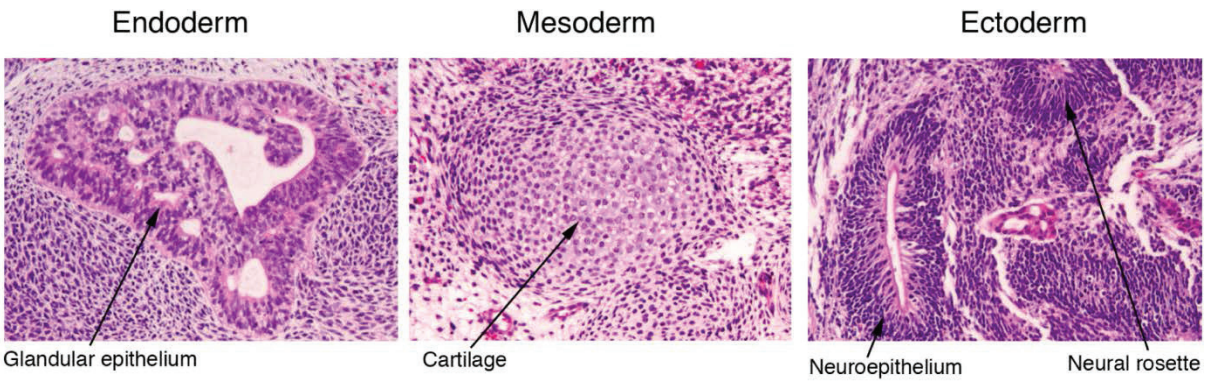
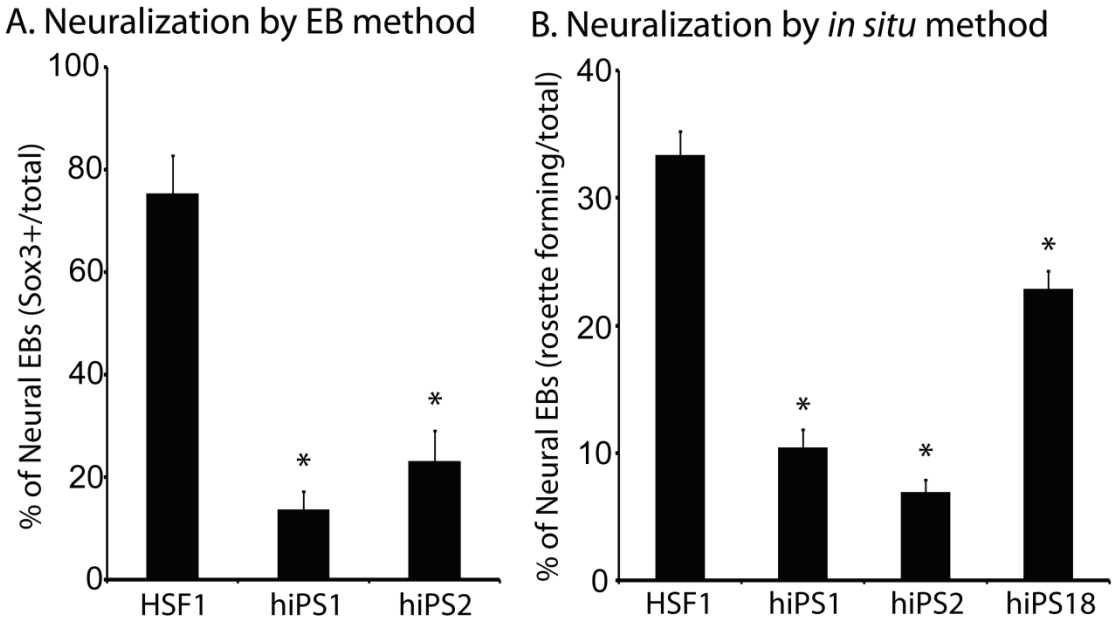
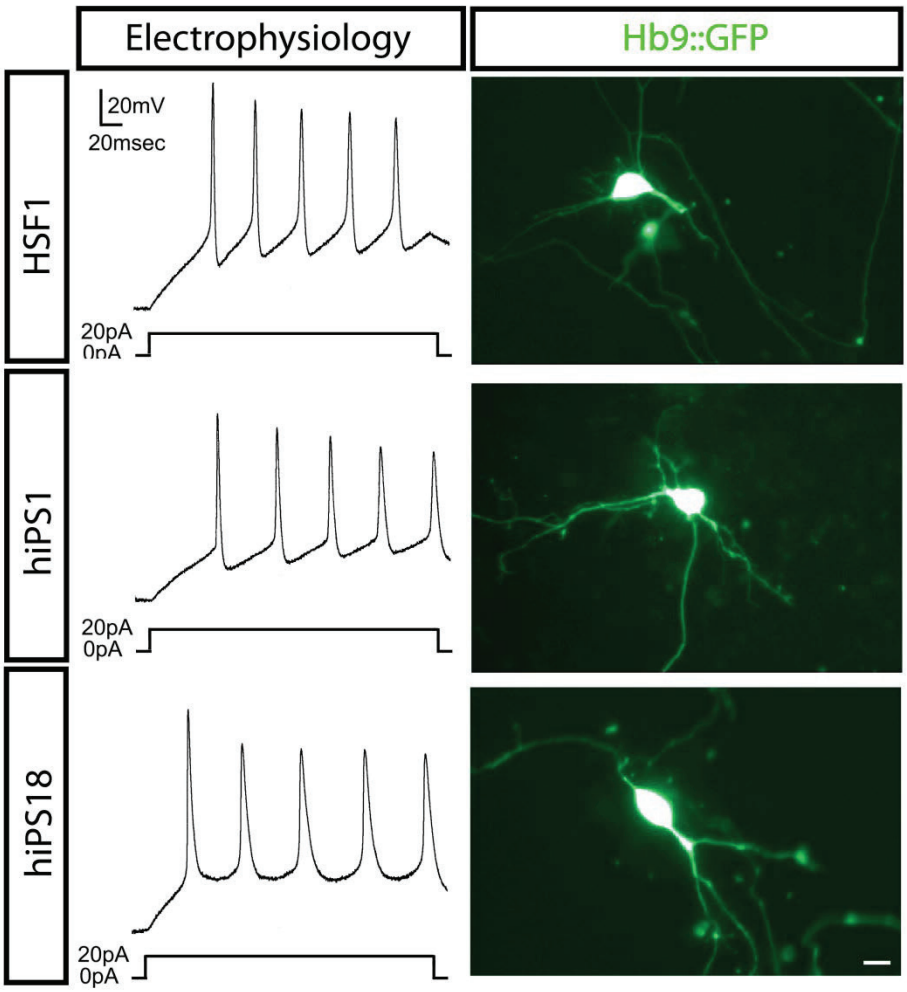



**Supporting Information Figure S1. In vivo differentiation demonstrates the pluripotency of human iPS cells.**
One million human iPS cells were injected into the testis of immunocompromised mice, and eight weeks later teratoma tumors were removed and subjected to histology by hematoxylin and eosin staining. Arrows indicate regions where cells typical of the indicated germ layers were found. Note that while human iPS cells always generated multipotent teratomas, injection of up to ten million parental fibroblasts never produced a single tumor of any kind (data not shown).




**Supporting Information Figure S2. HSF1 and human iPS appear to have different propensities for neural specification.**

Differentiation of embryoid bodies (EBs) made from HSF1 and human iPS cells drives a portion of them down a neural lineage as measured by expression of Sox3. Quantification of the percentage of EBs with at least 90% of cells expressing Sox3 highlights a difference in neuralization between HSF1 and human iPS1 and 2 after 2 weeks of differentiation (* denotes $p \leq 0.0003$ for each human iPS cell line versus HSF1)(a). The reduced efficiency of human IPS cells in forming neural progenitors was similarly observed when three different lines (hiPS1, 2, and 18) along with HSF1 were directly differentiated into neural rosettes without EB formation. These results suggest that the differences in neural formation are not simply due to variations in EB formation by HSF1 and these human iPS cell lines. Two weeks after replacing the standard hESC maintenance media (KO serum replacer, Invitrogen) to neural/ventralization media (as described in the text, Fig 1), the number of colonies that displayed neural rosette formation was quantified as a percentage of the total (* denotes $p \leq 0.0004$ for each human iPS line versus HSF1)(b). Data were collected from at least two independent experiments and represent the average across an $n \geq 6$. Error bars represent standard error of the mean. A two-tailed t-test was performed based on unequal variance to generate p values for each human iPS cell line versus HSF1.




**Supporting Information Figure S3. HSF1, hiPS1, and hiPS18 all generate mature, electrophysiologically active motor neurons.**
Rosettes derived from HSF1, hiPS1, and hiPS18 were differentiated for at least 50 days and transfected with the Hb9::GFP reporter. Whole cell patch clamp recordings from Hb9::GFP expressing HSF1 and human iPS-derived cells (Right) show repetitive firing after stimulation (Left). Results shown are representative of recordings made from at least 7 cells derived from HSF1 and hiPS1 and hiPS18.

# CHAPTER 3:

## Defining the nature of human pluripotent stem cell progeny

ORIGINAL ARTICLE

# Defining the nature of human pluripotent stem cell progeny

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**While it is clear that human pluripotent stem cells (hPSCs) can differentiate to generate a panoply of various cell types, it is unknown how closely *in vitro* development mirrors that which occurs *in vivo*. To determine whether human embryonic stem cells (hESCs) and human-induced pluripotent stem cells (hiPSCs) make equivalent progeny, and whether either makes cells that are analogous to tissue-derived cells, we performed comprehensive transcriptome profiling of purified PSC derivatives and their tissue-derived counterparts. Expression profiling demonstrated that hESCs and hiPSCs make nearly identical progeny for the neural, hepatic, and mesenchymal lineages, and an absence of re-expression from exogenous reprogramming factors in hiPSC progeny. However, when compared to a tissue-derived counterpart, the progeny of both hESCs and hiPSCs maintained expression of a subset of genes normally associated with early mammalian development, regardless of the type of cell generated. While pluripotent genes (*OCT4*, *SOX2*, *REX1*, and *NANOG*) appeared to be silenced immediately upon differentiation from hPSCs, genes normally unique to early embryos (*LIN28A*, *LIN28B*, *DPPA4*, and others) were not fully silenced in hPSC derivatives. These data and evidence from expression patterns in early human fetal tissue (3-16 weeks of development) suggest that the differentiated progeny of hPSCs are reflective of very early human development (< 6 weeks). These findings provide support for the idea that hPSCs can serve as useful *in vitro* models of early human development, but also raise important issues for disease modeling and the clinical application of hPSC derivatives.**

***Keywords*:** hESC; hiPSC; differentiation; human development; human fetal tissue; pluripotent

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## Introduction

hiPSCs are similar to hESCs in that they share the same pluripotency markers, display self-renewal, and are capable of differentiation into all three germ layers. The rapid development of hiPSC technology has delivered new hope for personalized medicine. In addition, hiPSCs may present a better tool for modeling disease as they can be derived from patients with various genetic diseases at virtually any age [1].

We and others have demonstrated that undifferentiated hiPSCs at early passages display a somewhat unique gene expression pattern from hESCs [2-4]. However, the functional relevance of any gene expression differences between hiPSCs and hESCs remains unclear. Recent evidence suggests that these differences may be explained by the fact that hiPSCs are more variant than hESCs at the epigenetic and transcriptional level [5-7]. It is also possible that the differences between hiPSCs and hESCs persist upon differentiation and that these differences influence the derivation of specified progeny, but this has not been formally tested. Recent work in murine and human iPSCs has shown that a residual epigenetic memory of the starting cell type is retained after reprogramming, and this memory appears to influence the efficiency of differentiation [8-11]. Furthermore, epigenetic differences between mouse iPSC lines diminish, as the cells are passaged [8], consistent with what was shown for gene expression between hiPSCs and hESCs [2, 12]. So far, differentiation efficiency appears to be as variable amongst hESC lines as it is for hiPSC lines [5, 13-15].

Despite varying efficiencies, all hPSC lines appear to have some potential to generate an array of functional

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cell types, including motor neurons, cardiomyocytes, dopaminergic neurons, hematopoietic cells, hepatocytes, etc. [16-21]. While it is clear that hiPSCs are capable of generating similar cell types as hESCs as determined by expression of a select number of markers, it remains to be seen if, on a global level, these derivatives are truly analogous. Furthermore, for hESCs or hiPSCs to be employed in therapy or as models of human development, it is also important to determine how well their differentiation mirrors natural development. Because both hiPSCs and hESCs undergo development *in vitro* instead of *in vivo*, it is not clear if current differentiation protocols accurately recapitulate the development that occurs in the human embryo. Nor is it clear whether cell types can be generated from hPSCs that mimic mature adult phenotypes.

To address these issues, we generated and purified derivatives of all three germ layers from hESCs and hiPSCs using established protocols. We compared the global gene expression pattern between the progeny of hESCs and hiPSCs, and primary tissue-derived equivalents from various stages of development. Our results show that the gene expression differences found between hiPSCs and hESCs in the undifferentiated state mostly dissipated after differentiation and derivatives from hiPSCs and hESCs were very similar to each other transcriptionally. On the other hand, a significant number of genes were different between PSC derivatives and their respective natural counterpart, regardless of the cell type generated. While *OCT4*, *SOX2*, *REX1*, and *NANOG* were effectively silenced, an alternative set of genes normally associated with the pluripotent state including *LIN28A*, *LIN28B*, and *DPPA4* remained expressed in pluripotent cell-derived progeny. We hypothesize and provide significant evidence that these cells derived from hPSCs are representative of cells found during human development prior to 6 weeks of development. Our data are consistent with the notion that ESC-derived cells represent early stages of development [22-25], but we demonstrate here that this also applies to hiPSC-derived cells. Furthermore, we attempt to place the PSC derivatives into a more precise developmental time frame than that has been established previously. These findings underscore the need to consider the maturity of cells produced from hPSCs for disease modeling or regenerative medicine.

## Results

*hESC and hiPSC lines are capable of generating derivatives representing all three embryonic germ layers*

Using previously established protocols, we differentiated hESCs and hiPSCs into derivatives representing all three embryonic germ layers: neural progenitor cells (NPCs) (ectoderm) [18], hepatocytes (endoderm) [20], and fibroblasts (FBs) (mesoderm). In our studies, the efficiency of derivation of various cell types from PSCs (including hESCs and FB-derived hiPSCs) was highly variable amongst various lines and across multiple experiments (data not shown and Karumbayaram *et al*. [18]). However, it was clear that all the PSC lines used here were able to generate a reasonable number of cells of the indicated type for analysis of gene expression and functional capacity.

*Generation of ectoderm*

When hESCs and hiPSCs were directed to generate NPCs and were isolated based on rosette morphology (p1), well-established neural stem cell markers (MUSASHI, PROMININ, PAX6, SOX2, and NESTIN) were induced as measured by RT-PCR (Supplementary information, Figure S1A). Immunocytochemistry confirmed the expression of these and other NPC markers at the protein level (Figure 1A and Supplementary information, Figure S1A′). By immunostaining, all the NPC markers labeled at least 80% of cells, demonstrating that the culture represented a homogenous pool of NPCs. While hESC and hiPSC-derived NPCs appeared to express these genes at a similar level to one another, there was variation in the level of protein expression on a per-cell basis observed between PSC-NPCs and those isolated from 16-week-old fetal brain. The transcription factor PAX6 was expressed at a lower level in PSC-NPCs when compared to 16-week-old fetal brain-derived NPCs (FNPC-16 Br) on a per-cell basis, while NESTIN expression was higher (Figure 1A). Because retinoic acid and smoothened agonist were used in the neural specification, it is possible that we induced a more posterior/ventral fate than NPCs isolated from fetal brain. To explore this possibility, the PCS-NPCs were also compared with NPCs isolated from 15.5-week-old fetal spinal cord (FNPC-15.5SC) and expanded under the same conditions. In fact, NESTIN and PAX6 were expressed in FNPC-15.5SCs at a similar level as in our PCS-NPCs (Figure 1A and Supplementary information, Figure S1A′). By immunostaining and morphology, both PSC-NPCs and 16-week-tissue-derived NPCs had the capacity to generate both Tuj1+ neurons and GFAP+ glia (Figure 1A′); however, the p1 PSC-NPCs mostly generated neurons (Tuj1+), while the 16-week-old-tissue-derived NPCs mostly produced glia (GFAP+) (Figure 1A″). Because neurogenesis precedes gliogenesis during *in vivo* development [26], these data suggest that PSC-NPCs may represent earlier developmental time points than the NPCs derived from 16-week-old fetal tissue.

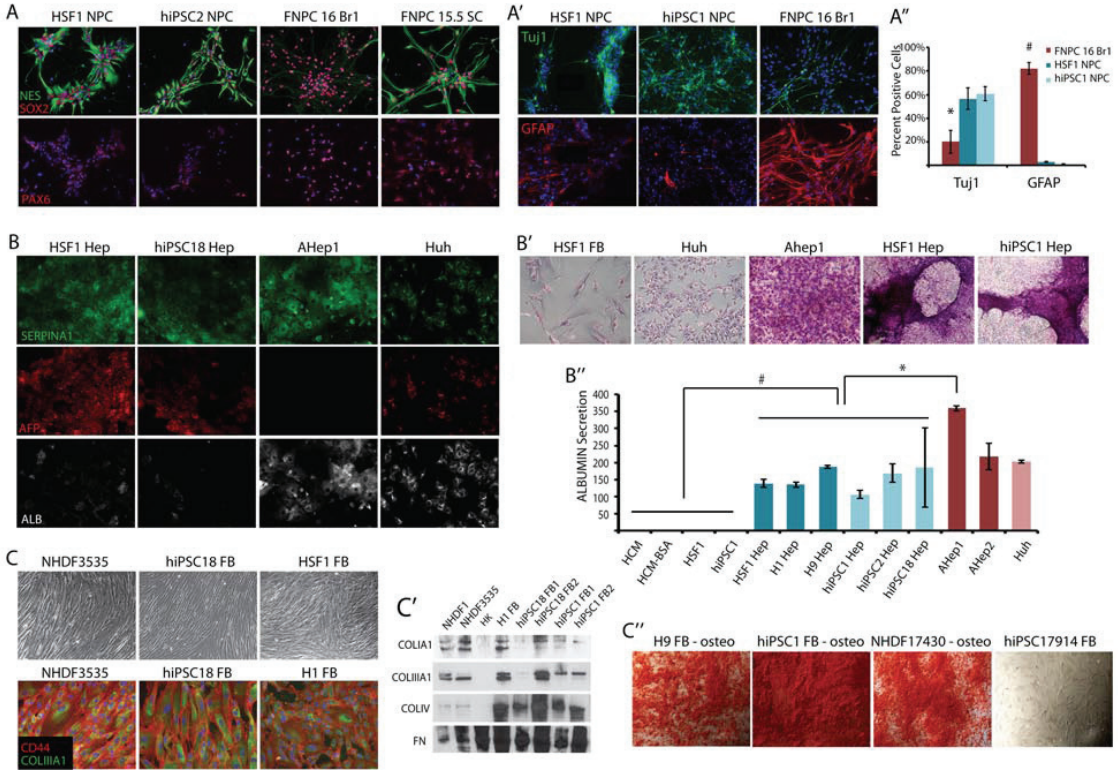



**Figure 1** hESC and hiPSC lines make cell types representing all three germ layers. hESC and hiPSC lines were directed to differentiate into either NPCs **(A)**, hepatocytes **(B)**, or fibroblasts **(C)**. **(A)** Immunofluorescence staining for SOX2 (red, top panel), NESTIN (green, top panel), and DNA (blue) and PAX6 (red, bottom panel). **(A′)** Immunofluorescence staining demonstrating that NPCs derived from hESCs, hiPSC, or natural sources could be differentiated into Tuj1+ neurons (green) and GFAP+ glia (red). **(A″)** Quantification of the percent of cells undergoing neuronal (Tuj1+) or glial (GFAP+) differentiation. Error bars represent standard error over 5-8 fields of view. $*P < 0.05$; $^{\#}P < 1.0E{-}06$. **(B)** Immunofluorescence staining of SERPINA1 (green), AFP (red), ALBUMIN (white), and DNA (blue). **(B′)** Periodic acid-schiff assay stain demonstrating glycogen storage in natural- and pluripotent-derived hepatocytes. **(B″)** ELISA measuring albumin secretion on confluent plates. Error bars represent standard error over two replicates. $*P < 0.01$; $^{\#}P < 0.05$. **(C)** Top, phase-contrast images of fusiform morphology displayed by pluripotent- and naturally derived fibroblasts. **(C)** Bottom, immunofluorescence staining of CD44 (red), COLIIIA1 (green), and DNA (blue). **(C′)** Western blot for secreted collagen proteins (COLIA1, COLIIIA1, and COLIV) and FIBRONECTIN (FN). HK, human keratinocyte. **(C″)** Alizarin Red stain following further differentiation of pluripotent cell- and tissue-derived fibroblasts down the osteogenic lineage.

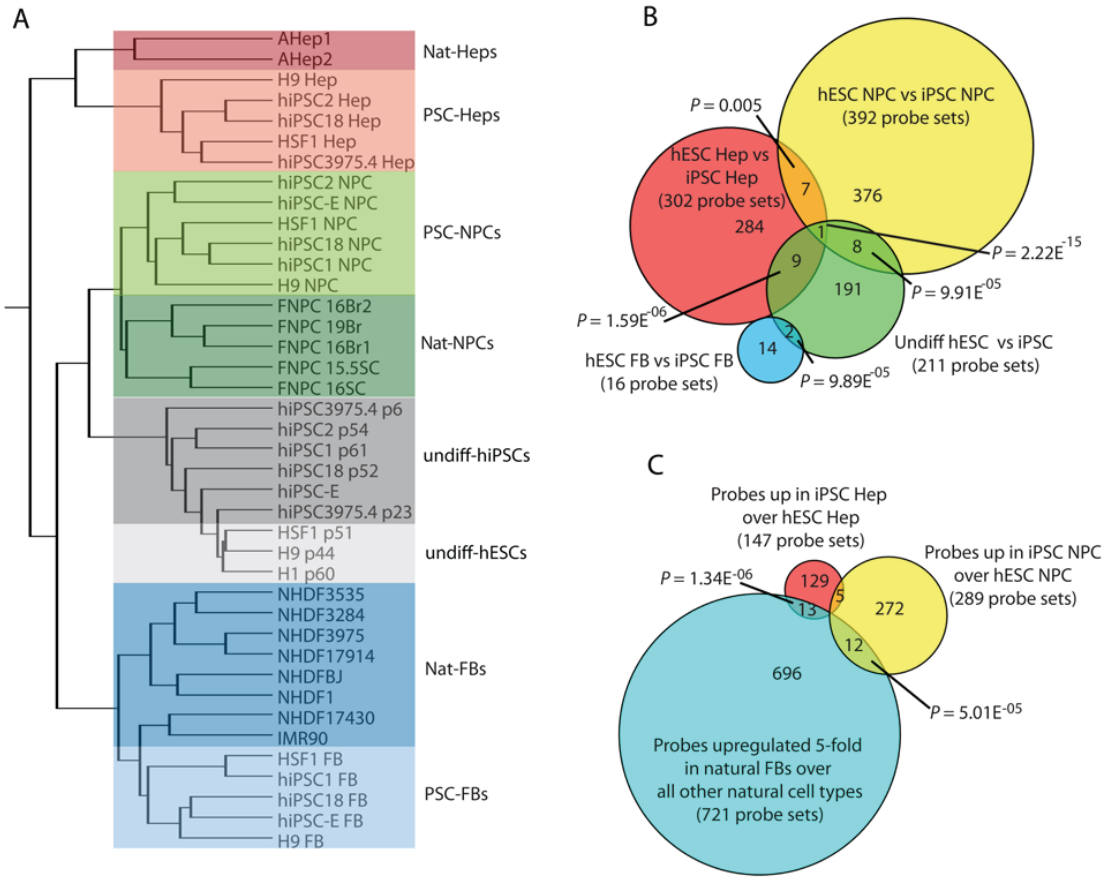
*Generation of endoderm*

For hepatocyte derivation, hESCs and hiPSCs were directed to undergo definitive endoderm formation, hepatic specification, hepatoblast expansion, and finally hepatic maturation as described previously [16, 20]. After 3 days of differentiation, definitive endoderm markers, *HNF3B* and *SOX17*, were induced, as demonstrated by RT-PCR (Supplementary information, Figure S1B). Following hepatic induction and expansion, the *SOX17* mRNA level declined, while various hepatic markers (*AFP*, *ALB*, *SERPINA1*, *CYP3A4*, and *CYP3A7*) were induced over time, as found in mature adult tissue-derived hepatocytes or a hepatocarcinoma cell line (Figure 1B and Supplementary information, Figure S1B). By morphology and immunostaining, the hepatocytes produced from PSCs were more similar to hepatoblasts, or immature hepatocytes that populate the developing early fetal liver (Figure 1B and Supplementary information, Figure S1B). These hepatic derivatives expressed a higher level of fetal hepatic genes such as *AFP* and *CYP3A7*, and a lower level of the more mature equivalents *ALBUMIN* and *CYP3A4*, when compared to counterparts made from adult liver

(Figure 1B and Supplementary information, Figure S1B). Again, this suggested that PSC-Heps could represent a younger developmental stage than those of adult liver. Regardless, the hepatocytes generated from PSCs were able to both store glycogen (Figure 1B′) and secrete albumin (Figure 1B″).

*Generation of mesoderm*

For FB generation, embryoid bodies (EBs) were first generated from hESCs and hiPSCs. The EBs were then plated in adherent conditions and grown in FB culturing media. Following several passages, these cultures began to display a homogenous fusiform morphology typical




D

| Affy Probe Set | | | hESC (3) | hiPSC (3) | Nat-Hep (2) | hiPSC-Hep (2) | Nat-FB (8) | hiPSC-FB (2) | Nat-NPC (5) | hiPSC-NPC (3) |
|---|---|---|---|---|---|---|---|---|---|---|
| 208286 | ***OCT4*** | Total | 11303.2 | 8332.5 | 216.8 | 159.4 | 126.8 | 173.8 | 123.4 | 160.9 |
| 214532 | ***OCT4*** | Total | 7223.5 | 6755.6 | 196.9 | 160.5 | 143.9 | 195.5 | 130.9 | 164.3 |
| 202431 | ***C-MYC*** | Total | 961.2 | 1570.9 | 876.3 | 752.9 | 1203.9 | 2198.5 | 683.0 | 591.3 |
| 213721 | ***SOX2*** | Total | 1384.7 | 1471.8 | 55.1 | 87.9 | 43.1 | 60.5 | 2498.7 | 2085.6 |
| 228038 | ***SOX2*** | Endogenous | 7792.5 | 6009.9 | 17.2 | 227.5 | 17.4 | 9.9 | 7734.2 | 6608.8 |
| 213722 | ***SOX2*** | Endogenous | 276.8 | 201.5 | 37.6 | 43.7 | 43.3 | 47.3 | 393.3 | 372.6 |
| 220184 | ***NANOG*** | Endogenous | 5685.4 | 6432.9 | 44.2 | 32.6 | 28.5 | 25.6 | 48.7 | 61.6 |
| 221841 | ***KLF4*** | Endogenous | 357.3 | 250.8 | 886.7 | 776.1 | 877.2 | 162.2 | 143.3 | 76.1 |

**Figure 2** Global gene expression analysis. **(A)** Hierarchical clustering analysis of global gene expression in undifferentiated hESCs, hiPSC, and their progeny compared to naturally derived cells. **(B)** Venn diagram summarizing the probe sets that were differentially expressed (*t*-test $P < 0.01$; fold change ≥1.54) between the progeny of hiPSCs versus the progeny of hESCs for each germ layer and the undifferentiated. **(C)** Venn diagram overlapping fibroblast signature probe sets (*t*-test between natural-FB and all other natural cell types; upregulated in FBs ≥5.0) with probe sets upregulated in iPSC progeny over ESC progeny for the NPC and Hep lineages. *P*-Values from **B** and **C** were measured by hypergeometric distribution or simulation as in [2]. **(D)** Normalized values from microarray probe sets for the reprogramming factors used to make the hiPSCs used in this study.

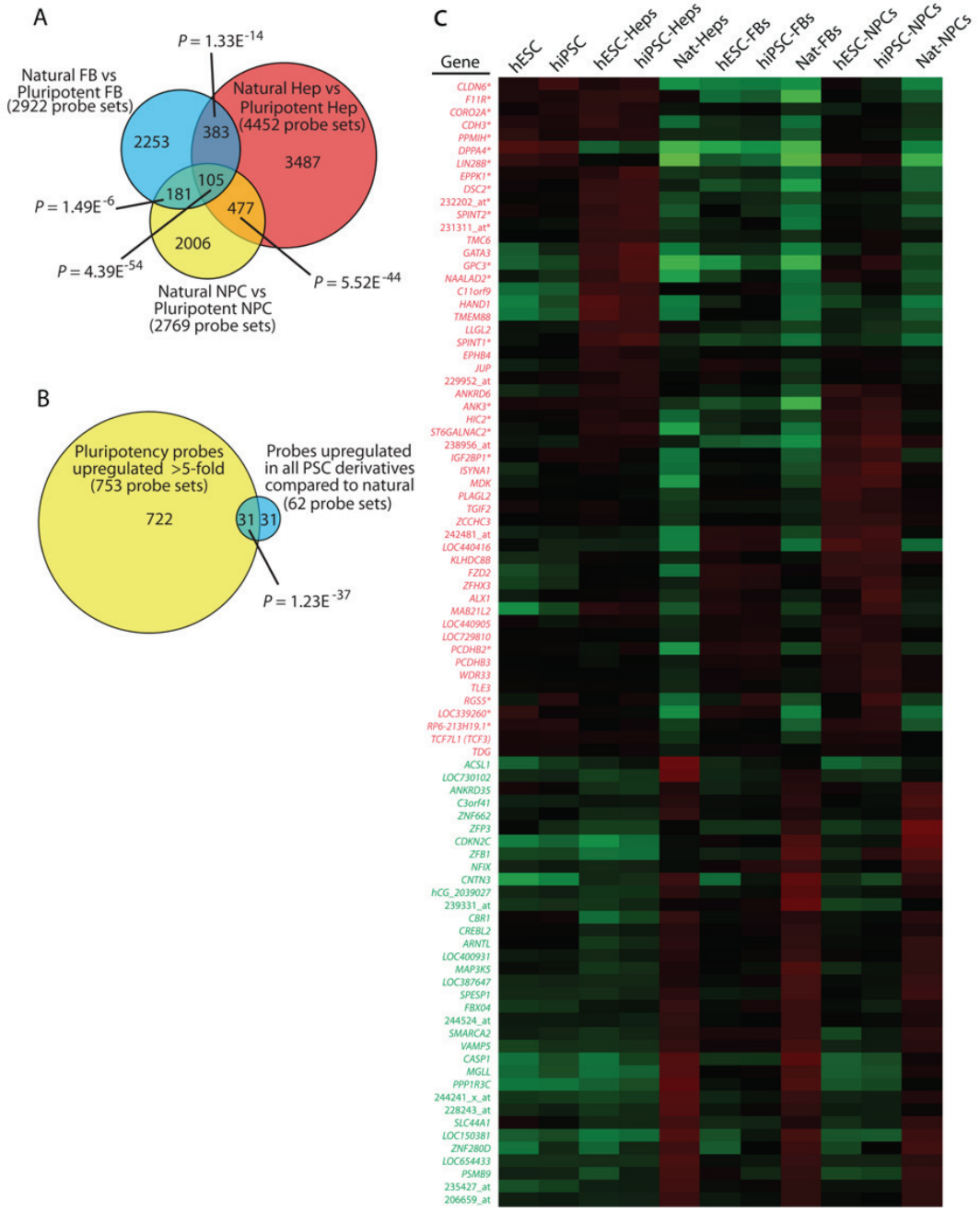



**Figure 3** Expression profiling identifies a conserved list of probe sets differentially expressed between pluripotent derivatives and their natural counterparts. **(A)** A *t*-test ($P < 0.01$) was performed to identify probe sets differentially expressed between PSC derivatives (FB, blue; Hep, red; NPC, yellow) and their respective natural counterparts (fold change ≥ 1.54). Venn diagram reveals the overlap of these differences across the different progeny. **(B)** Overlap of the 62 probe sets specifically upregulated in 3A with probe sets that demonstrate a significant difference between pluripotent cells and naturally derived somatic cells (fold upregulation ≥ 5). *P*-Values from **A** and **B** measured by hypergeometric distribution or simulation as in [2]. **(C)** A heat map was generated for the 88 unique genes or ESTs represented by the 105 probe sets shown in **A** that are differentially expressed between PSC derivatives and tissue derived cells. Note that signal shown represents value divided by the average of all samples and genes in red were consistently found upregulated in PSC progeny versus tissue-derived cells, while those in green were always downregulated. *Indicates genes expressed highly in the pluripotent stem cells (identified in **B**).

of FBs (Figure 1C). For comparison, FBs were derived from human dermis or lung at various developmental time points and were grown *in vitro* under the same conditions. When characterized at the RNA level, FB markers were expressed in PSC-FBs at a level comparable to FBs derived from the dermis of skin (Supplementary information, Figure S1C). Furthermore, using immunocytochemistry, two fibroblast markers, CD44 and COLIIIA1, were expressed in PSC-FBs at levels comparable to that observed in a neonatal dermal FB line (Figure 1C). Functionally, the PSC-FBs secreted a profile of collagens similar to that secreted by dermal FBs, while human keratinocytes did not (Figure 1C′). In addition, the PSC-FBs, like their natural counterparts, were not necessarily terminally differentiated, as they still retained the ability to undergo osteogenic specification (Figure 1C″).

*Global characterization of PSC derivatives by gene expression profiling*

To more precisely determine the identity of the derivatives of PSCs, each of the indicated cell types was purified and profiled for gene expression. We analyzed the transcriptional profiles of PSC derivatives as well as natural counterparts, and performed unsupervised hierarchical clustering (Figure 2A). From this clustering analysis we made several important observations.

First, most of the gene expression differences observed between hiPSCs and hESCs in the undifferentiated state were not found when hESC and hiPSC were differentiated, evident by the fact that hESC- and hiPSC-derived progeny did not segregate from one another. Second, while a small number of significant differences was detected between hESC progeny and that of hiPSCs for each lineage (roughly 300), these differences were not detected when a more stringent statistical measure (false discovery rate (FDR)) was applied (Figure 2B and data not shown). Furthermore, very few of these differences overlapped with the list of genes differentially expressed between undifferentiated hESCs and hiPSCs (Figure 2B), and none of them could be categorized with gene ontological (GO) analysis. Whether these differences serve to functionally distinguish hESC from hiPSC derivatives will require extensive investigation of differentiated progeny from hiPSCs, reflecting a variety of different somatic cell types.

Recent evidence suggests that murine and human iPSCs retain an epigenetic signature from their cell of origin that influences their differentiation potential [8-11]. All of the hiPSCs used in our analysis were derived from FBs, making similar types of analysis in our model system impossible. We did, however, look for residual expression of FB-specific genes in hiPSC derivatives versus hESC derivatives and found a handful of genes that might represent residual gene expression from the target cell of reprogramming (Figure 2C). We also ruled out that any gene expression difference was due to expression of integrated reprogramming factors in the hiPSCs because the reprogramming factors were not significantly expressed in these progeny, except for *SOX2*, as would be expected for NPCs (Figure 2D).

The most striking observation made from our transcriptome analysis was that, while PSC derivatives representing the three germ layers clustered with their respective natural counterparts, within each germ layer cluster, the PSC progeny were always distinguished from their naturally derived counterparts (Figure 2A). This finding suggested that, with the methods employed here and commonly applied elsewhere, PSC derivatives are similar but not identical to tissue-derived counterparts. This led to further investigation of the transcriptional differences between hPSC derivatives and their tissue-derived counterparts, and whether these differences are reflective of differences in developmental maturity or arise due to development performed *in vitro*.

*PSC derivatives and tissue-derived counterparts are distinguished by their gene expression*

The genes differentially expressed between PSC derivatives and their tissue-derived counterparts were compared using a *t*-test ($P < 0.01$) and requiring at least a 1.54-fold expression difference. Out of 36 749 probe sets that were expressed in at least 10% of samples, 2 922 were differentially expressed between PSC-FBs and dermal/lung FBs; 4 452 were differentially expressed between PSC-Heps and adult hepatocytes; and 2 769 were differentially expressed between PSC-NPCs and 16-week-old fetal NPCs. GO analysis of the genes differentially expressed in each germ layer representative yielded many categories for hepatocytes, but few for NPCs and FBs (Supplementary information, Figure S2D). Surprisingly, when superimposing these pools of differentially expressed probe sets and taking direction of differential expression into account, 105 were found to be differentially expressed between all PSC derivatives and their natural counterparts (Figure 3A), suggesting that all types of PSC derivatives share common differences with tissue-derived cells. No GO terms were conserved across these 105 probe sets (Supplementary information, Figure S2D). A more stringent analysis employing FDR correction produced a smaller list of probe sets, but the differences still overlapped significantly across the three germ layers and were entirely inclusive with the non-FDR corrected analysis (Supplementary information, Figure S2A).

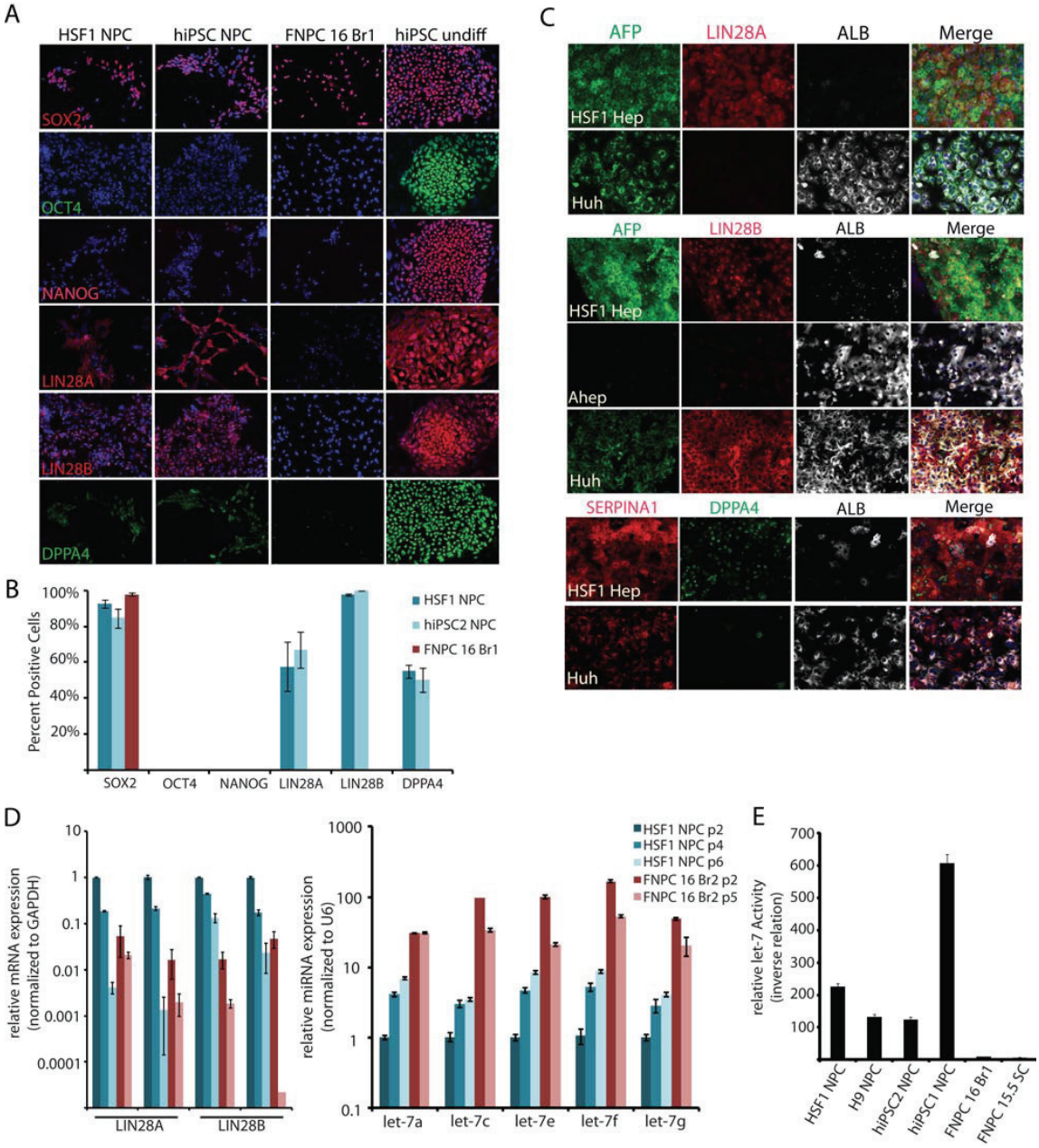



**Figure 4** Expression and activity of LIN28 and DPPA4 in PSC derivatives. **(A)** NPCs made from PSCs and brain were stained with pluripotency markers SOX2, OCT4, NANOG, LIN28A, LIN28B, and DPPA4. Undifferentiated hiPSCs were stained as a positive control for the pluripotency markers. **(B)** Quantification of the percent of FNPCs and PSC-NPCs expressing the indicated pluripotency markers. **(C)** HSF1-derived hepatocytes and control cells were immunostained with antibodies recognizing ALBUMIN, AFP, or SERPINA1 to highlight both immature and mature cells and either LIN28A, LIN28B, and DPPA4 to demonstrate that these pluripotency factors are not silenced immediately upon differentiation. Hepatocytes taken from adult human liver did not express any of these pluripotency genes, while Huh, a hepatocarcinoma cell line expressed LIN28B. **(D)** Real-time PCR for *LIN28A* and *LIN28B* mRNA (left) and *let-7* miRNA family members (right). mRNA expression was normalized to GAPDH, while miRNA expression was normalized to U6. Error bars represent standard error over three or four replicates. **(E)** To determine the relative let-7 activity in the indicated cell types, each was transfected with let-7 reporter and constitutive reporter as a transfection control. Dual luciferase assays were performed 48 h after transfection in triplicate. Assay shown was representative of three independent experiments.

From this pool of 105 differentially regulated probe sets, 62 were upregulated in all PSC derivatives versus their tissue-derived counterparts. Notably, 31 of these 62 probe sets overlap with probe sets that are highly upregulated in undifferentiated PSCs versus specified somatic cells (Figure 3B). This indicates that the PSC progeny continued to express a significant subset of genes associated with either pluripotency or early embryonic development. Many of these same genes were also expressed at a higher level in PSC derivatives versus tissue-derived cells generated independently by other groups, suggesting that these observations were not specific to our methods or the particular cell lines used here (Supplementary information, Figure S2C). *OCT4*, *SOX2*, and *NANOG* were not amongst the 31 probe sets related to pluripotency that remained high in PSC derivatives, demonstrating that these genes were silenced upon differentiation, as has been shown extensively. Instead, most of the 31 probe sets appeared to be not only expressed in PSCs but also play roles in early embryonic development as judged by functional data from lower organisms [27-29]. The expression pattern of all genes specifically expressed in PSCs can be found in Supplementary information, Table S1.

The expression pattern across cell types for the 88 unique genes and unannotated probe sets differentially expressed between PSC derivatives and tissue-derived counterparts (represented by 105 probe sets) is represented in Figure 3C as a heat map. Of the 53 genes that were higher in PSC derivatives (in red), 22 were also strongly expressed in undifferentiated PSCs relative to somatic cells (indicated with asterisk). This list included *LIN28B*, *DPPA4*, and *TCF7L1* (*TCF3*), all of which are known to play a role in ESCs and in very early mammalian development [28, 30-33]. Furthermore, 35 genes were downregulated in PSC derivatives compared to tissue-derived cells (in green), perhaps reflecting a state of incomplete specification, regardless of the cell type generated.

*LIN28* was first discovered as a regulator of developmental timing in *C. elegans* [29, 34]. *LIN28A* and *LIN28B* are highly expressed in undifferentiated hPSCs, but are thought to be silenced, as tissues are specified and mature. *LIN28A* has also been employed as a reprogramming factor in the generation of hiPSCs [35], suggesting that it can play a functional role in maintaining or inducing immature cell fate. *LIN28B* was expressed in all hESC- and hiPSC-derivatives, whereas *LIN28A* was found at a high level in PSC-NPCs and PSC-Heps, but not in PSC-FBs. *LIN28A*, *LIN28B*, and *DPPA4* were also frequently expressed at a high level in PSC derivatives generated by other groups (Supplementary information, Figure S2C), indicating that expression of these genes is not confined to just the hESC and hiPSC derivatives produced in our lab, but is perhaps an attribute of PSC derivatives in general.

To determine whether the changes detected in the RNA profiling data were just due to residual-stabilized RNA from the pluripotent state, the expression of LIN28A, LIN28B, and DPPA4 in PSC derivatives was also examined at the protein level. In fact, DPPA4, LIN28A, and LIN28B were all expressed at the protein level in PSC-NPCs (Figure 4A and 4B) and PSC-Heps (Figure 4C), but not in their tissue-derived counterparts. On the other hand, none of the other classic pluripotency factors (OCT4, NANOG, and REX1/ZFP42) were expressed in any of the PSC derivatives at the RNA or protein levels (Figure 4A-4C, and data not shown).

*The LIN28/let-7 circuit in PSC derivatives*

LIN28 has been shown to act as an RNA-binding protein that regulates miRNA maturation, particularly the let-7 family [27, 29-31, 36-40]. If LIN28 activity is higher in PSC derivatives, these cells would be expected to have low levels of mature let-7. To determine if the increased LIN28 expression in PSC-NPCs correlated with low levels of mature let-7, RT-PCR was used to probe the relative levels of mature let-7 miRNA family members. As expected, PSC-NPCs were found to have very low relative levels of mature let-7 family members compared to tissue-derived NPCs (Figure 4D).

To determine if the low levels of let-7 expression in PSC-NPCs also correlated with low activity of these miRNAs, cells were transfected with a reporter that drives constitutive expression of the Renilla luciferase gene with let-7 seed sequences added to its 3′ UTR [41]. Therefore, higher luciferase activity is a result of decreased let-7 miRNA activity in the cells. NPCs generated from 16-week-old fetal brain or spinal cord showed very little reporter activity, indicating high let-7 activity, whereas PSC-NPCs displayed high reporter activity, indicating the opposite (Figure 4E). Considering the LIN28 expression pattern, and the let-7 activity assay, PSC derivatives not only had high LIN28 expression, but also high LIN28 activity, which in turn led to low let-7 activity. While the LIN28/let-7 pathway has been implicated in development of many species including mouse [27, 30], this appears to be the first demonstration that human PSC derivatives have high LIN28 expression and low let-7 activity. These data further suggest PSC derivatives are developmentally immature compared to their natural counterparts and that caution is warranted in clinical application of these cells, as many human cancers are defined by high LIN28 and low let-7 expression [27, 39].

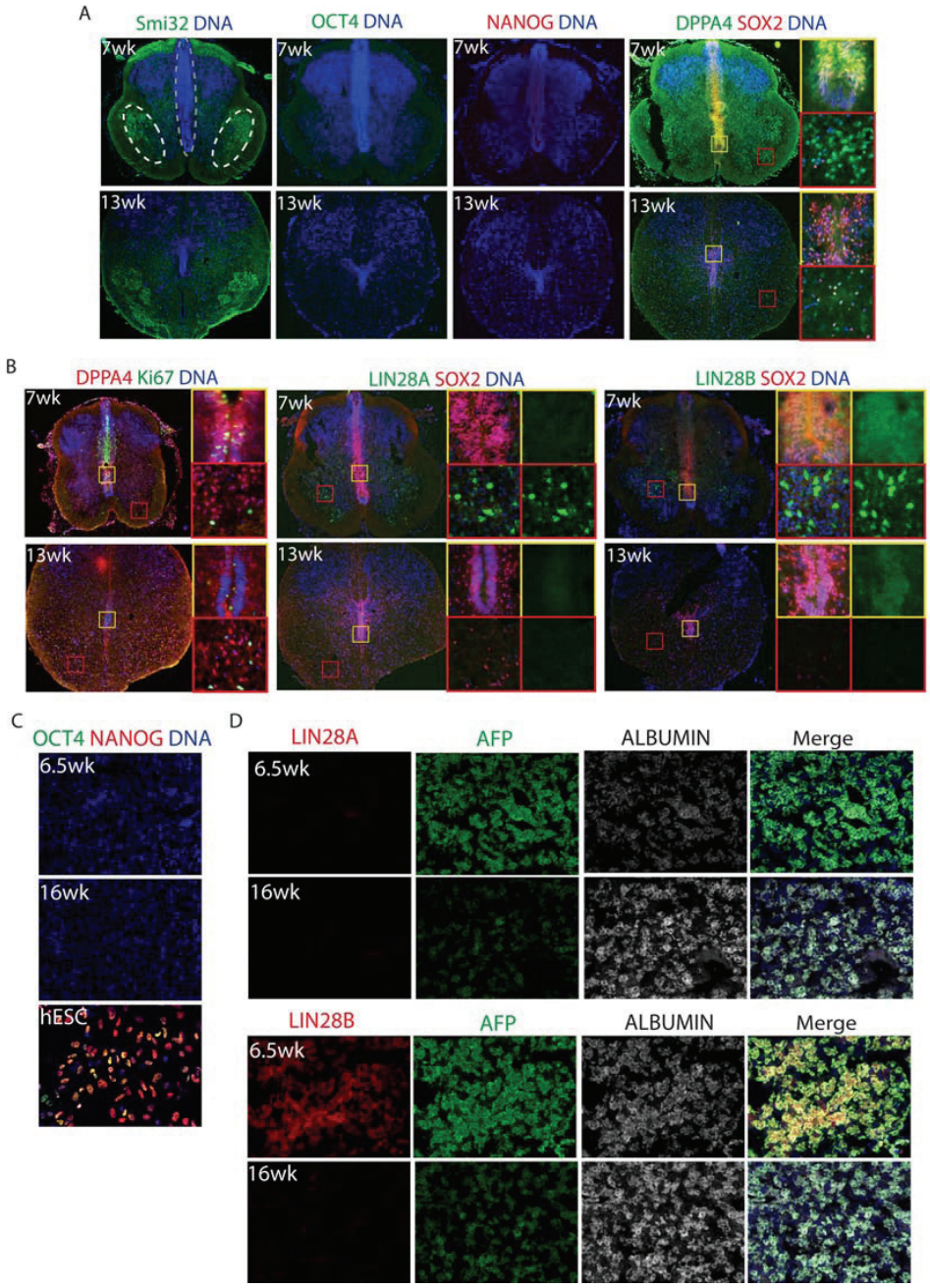



**Figure 5** Differentially expressed genes, *DPPA4*, *LIN28A*, and *LIN28B* are found in early fetal tissues. **(A)** Spinal cord tissues (7- and 13-week-old) were fixed, sectioned, and stained with the indicated markers. Smi32 was used to highlight the motor neuron pool (white circles). SOX2 labels the NPCs found along the midline (grey outline). **(B)** DPPA4 co-localized with proliferation marker Ki67 along the midline (yellow inset) at 7 weeks, but not in the dispersed lateral stain (red inset). At 13 weeks, DPPA4 was more dispersed and the number of Ki67-positive cells was decreased. LIN28A had a cytosolic staining pattern and was located in cells outside of the midline (red and yellow insets) at 7 weeks, but absent at 13 weeks. LIN28B had a nuclear staining pattern at 7 weeks. It was often co-localized with SOX2 along the midline at 7 weeks and was weaker at 13 weeks (yellow inset). **(C)** Fetal liver (6.5- and 16-weeks-old) were stained with the indicated pluripotency markers, showing the complete silencing of pluripotency genes. hESCs were used as a positive control. **(D)** Fetal liver (6.5- and 16-week-old) were stained with LIN28A, LIN28B, and the indicated liver markers.

*Expression of early embryonic genes in PSC derivatives suggests that they represent early stages of human development*

To determine whether expression of the *LIN28* genes and *DPPA4* in PSC derivatives was indicative of very early fetal development or simply an *in vitro* phenomenon, fetal tissues were also probed for expression of these genes. In the human fetal spinal cord, regional identity was established with Smi32, a marker of motor neurons, which was localized to bilateral pools on each side of the ventral cord (Figure 5A). The neural progenitor pool was localized at the midline and expressed SOX2 and Ki67 (Figure 5A and 5B). DPPA4 was also strongly expressed in the midline of the spinal cord along with SOX2 at 7 weeks of development, but was weaker by 13 weeks (Figure 5B). LIN28A was expressed in scattered cells in 7-week-old spinal cord, but was lost by 13 weeks. LIN28B, on the other hand, was strongly detected in 7-week-old human spinal cord cells outside of the midline and weakly expressed in the midline progenitor cells along with SOX2. Expression of LIN28B was significantly reduced, but not absent, in the spinal cord by 13 weeks of development (Figure 5B). The fact that midline progenitor cells of the human spinal cord did express DPPA4 and LIN28B at 7 weeks could further suggest that PSC-NPCs are more similar to an earlier stage of fetal development, or that multiple pools of diverse progenitors are present at this time point.

In the fetal liver, LIN28B, but not LIN28A, was detectable at 6.5 weeks (Figure 5D). Neither LIN28 protein was detectable in fetal liver by 16 weeks (Figure 5D). As expected, neither OCT4 nor NANOG was expressed in the spinal cord or fetal liver at any time point analyzed, consistent with the notion that these pluripotent genes are silenced very early in human development (Figure 5A and 5C). These data highlight the possibility that PSC derivatives differed from the tissue-derived cells as shown in Figures 2 and 3, because the cells were taken from tissues that were of at least 16 weeks of development. The presence of both LIN28 proteins and DPPA4 in 7-week-old spinal cord and liver, the fact that PSC-NPCs tended to be neurogenic as opposed to gliogenic, and that PSC-Heps express AFP instead of albumin, are all consistent with the notion that pluripotent derivatives are similar to cells found at 7 weeks of development or earlier.

*Are PSC-NPCs distinguished from fetal NPCs by time in vitro or time in vivo?*

Following the observation that early embryonic genes were expressed in younger fetal samples, but significantly reduced at later stages, we were prompted to ask two additional questions: (1) could additional time in culture bring the PSC derivatives closer to a natural counterpart; and (2) would younger fetal samples more closely resemble our PSC derivatives on a transcriptome level? To answer these questions, we performed a series of additional gene expression profiling experiments on the NPCs.

When PSC-NPCs were cultured for another month (each passage representing ~5-7 days in culture), both *LIN28A* and *LIN28B* mRNA were reduced as measured by RT-PCR analysis (Figure 4D). Furthermore, let-7 family members were upregulated with extended culture (Figure 4D). The reduction of LIN28A and LIN28B was also observed at the protein level, while DPPA4 did not seem to change with passaging (Figure 6A-6C). In order to determine if continued passaging brought PSC-NPCs globally closer to 16-week-old fetal spinal cord-derived NPCs, we performed two Pearson correlations comparing the transcriptomes of the indicated samples. When using only those probe sets identified by the original *t*-test (Figure 3A; 2 769 less 46 probes that did not make it past the new filtering), we observed a small increase in similarity between PSC-NPCs and fetal-derived NPCs (Figure 6E). In addition, we observed that a small, but statistically significant, number of the original 2 723 probe sets were "corrected" upon extended passaging, including *LIN28A* and *LIN28B* (Figure 6F). Ultimately, when all probe sets were considered, we observed only a modest increase in global transcriptome similarity with extended passaging (Figure 6D), suggesting that simply culturing PSC-NPCs does not generate cells that are equivalent to their tissue-derived counterparts.

To functionally determine whether passaging PSC-NPCs promotes their developmental maturity, we compared the differentiation potential of PSC-NPCs before and after continued culturing. This analysis indicated that culturing PSC-NPCs for an additional month did increase their gliogenic capacity from < 1% to ~15% (Figure 6G), but not to levels typical of any tissue-derived NPCs we have tested to date (~50% - 80% gliogenic with NPCs derived from fetal tissue at 6.5-19 weeks of development (Figure 1A″ and data not shown). These data further suggest that while continued culture can bring PSC-NPCs closer to their tissue-derived counterparts, this effect is small, and by itself is not sufficient to generate cells equivalent to tissue-derived NPCs. In addition, continued culture of PSC-NPCs in these conditions beyond 2 months (passage 4) led to the subsequent loss of NPC markers and differentiation capacity (data not shown). This suggests that, under these conditions, one cannot simply passage the cells indefinitely and expect continued developmental maturation *in vitro*.

To determine if PSC-NPCs would more closely resemble an NPC type isolated from younger fetal spinal

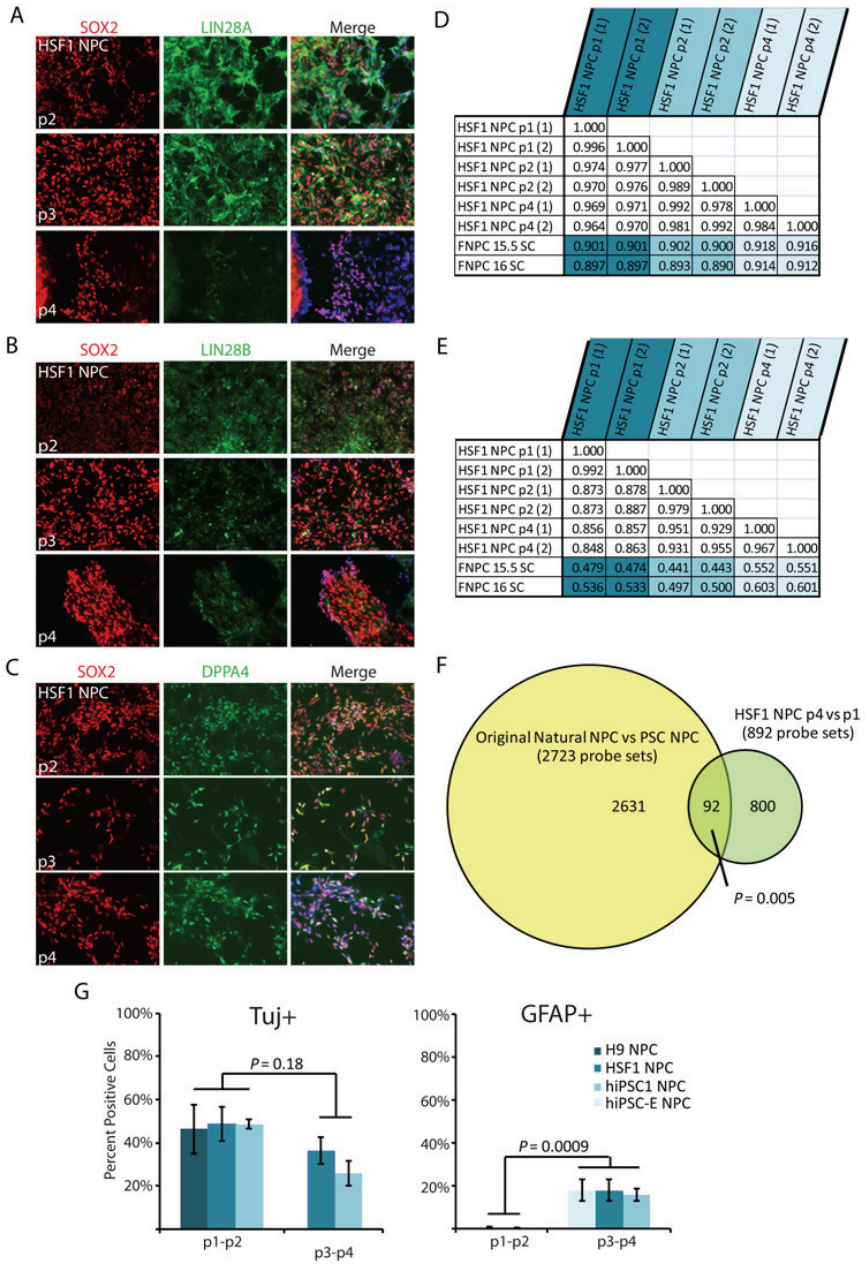



**Figure 6** Continued passaging of PSC-NPCs reduces LIN28 expression and corrects a small portion of the gene expression discrepancies. **(A-C)** Immunofluorescence staining of HSF1-derived NPCs over four passages. Each passage represents ~5-7 days in culture. **(D)** Pearson correlation comparing global gene expression between HSF1 NPCs over several passages and NPCs derived from 16-week-old fetal spinal cord. **(E)** Pearson correlation including only those probe sets identified as different between PSC-NPC and Nat-NPCs (analysis from Figure 3A). **(F)** Venn diagram demonstrating the original differences identified in Figure 3A overlap significantly with gene expression differences between p1 and p4 PSC-NPCs (*t*-test $P < 0.01$; fold change ≥ 1.54). Direction of differential expression was taken into account. Statistical analysis performed by hypergeometric distribution. Note: later analyses were performed by normalizing and filtering only samples of the neural lineage. As a result the original 2 769 probe sets identified by analysis in Figure 3A were reduced to 2 723. **(G)** Percent of PSC-NPCs at the indicated passage undergoing neuronal (Tuj1) or glial (GFAP) differentiation following 3 weeks of differentiation.

cord, we profiled additional fetal samples from 6.5 to 8 weeks of development. Pearson correlation of the global transcriptome demonstrated a dramatic increase in similarity between PSC-derived NPCs and NPCs from young fetal spinal cord compared to 16-week-old spinal cord (Figure 7A). Ideally, a comparison between cells derived from fetal tissue earlier than 6 weeks would determine whether PSC derivatives accurately reflect their counterparts found during very early fetal development, but because of the lack of access to such tissue, this question is difficult to answer. However, data on gene expression across whole human embryos from 3-5 weeks of development recently became available [42].

We identified a list of 1 645 probe sets differentially expressed between 3-week (stage 9) and 5-week (stage 14) embryos. We overlaid these identified probe sets with the list of probe sets differentially expressed between PSC derivatives and their natural counterparts (Figure 3A; 105 is reduced to 36 due to the more restrictive chip used by Fang *et al*. See Materials and Methods for details) and found significant overlap. *LIN28A* was the most differentially expressed probe set between stage 9 and stage 14 embryos. Knowing that *LIN28A* was differentially expressed in both the NPC and hepatocyte lineages, but not the FBs of our data, we repeated the analysis and excluded FBs (Figure 7C). Amongst the 53 probe sets conserved in our data and the Fang *et al*. [42] data are *DPPA4* and *LIN28A* (note: *LIN28B* is not represented on the Fang *et al*. array). The expression pattern for the 46 unique genes represented by those 53 probe sets is represented by two heat maps, one including the samples from Fang *et al*. [42] and the other including our own samples (Figure 7D). The fact that a significant number of genes that are normally downregulated between 3-5 weeks of development appear to distinguish PSC derivatives from their tissue-derived counterparts further suggests that PSC derivatives might accurately recapitulate cells found prior to 6 weeks of development.

## Discussion

Our data have revealed several important insights about differentiation from hPSCs. First, our data showed that hESCs and hiPSCs make specified derivatives that are nearly equivalent transcriptionally. This was surprising considering the vastly different circumstances by which hESCs and hiPSCs are derived, and in light of well-documented differences between them at the epigenetic and transcriptional levels in the undifferentiated state [2-6, 8, 9, 43, 44]. This similarity might be attributed to the fact that only high-passage hiPSCs (> 40) were used. However, even at high passage, a small number of genes still appeared to distinguish the undifferentiated hiPSC and hESC lines used. The fact that these differences were largely undetectable in the differentiated state could suggest that the progeny of these cell types are more similar than their parental cells or that the genes differentially expressed between them are not expressed in the specified progeny.

Second, upon differentiation, we did not detect appreciable re-expression of the exogenous reprogramming factors. Because the expression of *OCT4* was undetectable in any PSC derivative, it is unlikely that the loci representing the retroviral reprogramming factors were reactivated upon differentiation or even continued culture. This finding could suggest that, at least in the contexts analyzed here, concerns over re-expression of oncogenic factors from hiPSC lines generated by viral integration could be mitigated by specification of hiPSCs to even a progenitor state, such as NPC, hepatoblast, or FB.

Third, both hESCs and hiPSCs made progeny that continued to express a group of genes known to play roles in very early embryonic development. While the progeny of ESCs have been proposed to represent embryonic cell types because of the primitive nature of the starting cell types [23, 25, 45], to our knowledge human pluripotent cell-derived progeny have not yet been placed into such a narrow developmental context. The most logical interpretation of our data is that the PSC derivatives generated here represent cells similar to those found earlier than 6 weeks of development.

It remains possible, however, that current protocols to make hPSC derivatives generate cells that do not completely represent cells found in tissue at any developmental stage. With limited access to tissues representing the earliest stages of development, this possibility will remain unexplored for now. It is also possible that new culture conditions could be defined that improve differentiation *in vitro* to better recapitulate that which occurs *in vivo*. In addition, it is possible that experimentally manipulating the expression of early embryonic genes described here could be used to accelerate development *in vitro*. Regardless of the fact that PSC derivatives produced in other labs also express some of the same early embryonic genes suggest that many protocols lead to a consistent result, so perhaps a shift in differentiation strategy or expectations is required.

Our data also suggest that simple approaches, such as continued culture, can further the development of PSC-derived cells, though this method also has its limitations as described above. Nevertheless, it is tempting to speculate that, upon specification, the differentiation process is more or less pre-determined by mechanisms that lock cells into a process that takes a specific amount of time,

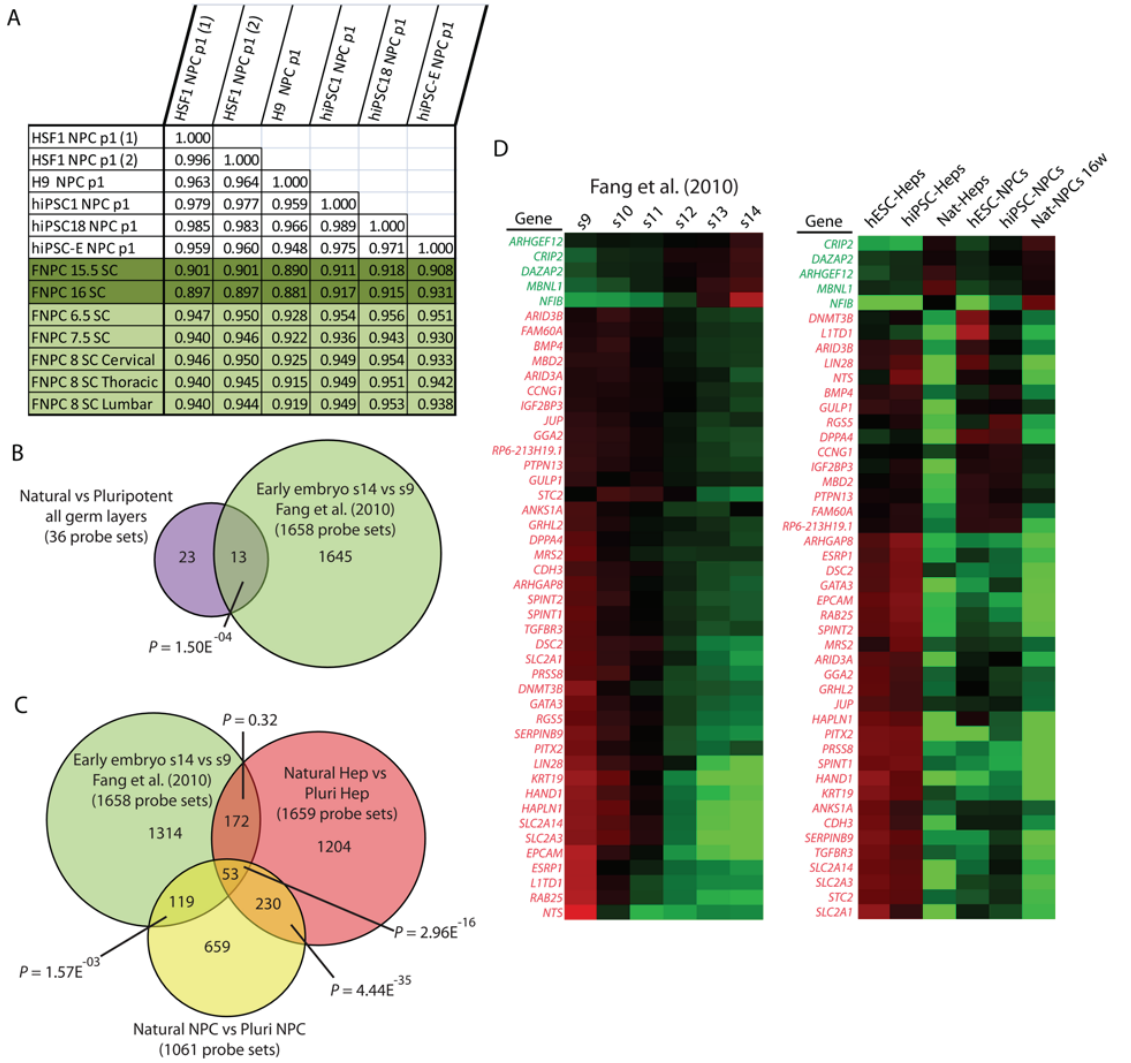



**Figure 7** Evidence that PSC derivatives reflect cell types found prior to 6 weeks of development. **(A)** Pearson correlation comparing global gene expression between PSC-NPCs and fetal spinal cord NPCs. **(B)** Comparison of the original probe sets identified as different between PSC derivatives and their natural counterpart (Figure 3A) and those differentially expressed between stage 9 and stage 14 embryos (Fang *et al*. [42], *t*-test $P < 0.01$, fold change ≥ 1.54). **(C)** Venn diagram comparing the probe sets different between PSC derivatives and their tissue-derived counterparts for the Hep and NPC lineages and those differentially expressed between stage 9 and stage 14 embryos. **(D)** Heat maps generated for the 46 unique genes represented by the 53 probe sets shown in **C**. Left, samples include the six stages of embryonic development represented in Fang *et al*. [42]. Right, samples include PSC-derived NPCs and Heps and their respective natural counterparts. Green probe sets represent those genes upregulated over the course of development, while red probe sets are those downregulated over the course of development.

or number of cell divisions complete, regardless of the culture conditions employed. This idea has significant support from studies with *in vitro* murine development [45], where differentiation of mESCs under minimal conditions allowed appropriate temporal and regional specification of neural tissue.

Recent work suggested that hPSC derivatives share hallmarks of gene expression with oncogenic cells [46].

Our data suggest that the presence of oncogenic hallmarks could be explained by persistent expression of genes in hPSC derivatives that are typical of the early embryo and known to be re-expressed in cancers. For example, while *LIN28* is not normally expressed in postnatal mammalian tissue, it was recently shown to be re-expressed in 15% of human cancers; and cancers with poor prognosis that are high in *LIN28* expression are low in let-7 family expression [39]. This study also showed that overexpression of *LIN28* drove transformation of FBs, consistent with its proposed role in reprogramming [35, 38]. Therefore, if the derivatives of PSCs are to be used clinically, it could be important to take into account the residual expression of the early embryonic genes, particularly, *LIN28*. Finally, one of the great benefits of iPS technology is the ability to model human diseases *in vitro* using patient-derived cells. Our data would suggest that it could be difficult to model human diseases in this context, unless a phenotype manifests very early in development.

## Materials and Methods

*Human tissue sourcing*

Primary human hepatocytes and FBs were acquired from Lonza (Switzerland). Fetal tissues were generated from discarded anonymized material obtained from elective terminations of first and second trimester pregnancies performed by the Family Planning Associates or the UCLA Medical Center. The fetal ages discussed throughout the paper represent the developmental age established 2 weeks after the first day of the last menstrual cycle. Tissues were harvested directly into sterile containers with PBS and transported on ice in PBS containing 5% FBS (Hyclone), 0.1% Ciprofloxacin HCl (10 µg/ml, Sigma), 1% amphotericin B (250 µg/ml, Invitrogen) and 1% penicillin-streptomycin (10 000 U/ml-10 000 µg/ml, Gibco) and processed the same day. Fetal spinal cord NPC lines were generated from 6-16-week-old spinal cords treated with collagenase and dispase for 1 h at 37 °C and plated on poly-ornithine/laminin-coated plates.

*Cell culture*

hESC and hiPSC were cultured as described previously [2, 47] in accordance with the UCLA ESCRO. Neural rosette derivation, NPC purification, and further differentiation to neurons and glia were performed as described [18]. Hepatocytes were differentiated using the four-stage protocol as described [20], with the exception of using FBS during the first 3 days of endoderm derivation. The AFP-GFP reporter [48] was transfected during differentiation using Lipofectamine 2000 (Invitrogen) and labeled cells were isolated using FACSA RIA (BD Biosciences). To generate FBs, EBs were cultured in adherent conditions using standard FB culturing media [47] and passaged until adopting typical FB morphology.

*Immunostaining and western blot*

Tissue acquired from embryonic spinal cord was fixed in 4% PFA for 1 followed by cryopreservation with sucrose and embedding in O.C.T. Compound (Sakura). Fetal livers were freshly embedded in O.C.T. Compound. Both tissues were sectioned on a CM3050S cryostat (Leica) at 6-10 µM thickness. Liver sections were fixed in formalin for 10 min at room temperature prior to immunostaining. Immunostaining was performed as described [18, 50]. Coverslips were fixed in 4% PFA, blocked for 1 h in 10% serum + 0.1% Tween 20 (or 0.1% Triton-X-100), then incubated overnight at 4 °C with primary antibodies. Following primary antibody incubation, the coverslips/cryosections were incubated with Alexa Fluor secondary antibodies (Invitrogen) at room temperature for 1 h and mounted in Prolong Gold with DAPI (Invitrogen). All imaging was performed on Zeiss Axio Imager A1. Antibodies used include the following: mouse anti-DPPA4 (Abnova), goat anti-ALB (Bethyl Laboratories), rabbit anti-SOX2 (Biolegend), rabbit anti-LIN28A, rabbit anti-LIN28B, and rabbit anti-Nanog (Cell Signaling Technology), rabbit anti-PAX6, rabbit anti-Tuj1 (Covance), rabbit anti-GFAP (DAKO), anti-CD44 (Developmental Studies Hybridoma Bank), mouse anti-NESTIN (Neuromics), goat anti-COL3A1, mouse anti-AFP, mouse anti-OCT3/4 (Santa Cruz Biotechnology Inc.), rabbit anti-SERPINA1 (Sigma Aldrich), and rabbit anti-Ki67 (Abcam). Western blot analysis was performed using standard procedures as described [50].

*Assays for function*

For periodic acid-schiff assay, cells were fixed with formalin-ethanol fixative and stained using standard protocol described in the manufacturer's manual (Sigma Aldrich). For albumin ELISA assay, media were collected from confluent wells and subjected to ELISA assay according to the manufacturer's protocol (Bethyl Laboratories). For Alizarin Red staining, natural and PSC-FBs were subjected to osteogenic induction and were stained with Alizarin Red as described [51]. Student's *t*-tests for the ELISA and NPC differentiation assays were performed in Excel.

*Reporter assay*

Cells transfected with the psiCHECK2- let-7 8× luciferase reporter (Addgene #20932) or psiCHECK2 control reporter (Promega) were lysed 72 h post-transfection and subjected to dual-glo luciferase assay as described in the manufacturer's protocol (Promega). The Renilla luciferase gene was driven by T7 promoter and contained eight let-7 targeting sequences in the 3′ UTR, and Firefly luciferase driven by a constitutive promoter as a transfection control. Luciferase assays were carried out in a GloMax 96 Microplate Luminometer (Promega).

*Expression analysis*

RNA isolation, reverse transcription, and real-time PCR were performed as described (Lowry *et al*. [47]). Microarray profiling was performed with Affymetrix Human HG-U133 2.0 Plus arrays as described [47, 52]. Data were normalized with Robust Multi-chip Algorithm in Genespring. Probe sets that were not expressed at a raw value of > 50 in at least 10% of samples were eliminated from further analysis. Note that outside data were normalized and analyzed separately, but with identical methods described above. Later, analysis on the NPC lineage was performed after separate normalization and filtering of only relevant samples. Outside data sets were collected from the Gene Expression Omnibus (NCBI) and includes: GSE19735 (Rafii), comparing hESC-derived endothelial cell (EC) to human umbilical vein ECs (HUVECs) and

human smooth muscle cells; GSE14897 (Duncan), comparing undifferentiated hESCs and hiPSCs to hepatocytes made from each; GSE20013 (Wilson), comparing ECs made from hESCs to ECs made form tissue (HUVEC); and GSE18887 (Fang), comparing entire human embryo samples from 3-5 weeks of development. All outside data were analyzed separately, but by employing the same methods. Fang *et al*. [42] utilized a U133 chip, which had fewer probe sets than our U133 2.0 plus chip. Only those probe sets that made it past the filtering on both chips were included in this analysis. Hierarchical Euclidian clustering was performed with complete linkage. Gene expression differences were judged to be significant if the *P*-value of the fold change was < 0.01 and at least 1.54-fold different between indicated samples. Gene expression differences were also judged by Benjamini-Hochberg correction for FDR at a *P*-value of < 0.05 and 1.54-fold change (Supplementary information, Figure S2). Further statistical analysis for hypergeometric distribution and three-way simulation was performed with R, package 2.9.2 as described [12]. Heat maps were generated by averaging the raw value of like samples and representing it as a ratio of the average of the raw value of all samples (Cluster 3.0, Java TreeView). Pearson correlations were generated in Excel.

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(**Supplementary information** is linked to the online version of the paper on the *Cell Research* website.)

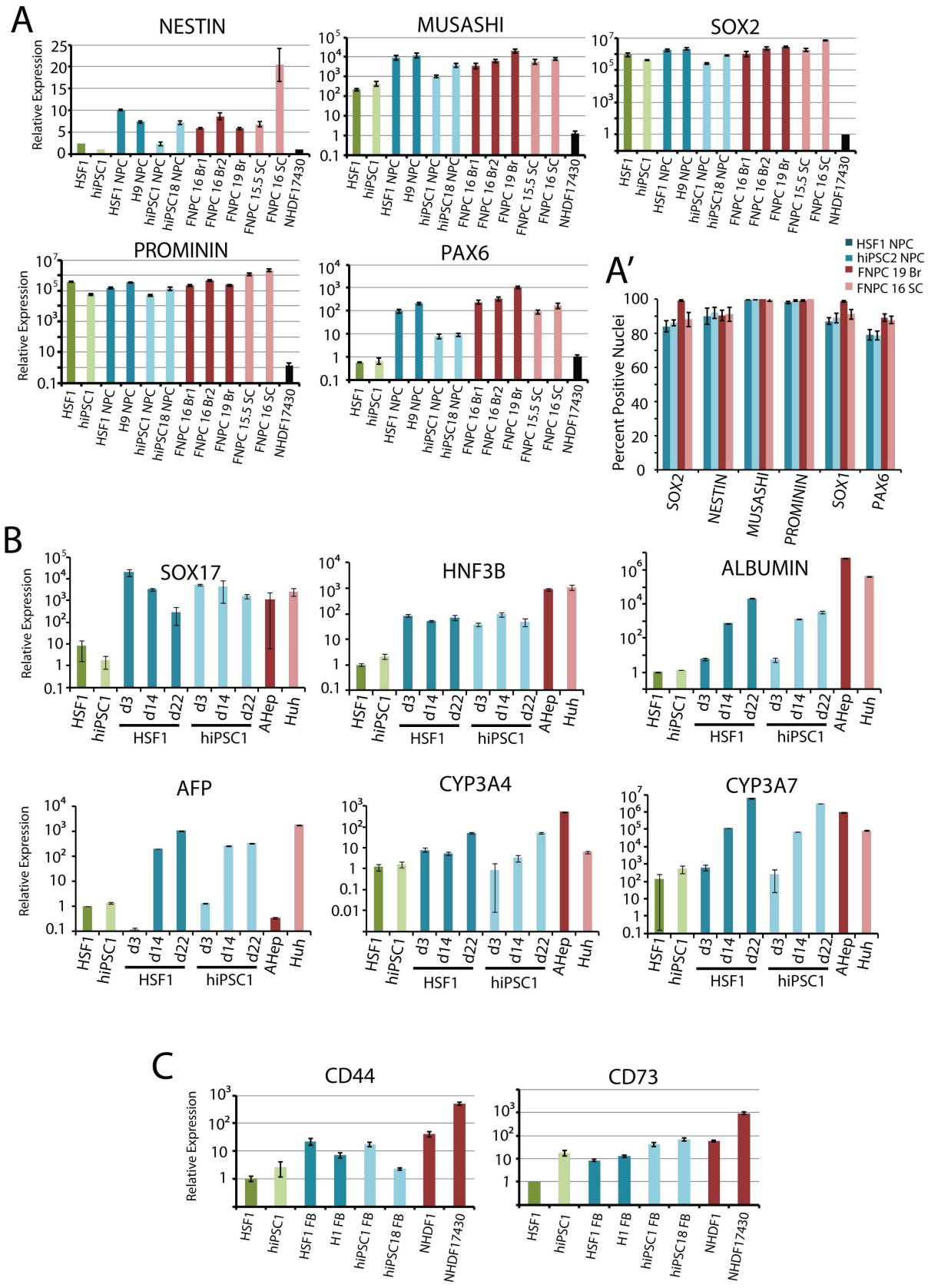
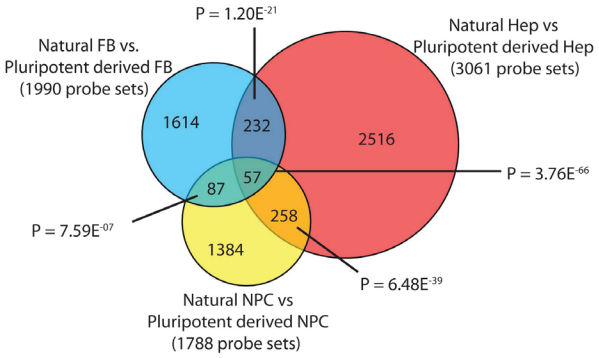
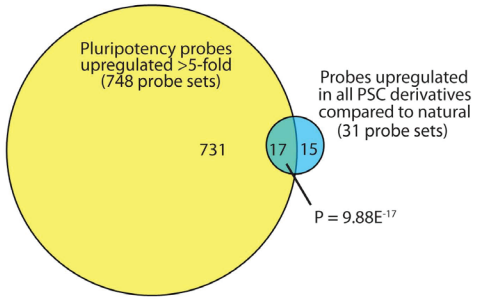

A
NESTIN
MUSASHI
SOX2
PROMININ
PAX6
Relative Expression
HSF1
hiPSC1
HSF1 NPC
H9 NPC
hiPSC1 NPC
hiPSC18 NPC
FNPC 16 Br1
FNPC 16 Br2
FNPC 19 Br
FNPC 15.5 SC
FNPC 16 SC
NHDF17430
A′
HSF1 NPC
hiPSC2 NPC
FNPC 19 Br
FNPC 16 SC
Percent Positive Nuclei
SOX2
NESTIN
MUSASHI
PROMININ
SOX1
PAX6
B
SOX17
HNF3B
ALBUMIN
AFP
CYP3A4
CYP3A7
d3
d14
d22
AHep
Huh
HSF1
hiPSC1
C
CD44
CD73
HSF1 FB
H1 FB
hiPSC1 FB
hiPSC18 FB
NHDF1

**Figure S1** hESC and hiPSC lines make cell types representing all three germ layers. **A,** RT-PCRs demonstrating mRNA expression of NPC markers (SOX2, NESTIN, MUSASHI, PROMININ, and PAX6) in NPCs derived from PSCs, fetal brain and fetal spinal cord.Error bars represent standard error over 4 replicates. Values set relative to NHDF17430. **A',** Quantification of the percent positive cells in NPC cultures expressing SOX2,PAX6, MMUSASHI, PROMININ, and NESTIN). Error bars represent standard error over 10 fields of view. **B,** RT-PCRs of definitive endoderm markers (SOX17 and HNF3B), fetal hepatocyte markers (AFP and CYP3A7) and adult hepatocyte markers (ALBUMINand CYP3A4) following 3, 14 and 22 days of directed-differentiation. Error bars represent standard error over 4 replicates. Values set relative to HSF1. **C,** RT-PCR of fibroblast markers (CD44 and CD73) following differentiation of pluripotent stem cells to fibroblasts.Error bars represent standard error over 4 replicates. Values set relative to HSF1.

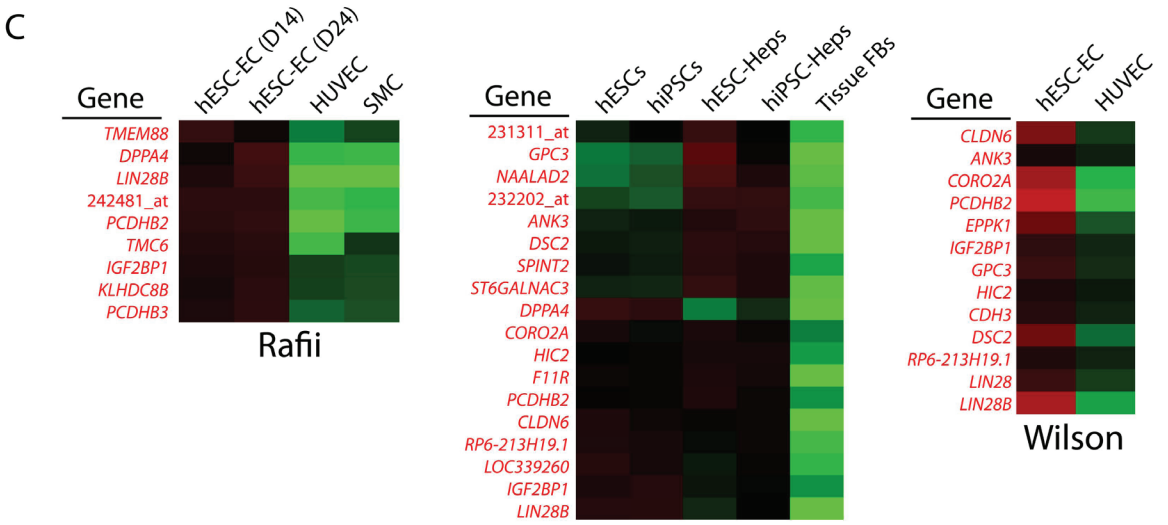
A




B




C




D

| Go Terms: Biological Processes | | |
|---|---|---|
| **NPC** | **Hep** | **FB** |
| **homophilic cell adhesion; nervous system development;** developmental process; calcium-dependent cell-cell adhesion | **nervous system development**; oxidation reduction; acute inflammatory response; metabolic process; cofactor metabolic process; cellular lipid metabolic process; regulation of RNA metabolic process; regulation of nucleobase, nucleoside, nucleotide; regulation of transcription, DNA-dependent; regulation of transcription; aromatic compound metabolic process; regulation of gene expression; biphenyl metabolic process; bilirubin conjugation; humoral immune response mediated circulating immunoglobulin; activation of plasma proteins during accute inflammatory response; complement activation, classical pathway; complement activation; lipid metabolic process; regulation of macromolecule biosynthetic process; tetrapyrrole catabolic process; porphyrin catabolic process; cellular metabolic process; regulation of biosynthetic process; transcription; regulation of cellular metabolic process; benzene and derivative metabolic process; negative regulation of biosynthetic process; heterocycle metabolic process; regulation of metabolic process; steroid metabolic process; negative regulation of gene expression; biosynthetic process; negative regulation of transcription; negative regulation of nucleobase, nucleoside, nucleotide; regulation of macromolecule metabolic process; tetrapyrrole metabolic process; porphyrin metabolic process | **homophilic cell adhesion;** cell-cell adhesion |

**Figure S2** Gene expression differences between PSC and tissue derivatives are conserved regardless of statistical analyses employed or lab. **A and B,** A similar analysis as shown in Figure 3A and B, but instead using FDR ($p \leq 0.05$) and fold change $\geq 1.54$ as criteria to determine genes that were differentially expressed. **C,** The same analysis shown in Figure 3C was performed with 3 outside datasets. Outside data sets included: GSE19735 (Rafii), comparing hESC-derived endothelial cell (EC) to human umbilical vein endothelial cells (HUVEC) and human smooth muscle cells (SMC); GSE14897 (Duncan), comparing undifferentiated hESCs and hiPSCs to hepatocytes made from each; and GSE20013 (Wilson), comparing endothelial cells made from hESCs to endothelial cells made form tissue (HUVEC). **D,** Gene Ontology Biological Processes terms identified as different between PSC derivatives and their natural counterparts ($p<0.01$). Underlined terms were identified in 2 of 3 germ layers. Note: no term was identified in all three germ layers.

| Probe Set ID | Gene Symbol | hESC (3) | | hiPSC (6) | | PSC-Heps (5) | | Adult-Hep (2) | |
|---|---|---|---|---|---|---|---|---|---|
| | | *avg* | *stderr* | *avg* | *stderr* | *avg* | *stderr* | *avg* | *stderr* |
| 214829_at | AASS | 2936 | 215 | 1982 | 316 | 220 | 13 | 314 | 106 |
| 237974_at | ABHD12B | 529 | 72 | 204 | 98 | 8 | 0 | 8 | 1 |
| 203872_at | ACTA1 | 897 | 106 | 1368 | 519 | 657 | 280 | 18 | 0 |
| 205132_at | ACTC1 | 2724 | 398 | 3937 | 690 | 4382 | 1100 | 125 | 14 |
| 236126_at | ACVR2B | 3084 | 42 | 2631 | 168 | 1713 | 141 | 211 | 48 |
| 1553179_at | ADAMTS19 | 314 | 16 | 316 | 34 | 135 | 35 | 36 | 3 |
| 213217_at | ADCY2 | 1071 | 44 | 1540 | 266 | 223 | 36 | 50 | 3 |
| 205268_s_at | ADD2 | 624 | 78 | 789 | 126 | 35 | 3 | 44 | 5 |
| 228771_at | ADRBK2 | 866 | 38 | 644 | 81 | 575 | 46 | 55 | 13 |
| 223075_s_at | AIF1L | 1006 | 59 | 1435 | 179 | 379 | 43 | 46 | 6 |
| 228342_s_at | ALPK3 | 215 | 32 | 235 | 27 | 1319 | 146 | 169 | 53 |
| 215783_s_at | ALPL | 159 | 9 | 460 | 110 | 61 | 11 | 71 | 12 |
| 206385_s_at | ANK3 | 1363 | 85 | 1379 | 126 | 1438 | 94 | 290 | 164 |
| 209369_at | ANXA3 | 1028 | 141 | 1699 | 312 | 3550 | 863 | 939 | 427 |
| 218261_at | AP1M2 | 473 | 19 | 459 | 71 | 392 | 52 | 73 | 6 |
| 228415_at | AP1S2 | 9974 | 891 | 8598 | 1058 | 1932 | 179 | 408 | 30 |
| 203381_s_at | APOE | 3165 | 72 | 2766 | 162 | 8399 | 1069 | 12644 | 6762 |
| 203946_s_at | ARG2 | 1026 | 28 | 873 | 93 | 1180 | 84 | 303 | 221 |
| 37117_at | ARHGAP8 | 976 | 48 | 873 | 100 | 2696 | 355 | 142 | 28 |
| 218964_at | ARID3B | 936 | 89 | 1249 | 96 | 805 | 64 | 101 | 6 |
| 209464_at | AURKB | 1012 | 38 | 915 | 69 | 140 | 18 | 97 | 62 |
| 1555962_at | B3GNT7 | 509 | 69 | 524 | 56 | 184 | 33 | 40 | 2 |
| 222891_s_at | BCL11A | 1297 | 46 | 1032 | 123 | 424 | 75 | 18 | 1 |
| 227920_at | BEND3 | 1124 | 37 | 873 | 82 | 188 | 20 | 110 | 20 |
| 230896_at | BEND4 | 1497 | 181 | 824 | 94 | 22 | 6 | 11 | 0 |
| 218332_at | BEX1 | 4164 | 274 | 3931 | 216 | 5073 | 467 | 85 | 34 |
| 224367_at | BEX2 | 1514 | 149 | 1393 | 143 | 748 | 100 | 58 | 21 |
| 1554020_at | BICD1 | 791 | 108 | 633 | 93 | 216 | 22 | 40 | 29 |
| 205733_at | BLM | 512 | 28 | 495 | 28 | 93 | 11 | 45 | 13 |
| 231953_at | BPTF | 695 | 15 | 540 | 45 | 68 | 5 | 42 | 13 |
| 209642_at | BUB1 | 1695 | 60 | 1413 | 64 | 264 | 70 | 95 | 83 |
| 203755_at | BUB1B | 2938 | 32 | 2576 | 127 | 390 | 66 | 215 | 179 |
| 228281_at | C11orf82 | 721 | 52 | 762 | 23 | 135 | 21 | 86 | 53 |
| 218614_at | C12orf35 | 2753 | 139 | 1609 | 139 | 1395 | 132 | 1001 | 533 |
| 227928_at | C12orf48 | 285 | 23 | 307 | 31 | 55 | 9 | 40 | 29 |
| 1570153_at | C13orf38 | 984 | 306 | 716 | 144 | 20 | 3 | 21 | 10 |
| 220536_at | C14orf115 | 609 | 7 | 501 | 41 | 103 | 7 | 104 | 22 |
| 226456_at | C16orf75 | 2096 | 64 | 1457 | 123 | 370 | 45 | 145 | 76 |
| 223631_s_at | C19orf33 | 332 | 57 | 333 | 111 | 623 | 94 | 158 | 6 |
| 219010_at | C1orf106 | 1645 | 59 | 1271 | 83 | 2317 | 188 | 390 | 154 |
| 220011_at | C1orf135 | 773 | 45 | 693 | 30 | 161 | 11 | 76 | 14 |
| 1554340_a_at | C1orf187 | 409 | 15 | 376 | 70 | 28 | 8 | 14 | 0 |
| 219004_s_at | C21orf45 | 1586 | 22 | 1361 | 53 | 262 | 32 | 181 | 64 |
| 232067_at | C6orf168 | 315 | 30 | 424 | 50 | 241 | 70 | 21 | 9 |
| 230903_s_at | C8orf42 | 1308 | 144 | 1328 | 137 | 451 | 46 | 414 | 114 |
| 243610_at | C9orf135 | 2099 | 301 | 1926 | 363 | 54 | 14 | 12 | 2 |
| 209726_at | CA11 | 787 | 87 | 519 | 93 | 120 | 19 | 21 | 2 |
| 219464_at | CA14 | 642 | 19 | 469 | 60 | 113 | 17 | 48 | 7 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 225627_s_at | CACHD1 | 2449 | 113 | 1967 | 216 | 1664 | 267 | 109 | 20 |
| 204811_s_at | CACNA2D2 | 484 | 46 | 386 | 80 | 148 | 15 | 79 | 17 |
| 205625_s_at | CALB1 | 412 | 105 | 465 | 87 | 106 | 26 | 15 | 0 |
| 214636_at | CALCB | 136 | 74 | 211 | 142 | 26 | 8 | 18 | 4 |
| 226473_at | CBX2 | 1346 | 36 | 959 | 100 | 459 | 88 | 73 | 10 |
| 239233_at | CCDC88A | 72 | 6 | 57 | 8 | 9 | 1 | 9 | 1 |
| 227228_s_at | CCDC88C | 339 | 27 | 398 | 46 | 294 | 35 | 54 | 15 |
| 210381_s_at | CCKBR | 198 | 28 | 318 | 104 | 461 | 209 | 34 | 1 |
| 228729_at | CCNB1 | 2322 | 78 | 1721 | 119 | 356 | 41 | 177 | 156 |
| 202705_at | CCNB2 | 2785 | 63 | 2362 | 84 | 458 | 55 | 226 | 195 |
| 209583_s_at | CD200 | 1809 | 39 | 1618 | 281 | 222 | 51 | 31 | 17 |
| 209771_x_at | CD24 | 17099 | 52 | 14710 | 602 | 15262 | 1043 | 9532 | 1357 |
| 204695_at | CDC25A | 1369 | 23 | 1255 | 91 | 253 | 15 | 101 | 12 |
| 203967_at | CDC6 | 611 | 52 | 652 | 33 | 156 | 13 | 99 | 78 |
| 204510_at | CDC7 | 1398 | 99 | 1268 | 93 | 291 | 27 | 127 | 76 |
| 224428_s_at | CDCA7 | 2738 | 36 | 2073 | 188 | 547 | 102 | 113 | 95 |
| 201131_s_at | CDH1 | 4756 | 206 | 3161 | 353 | 5044 | 640 | 2050 | 886 |
| 203256_at | CDH3 | 1688 | 6 | 1409 | 135 | 1869 | 250 | 94 | 34 |
| 226185_at | CDS1 | 368 | 26 | 282 | 29 | 608 | 74 | 64 | 17 |
| 228868_x_at | CDT1 | 615 | 1 | 496 | 38 | 168 | 24 | 47 | 25 |
| 219505_at | CECR1 | 1049 | 148 | 866 | 125 | 544 | 47 | 90 | 2 |
| 223729_at | CECR2 | 982 | 30 | 815 | 68 | 213 | 10 | 194 | 69 |
| 231772_x_at | CENPH | 927 | 31 | 659 | 79 | 138 | 17 | 59 | 32 |
| 221378_at | CER1 | 492 | 154 | 501 | 156 | 60 | 23 | 33 | 1 |
| 225817_at | CGNL1 | 1538 | 74 | 1089 | 123 | 1192 | 44 | 1574 | 903 |
| 218829_s_at | CHD7 | 836 | 228 | 1447 | 138 | 1049 | 71 | 196 | 11 |
| 205394_at | CHEK1 | 1163 | 37 | 849 | 90 | 148 | 19 | 95 | 75 |
| 226803_at | CHMP4C | 212 | 29 | 402 | 61 | 615 | 139 | 761 | 41 |
| 206533_at | CHRNA5 | 175 | 20 | 157 | 8 | 19 | 2 | 16 | 4 |
| 224400_s_at | CHST9 | 163 | 37 | 149 | 42 | 384 | 67 | 242 | 76 |
| 202712_s_at | CKMT1A/B | 926 | 60 | 553 | 90 | 51 | 5 | 52 | 4 |
| 205328_at | CLDN10 | 715 | 32 | 845 | 144 | 47 | 5 | 94 | 0 |
| 237810_at | CLDN6 | 1051 | 312 | 2111 | 534 | 1113 | 85 | 28 | 1 |
| 202790_at | CLDN7 | 894 | 62 | 684 | 49 | 1444 | 208 | 507 | 192 |
| 219301_s_at | CNTNAP2 | 1404 | 132 | 1184 | 222 | 425 | 57 | 103 | 2 |
| 223796_at | CNTNAP3 | 284 | 43 | 256 | 48 | 33 | 7 | 26 | 7 |
| 213050_at | COBL | 787 | 154 | 826 | 188 | 705 | 69 | 864 | 421 |
| 205229_s_at | COCH | 838 | 53 | 1292 | 322 | 187 | 33 | 42 | 18 |
| 213492_at | COL2A1 | 159 | 10 | 908 | 576 | 3865 | 944 | 43 | 6 |
| 213992_at | COL4A6 | 333 | 36 | 486 | 44 | 3188 | 414 | 20 | 3 |
| 227177_at | CORO2A | 211 | 14 | 302 | 46 | 483 | 104 | 149 | 42 |
| 224344_at | COX6A1 | 2567 | 865 | 1506 | 544 | 57 | 6 | 46 | 0 |
| 208146_s_at | CPVL | 598 | 20 | 543 | 48 | 726 | 163 | 38 | 19 |
| 239205_s_at | CR1 /CR1L | 74 | 37 | 175 | 33 | 10 | 1 | 9 | 1 |
| 239206_at | CR1L | 62 | 32 | 108 | 14 | 15 | 1 | 14 | 0 |
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| 206224_at | CST1 | 131 | 23 | 524 | 393 | 1492 | 323 | 22 | 4 |
| 210074_at | CTSL2 | 2509 | 180 | 1948 | 169 | 1804 | 197 | 132 | 20 |
| 203917_at | CXADR | 4296 | 116 | 4419 | 278 | 2238 | 36 | 1209 | 445 |

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| 239155_at | CXADRP1 | 663 | 22 | 533 | 54 | 315 | 35 | 285 | 10 |
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| 206424_at | CYP26A1 | 1367 | 155 | 1472 | 343 | 103 | 23 | 33 | 4 |
| 223385_at | CYP2S1 | 291 | 25 | 778 | 229 | 94 | 15 | 42 | 5 |
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| 206588_at | DAZL | 112 | 20 | 76 | 26 | 6 | 0 | 6 | 0 |
| 205399_at | DCLK1 | 1595 | 157 | 1347 | 183 | 38 | 7 | 20 | 3 |
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| 219945_at | DDX25 | 497 | 53 | 374 | 62 | 54 | 8 | 34 | 3 |
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| 205603_s_at | DIAPH2 | 2259 | 737 | 1789 | 142 | 151 | 9 | 236 | 37 |
| 203764_at | DLGAP5 | 2130 | 39 | 1815 | 78 | 207 | 40 | 134 | 120 |
| 209560_s_at | DLK1 | 108 | 14 | 721 | 464 | 6810 | 625 | 14 | 0 |
| 226926_at | DMKN | 1084 | 21 | 1176 | 174 | 716 | 90 | 94 | 8 |
| 222640_at | DNMT3A | 1967 | 36 | 1824 | 60 | 950 | 46 | 214 | 6 |
| 220668_s_at | DNMT3B | 12326 | 579 | 8911 | 479 | 660 | 118 | 134 | 16 |
| 240301_at | DPPA2 | 359 | 54 | 150 | 18 | 46 | 7 | 26 | 3 |
| 231385_at | DPPA3 | 240 | 14 | 43 | 13 | 304 | 61 | 10 | 0 |
| 232985_s_at | DPPA4 | 9192 | 358 | 7635 | 379 | 447 | 80 | 30 | 4 |
| 226817_at | DSC2 | 1051 | 63 | 871 | 114 | 2545 | 162 | 401 | 22 |
| 219000_s_at | DSCC1 | 1227 | 71 | 1044 | 72 | 126 | 11 | 89 | 36 |
| 217901_at | DSG2 | 4138 | 153 | 3471 | 256 | 5354 | 711 | 2051 | 300 |
| 218585_s_at | DTL | 2142 | 109 | 2321 | 161 | 307 | 31 | 180 | 146 |
| 221586_s_at | E2F5 | 960 | 17 | 1003 | 86 | 332 | 58 | 117 | 60 |
| 204271_s_at | EDNRB | 1710 | 132 | 3166 | 671 | 930 | 122 | 143 | 118 |
| 219232_s_at | EGLN3 | 384 | 21 | 381 | 51 | 113 | 7 | 172 | 111 |
| 227180_at | ELOVL7 | 312 | 13 | 253 | 40 | 473 | 127 | 73 | 3 |
| 226789_at | EMB | 906 | 15 | 783 | 110 | 242 | 78 | 106 | 29 |
| 229080_at | EMID2 | 240 | 31 | 306 | 43 | 427 | 73 | 52 | 0 |
| 220161_s_at | EPB41L4B | 1251 | 52 | 1234 | 144 | 349 | 16 | 2354 | 236 |
| 229292_at | EPB41L5 | 461 | 52 | 362 | 40 | 429 | 55 | 614 | 283 |
| 201839_s_at | EPCAM | 7357 | 195 | 5443 | 524 | 9719 | 1314 | 438 | 20 |
| 205977_s_at | EPHA1 | 421 | 34 | 427 | 35 | 392 | 72 | 180 | 77 |
| 229288_at | EPHA7 | 124 | 7 | 519 | 130 | 690 | 106 | 18 | 4 |
| 220013_at | EPHX3 | 527 | 33 | 430 | 88 | 166 | 27 | 50 | 3 |
| 232165_at | EPPK1 | 478 | 12 | 473 | 95 | 970 | 216 | 96 | 7 |
| 226213_at | ERBB3 | 1538 | 70 | 1024 | 143 | 1494 | 330 | 2774 | 1251 |
| 219650_at | ERCC6L | 531 | 54 | 466 | 25 | 68 | 8 | 71 | 32 |
| 225846_at | ESRP1 | 3037 | 150 | 2478 | 340 | 2209 | 75 | 44 | 2 |
| 219395_at | ESRP2 | 593 | 7 | 522 | 68 | 1168 | 88 | 964 | 335 |
| 223000_s_at | F11R | 4770 | 216 | 4284 | 316 | 6440 | 408 | 3156 | 616 |
| 213506_at | F2RL1 | 2044 | 161 | 2561 | 702 | 9854 | 2230 | 1272 | 465 |
| 219429_at | FA2H | 246 | 27 | 164 | 27 | 108 | 17 | 58 | 2 |
| 202345_s_at | FABP5 | 10904 | 434 | 10457 | 384 | 4683 | 1258 | 523 | 452 |
| 235177_at | FAM119A | 1700 | 74 | 1400 | 88 | 495 | 54 | 318 | 87 |
| 230067_at | FAM124A | 837 | 37 | 698 | 80 | 43 | 4 | 37 | 12 |
| 238018_at | FAM150B | 177 | 3 | 398 | 78 | 29 | 3 | 107 | 50 |
| 235048_at | FAM169A | 867 | 16 | 880 | 53 | 533 | 65 | 213 | 88 |
| 242348_at | FAM19A4 | 55 | 6 | 50 | 5 | 246 | 172 | 8 | 0 |
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| 220147_s_at | FAM60A | 6725 | 54 | 6074 | 516 | 2210 | 247 | 384 | 179 |
| 225687_at | FAM83D | 1509 | 60 | 1425 | 82 | 144 | 23 | 161 | 116 |
| 235269_at | FAM83F | 175 | 20 | 212 | 30 | 477 | 74 | 48 | 11 |
| 226129_at | FAM83H | 453 | 16 | 519 | 55 | 944 | 56 | 469 | 9 |
| 225864_at | FAM84B | 997 | 70 | 984 | 88 | 1669 | 130 | 350 | 116 |
| 242560_at | FANCD2 | 1740 | 72 | 1669 | 112 | 362 | 28 | 118 | 60 |
| 220615_s_at | FAR2 | 513 | 31 | 491 | 73 | 307 | 74 | 15 | 1 |
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| 205110_s_at | FGF13 | 1380 | 148 | 2101 | 215 | 469 | 93 | 86 | 11 |
| 223761_at | FGF19 | 154 | 13 | 222 | 85 | 62 | 7 | 38 | 11 |
| 203638_s_at | FGFR2 | 1139 | 107 | 935 | 102 | 1324 | 60 | 583 | 469 |
| 1570515_a_at | FILIP1 | 85 | 13 | 76 | 20 | 20 | 3 | 7 | 1 |
| 1553991_s_at | FLJ20674 | 942 | 67 | 557 | 60 | 76 | 9 | 290 | 18 |
| 1564413_at | FLJ36116 | 474 | 57 | 799 | 371 | 587 | 195 | 42 | 3 |
| 210287_s_at | FLT1 | 82 | 23 | 59 | 14 | 15 | 2 | 11 | 1 |
| 222906_at | FLVCR1 | 1358 | 175 | 1335 | 145 | 785 | 171 | 153 | 14 |
| 228463_at | FOXA3 | 324 | 38 | 243 | 14 | 547 | 32 | 449 | 7 |
| 241612_at | FOXD3 | 191 | 15 | 198 | 25 | 12 | 1 | 12 | 0 |
| 231407_s_at | FOXH1 | 1138 | 36 | 753 | 89 | 182 | 24 | 39 | 1 |
| 202724_s_at | FOXO1 | 1038 | 40 | 696 | 62 | 469 | 80 | 227 | 27 |
| 226145_s_at | FRAS1 | 2024 | 194 | 1784 | 287 | 4479 | 322 | 88 | 44 |
| 209864_at | FRAT2 | 1668 | 53 | 1258 | 102 | 471 | 59 | 260 | 46 |
| 230964_at | FREM2 | 535 | 60 | 412 | 69 | 1943 | 162 | 30 | 20 |
| 203697_at | FRZB | 136 | 22 | 292 | 88 | 2791 | 224 | 15 | 1 |
| 221245_s_at | FZD5 | 2433 | 28 | 2559 | 196 | 3828 | 735 | 1528 | 529 |
| 227830_at | GABRB3 | 2725 | 101 | 2266 | 163 | 96 | 8 | 40 | 3 |
| 214240_at | GAL | 4289 | 628 | 4936 | 816 | 97 | 12 | 548 | 453 |
| 203397_s_at | GALNT3 | 587 | 217 | 507 | 72 | 138 | 26 | 81 | 19 |
| 213280_at | GARNL4 | 831 | 34 | 625 | 91 | 267 | 32 | 105 | 54 |
| 230788_at | GCNT2 | 1903 | 45 | 1569 | 80 | 1475 | 65 | 558 | 1 |
| 220053_at | GDF3 | 1112 | 149 | 1167 | 687 | 96 | 31 | 59 | 4 |
| 206102_at | GINS1 | 3530 | 92 | 3173 | 173 | 538 | 52 | 284 | 193 |
| 221521_s_at | GINS2 | 1515 | 82 | 1476 | 67 | 209 | 30 | 130 | 62 |
| 1569886_a_at | GLB1L3 | 194 | 9 | 105 | 30 | 6 | 0 | 6 | 0 |
| 204836_at | GLDC | 2983 | 325 | 2976 | 330 | 3566 | 650 | 1970 | 263 |
| 220108_at | GNA14 | 475 | 100 | 1035 | 110 | 54 | 7 | 76 | 2 |
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| 209220_at | GPC3 | 374 | 55 | 614 | 60 | 9377 | 1086 | 42 | 11 |
| 204984_at | GPC4 | 3755 | 202 | 3711 | 593 | 383 | 68 | 49 | 17 |
| 206696_at | GPR143 | 249 | 11 | 201 | 25 | 104 | 11 | 40 | 13 |
| 223423_at | GPR160 | 960 | 67 | 714 | 45 | 515 | 136 | 49 | 1 |
| 207183_at | GPR19 | 665 | 52 | 559 | 59 | 47 | 10 | 14 | 1 |
| 206002_at | GPR64 | 412 | 11 | 305 | 31 | 42 | 7 | 115 | 4 |
| 203632_s_at | GPRC5B | 2138 | 45 | 1764 | 150 | 1766 | 498 | 870 | 239 |
| 206204_at | GRB14 | 385 | 26 | 451 | 44 | 182 | 27 | 2154 | 1437 |
| 205862_at | GREB1 | 144 | 15 | 187 | 41 | 295 | 54 | 76 | 57 |
| 219388_at | GRHL2 | 199 | 34 | 166 | 34 | 164 | 28 | 27 | 4 |
| 229377_at | GRTP1 | 668 | 166 | 391 | 57 | 352 | 45 | 985 | 564 |
| 1255_g_at | GUCA1A | 813 | 63 | 360 | 98 | 104 | 17 | 16 | 2 |
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| 223541_at | HAS3 | 896 | 97 | 845 | 50 | 154 | 31 | 162 | 100 |
| 239781_at | hCG_1815504 | 571 | 36 | 349 | 30 | 27 | 3 | 32 | 5 |
| 227350_at | HELLS | 2866 | 60 | 2053 | 247 | 406 | 69 | 129 | 72 |
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| 219743_at | HEY2 | 548 | 32 | 364 | 13 | 129 | 17 | 75 | 41 |
| 212964_at | HIC2 | 990 | 40 | 817 | 91 | 1601 | 118 | 93 | 5 |
| 239975_at | HLA-DPB2 | 1348 | 251 | 731 | 169 | 14 | 3 | 11 | 1 |
| 208025_s_at | HMGA2 | 2761 | 223 | 3488 | 340 | 1927 | 396 | 176 | 160 |
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| 225792_at | HOOK1 | 705 | 49 | 537 | 44 | 277 | 36 | 719 | 340 |
| 203914_x_at | HPGD | 191 | 37 | 416 | 103 | 2660 | 638 | 3283 | 402 |
| 219983_at | HRASLS | 368 | 29 | 296 | 27 | 231 | 37 | 15 | 7 |
| 1552767_a_at | HS6ST2 | 293 | 37 | 662 | 269 | 1341 | 129 | 33 | 16 |
| 204130_at | HSD11B2 | 107 | 11 | 99 | 11 | 34 | 4 | 25 | 5 |
| 210029_at | IDO1 | 2459 | 290 | 1164 | 370 | 49 | 3 | 40 | 7 |
| 230960_at | IGDCC3 | 276 | 14 | 655 | 114 | 2426 | 530 | 23 | 6 |
| 227377_at | IGF2BP1 | 750 | 43 | 1256 | 173 | 991 | 178 | 67 | 13 |
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| 216493_s_at | IGF2BP3 | 983 | 24 | 543 | 93 | 102 | 9 | 37 | 2 |
| 227760_at | IGFBPL1 | 464 | 27 | 602 | 106 | 164 | 40 | 23 | 1 |
| 227997_at | IL17RD | 1566 | 61 | 2333 | 412 | 435 | 68 | 143 | 108 |
| 203126_at | IMPA2 | 1333 | 47 | 1221 | 128 | 760 | 89 | 270 | 64 |
| 213447_at | IPW | 3908 | 155 | 3757 | 293 | 720 | 133 | 316 | 9 |
| 203474_at | IQGAP2 | 730 | 52 | 627 | 46 | 848 | 146 | 1669 | 849 |
| 227297_at | ITGA9 | 98 | 20 | 194 | 33 | 344 | 36 | 67 | 53 |
| 221051_s_at | ITGB1BP3 | 946 | 40 | 957 | 115 | 275 | 115 | 47 | 5 |
| 202747_s_at | ITM2A | 966 | 72 | 532 | 67 | 196 | 27 | 39 | 7 |
| 203298_s_at | JARID2 | 4897 | 157 | 4373 | 191 | 1018 | 68 | 234 | 26 |
| 229139_at | JPH1 | 549 | 26 | 644 | 40 | 254 | 20 | 24 | 2 |
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| 205206_at | KAL1 | 2053 | 84 | 2543 | 348 | 410 | 123 | 46 | 24 |
| 239835_at | KBTBD8 | 708 | 42 | 543 | 50 | 202 | 24 | 64 | 40 |
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| 1552897_a_at | KCNG3 | 407 | 14 | 217 | 46 | 27 | 8 | 11 | 1 |
| 219615_s_at | KCNK5 | 271 | 27 | 231 | 20 | 85 | 15 | 184 | 79 |
| 220116_at | KCNN2 | 739 | 25 | 631 | 60 | 362 | 43 | 85 | 48 |
| 58916_at | KCTD14 | 399 | 13 | 362 | 30 | 126 | 17 | 446 | 206 |
| 203934_at | KDR | 927 | 242 | 677 | 146 | 557 | 90 | 38 | 15 |
| 231887_s_at | KIAA1274 | 406 | 24 | 459 | 31 | 142 | 14 | 62 | 5 |
| 235301_at | KIAA1324L | 379 | 32 | 333 | 27 | 334 | 16 | 36 | 7 |
| 228565_at | KIAA1804 | 688 | 50 | 745 | 115 | 203 | 43 | 234 | 86 |
| 232069_at | KIF26A | 370 | 20 | 307 | 14 | 217 | 46 | 72 | 21 |
| 242517_at | KISS1R | 310 | 25 | 166 | 32 | 419 | 160 | 45 | 12 |
| 213610_s_at | KLHL23 | 1825 | 92 | 1431 | 101 | 439 | 44 | 220 | 76 |
| 220239_at | KLHL7 | 1441 | 85 | 1510 | 63 | 230 | 17 | 113 | 52 |
| 206541_at | KLKB1 | 320 | 31 | 401 | 52 | 54 | 5 | 2822 | 2468 |
| 244264_at | KLRG2 | 238 | 26 | 342 | 98 | 74 | 8 | 35 | 1 |

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| 201596_x_at | KRT18 | 4494 | 302 | 5600 | 770 | 12374 | 954 | 14490 | 4342 |
| 201650_at | KRT19 | 1051 | 30 | 802 | 138 | 6793 | 511 | 1104 | 308 |
| 209008_x_at | KRT8 | 1472 | 222 | 2030 | 438 | 7133 | 277 | 7813 | 2389 |
| 219955_at | L1TD1 | 12955 | 366 | 11925 | 449 | 196 | 33 | 32 | 4 |
| 227048_at | LAMA1 | 453 | 22 | 487 | 32 | 4531 | 1084 | 32 | 22 |
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| 204891_s_at | LCK | 769 | 64 | 635 | 100 | 41 | 2 | 36 | 2 |
| 206309_at | LECT1 | 813 | 16 | 901 | 124 | 27 | 2 | 25 | 3 |
| 206268_at | LEFTY1 | 1272 | 91 | 4740 | 1577 | 34 | 6 | 53 | 24 |
| 206012_at | LEFTY2 | 790 | 46 | 2988 | 1390 | 142 | 27 | 48 | 1 |
| 219823_at | LIN28 | 14338 | 239 | 11441 | 925 | 5595 | 633 | 18 | 4 |
| 229349_at | LIN28B | 5194 | 135 | 4153 | 246 | 1749 | 119 | 10 | 1 |
| 227933_at | LINGO1 | 1703 | 62 | 867 | 133 | 186 | 17 | 72 | 3 |
| 230195_at | LOC100131138 | 1004 | 247 | 1340 | 379 | 163 | 17 | 13 | 3 |
| 226809_at | LOC100216479 | 703 | 264 | 661 | 119 | 342 | 71 | 24 | 8 |
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| 1561101_at | LOC153469 | 419 | 40 | 371 | 75 | 28 | 2 | 24 | 3 |
| 228051_at | LOC202451 | 498 | 30 | 402 | 70 | 87 | 14 | 46 | 8 |
| 1557369_a_at | LOC285401 | 354 | 37 | 204 | 47 | 24 | 1 | 25 | 1 |
| 1564359_a_at | LOC339260 | 502 | 22 | 223 | 51 | 184 | 30 | 10 | 2 |
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| 232001_at | LOC439949 | 489 | 55 | 479 | 60 | 47 | 3 | 37 | 7 |
| 229669_at | LOC440416 | 945 | 58 | 561 | 129 | 566 | 68 | 75 | 55 |
| 1557765_at | LOC643401 | 99 | 32 | 50 | 9 | 78 | 10 | 8 | 0 |
| 1568780_at | LOC649305 | 583 | 15 | 462 | 59 | 515 | 185 | 55 | 16 |
| 239319_at | LOC728342 | 147 | 22 | 158 | 40 | 45 | 8 | 25 | 2 |
| 242070_at | LOC728485 | 313 | 65 | 319 | 48 | 59 | 8 | 28 | 0 |
| 229978_at | LOC729993 | 864 | 69 | 887 | 211 | 288 | 79 | 33 | 0 |
| 232111_at | LOC730125 | 936 | 179 | 1042 | 88 | 64 | 12 | 32 | 1 |
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| 220317_at | LRAT | 305 | 6 | 83 | 31 | 12 | 1 | 12 | 1 |
| 226884_at | LRRN1 | 3515 | 118 | 2651 | 201 | 1718 | 191 | 15 | 1 |
| 208190_s_at | LSR | 637 | 44 | 924 | 155 | 2106 | 113 | 1442 | 284 |
| 223413_s_at | LYAR | 1925 | 44 | 1480 | 172 | 261 | 24 | 255 | 135 |
| 232151_at | MACC1 | 216 | 22 | 181 | 36 | 85 | 24 | 26 | 3 |
| 203362_s_at | MAD2L1 | 3445 | 109 | 3179 | 175 | 538 | 67 | 314 | 262 |
| 224650_at | MAL2 | 2195 | 117 | 1763 | 156 | 1502 | 126 | 1055 | 254 |
| 213927_at | MAP3K9 | 334 | 8 | 283 | 30 | 506 | 40 | 148 | 16 |
| 202890_at | MAP7 | 2125 | 43 | 1281 | 188 | 869 | 109 | 664 | 172 |
| 235141_at | MARVELD2 | 1453 | 79 | 1398 | 218 | 3387 | 453 | 2879 | 1095 |
| 239350_at | MARVELD3 | 446 | 5 | 277 | 51 | 323 | 16 | 137 | 42 |
| 242260_at | MATR3 | 662 | 57 | 371 | 63 | 77 | 7 | 59 | 23 |
| 214397_at | MBD2 | 825 | 25 | 658 | 52 | 427 | 43 | 11 | 1 |
| 220651_s_at | MCM10 | 1126 | 43 | 1058 | 41 | 90 | 16 | 81 | 57 |
| 201555_at | MCM3 | 3310 | 117 | 3009 | 68 | 635 | 57 | 438 | 112 |
| 222037_at | MCM4 | 727 | 24 | 793 | 41 | 130 | 14 | 97 | 41 |
| 216237_s_at | MCM5 | 1970 | 57 | 1935 | 97 | 339 | 42 | 201 | 81 |
| 229797_at | MCOLN3 | 309 | 37 | 230 | 50 | 414 | 105 | 16 | 1 |
| 212693_at | MDN1 | 2023 | 43 | 2019 | 232 | 418 | 46 | 494 | 87 |
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| 228658_at | MIAT | 899 | 47 | 386 | 109 | 54 | 19 | 15 | 2 |
| 232291_at | MIRHG1 | 1082 | 43 | 680 | 155 | 172 | 49 | 81 | 6 |
| 218883_s_at | MLF1IP | 3956 | 235 | 3451 | 234 | 470 | 51 | 145 | 77 |
| 223700_at | MND1 | 586 | 47 | 500 | 32 | 60 | 10 | 29 | 15 |
| 238778_at | MPP7 | 131 | 13 | 111 | 16 | 98 | 17 | 52 | 13 |
| 213924_at | MPPE1 | 480 | 42 | 580 | 92 | 197 | 27 | 72 | 0 |
| 218536_at | MRS2 | 1760 | 221 | 1674 | 169 | 1051 | 227 | 338 | 8 |
| 209421_at | MSH2 | 2327 | 94 | 2172 | 133 | 348 | 53 | 197 | 59 |
| 223311_s_at | MTA3 | 1945 | 92 | 1371 | 129 | 521 | 39 | 240 | 4 |
| 203345_s_at | MTF2 | 2838 | 76 | 2335 | 219 | 688 | 39 | 306 | 15 |
| 209757_s_at | MYCN | 3159 | 139 | 2783 | 357 | 868 | 217 | 17 | 1 |
| 229464_at | MYEF2 | 699 | 26 | 665 | 70 | 161 | 18 | 22 | 6 |
| 218966_at | MYO5C | 728 | 16 | 1026 | 199 | 687 | 135 | 540 | 159 |
| 228242_at | N4BP2 | 1458 | 17 | 1074 | 117 | 943 | 59 | 189 | 14 |
| 222161_at | NAALAD2 | 175 | 61 | 104 | 16 | 910 | 114 | 10 | 3 |
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| 219368_at | NAP1L2 | 914 | 55 | 649 | 75 | 45 | 9 | 73 | 12 |
| 204749_at | NAP1L3 | 2189 | 381 | 2242 | 221 | 213 | 69 | 89 | 82 |
| 201970_s_at | NASP | 6774 | 74 | 5543 | 336 | 1667 | 234 | 834 | 81 |
| 238697_at | NCRNA00086 | 298 | 51 | 193 | 37 | 613 | 89 | 22 | 8 |
| 203961_at | NEBL | 262 | 38 | 327 | 36 | 245 | 16 | 150 | 8 |
| 203413_at | NELL2 | 1279 | 37 | 1309 | 82 | 111 | 26 | 44 | 3 |
| 204702_s_at | NFE2L3 | 526 | 20 | 597 | 78 | 267 | 79 | 23 | 13 |
| 215228_at | NHLH2 | 108 | 24 | 145 | 32 | 33 | 5 | 16 | 1 |
| 221933_at | NLGN4X | 3114 | 161 | 3385 | 379 | 354 | 127 | 25 | 7 |
| 221690_s_at | NLRP2 | 1464 | 726 | 1088 | 446 | 1057 | 367 | 22 | 1 |
| 206023_at | NMU | 2029 | 114 | 1623 | 197 | 55 | 14 | 26 | 4 |
| 230916_at | NODAL | 900 | 39 | 997 | 163 | 309 | 108 | 68 | 1 |
| 206801_at | NPPB | 632 | 204 | 548 | 177 | 1899 | 703 | 57 | 9 |
| 205440_s_at | NPY1R | 143 | 31 | 71 | 18 | 64 | 16 | 9 | 3 |
| 228894_at | NR6A1 | 1154 | 57 | 1084 | 89 | 778 | 168 | 76 | 11 |
| 227494_at | NR6A1 | 727 | 58 | 915 | 121 | 772 | 162 | 109 | 7 |
| 206291_at | NTS | 2103 | 646 | 3794 | 903 | 746 | 224 | 15 | 6 |
| 213947_s_at | NUP210 | 547 | 26 | 547 | 54 | 293 | 39 | 202 | 87 |
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| 209925_at | OCLN | 198 | 28 | 184 | 31 | 624 | 148 | 578 | 430 |
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| 219073_s_at | OSBPL10 | 907 | 20 | 995 | 164 | 855 | 106 | 87 | 73 |
| 242128_at | OTX2 | 2717 | 173 | 1782 | 294 | 551 | 161 | 9 | 1 |
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| 228245_s_at | OVOS/OVOS2 | 622 | 97 | 513 | 100 | 130 | 43 | 32 | 10 |
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| 214834_at | PAR5 | 90 | 18 | 44 | 8 | 7 | 0 | 7 | 0 |
| 235165_at | PARD6B | 566 | 33 | 420 | 61 | 1205 | 169 | 352 | 219 |
| 223435_s_at | PCDHA1 | 756 | 21 | 943 | 128 | 834 | 129 | 48 | 2 |
| 231725_at | PCDHB2 | 570 | 95 | 584 | 46 | 588 | 91 | 23 | 8 |
| 223629_at | PCDHB5 | 657 | 146 | 702 | 219 | 488 | 94 | 66 | 5 |
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| 225958_at | PHC1 | 4691 | 285 | 3371 | 261 | 369 | 44 | 152 | 21 |
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| 201578_at | PODXL | 9923 | 543 | 8961 | 415 | 5476 | 453 | 157 | 101 |
| 205909_at | POLE2 | 849 | 15 | 791 | 61 | 99 | 13 | 164 | 28 |
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| 208286_x_at | POU5F1 (OCT4) | 11303 | 544 | 8105 | 983 | 170 | 15 | 217 | 56 |
| 214532_x_at | POU5F1B | 7223 | 102 | 6228 | 540 | 173 | 11 | 197 | 38 |
| 210265_x_at | POU5F1P3 | 4766 | 233 | 3359 | 362 | 46 | 4 | 56 | 10 |
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| 220714_at | PRDM14 | 2002 | 213 | 996 | 223 | 72 | 4 | 65 | 10 |
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| 210758_at | PSIP1 | 1961 | 103 | 1526 | 101 | 199 | 15 | 131 | 32 |
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| 224578_at | RCC2 | 5617 | 91 | 5182 | 289 | 1941 | 128 | 534 | 214 |
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| 232060_at | ROR1 | 2157 | 273 | 1905 | 226 | 738 | 119 | 409 | 394 |
| 226482_s_at | RP11-544M22.4 | 1482 | 104 | 1029 | 187 | 589 | 51 | 965 | 179 |
| 218499_at | RP6-213H19.1 | 2967 | 127 | 3211 | 392 | 1778 | 190 | 395 | 29 |
| 219370_at | RPRM | 794 | 10 | 1460 | 321 | 148 | 16 | 88 | 28 |
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| 222939_s_at | SLC16A10 | 457 | 16 | 565 | 107 | 393 | 96 | 116 | 32 |
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| 202499_s_at | SLC2A3 | 4738 | 353 | 5842 | 1062 | 14179 | 1069 | 119 | 103 |
| 209267_s_at | SLC39A8 | 1077 | 95 | 1192 | 186 | 383 | 55 | 730 | 249 |
| 223748_at | SLC4A11 | 709 | 63 | 406 | 66 | 174 | 13 | 149 | 45 |
| 232263_at | SLC6A15 | 217 | 32 | 275 | 35 | 30 | 2 | 12 | 2 |
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| 222071_s_at | SLCO4C1 | 250 | 28 | 165 | 33 | 242 | 98 | 195 | 117 |
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| 210715_s_at | SPINT2 | 2076 | 66 | 1621 | 147 | 4158 | 446 | 228 | 30 |
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| 230185_at | THAP9 | 513 | 35 | 398 | 30 | 114 | 13 | 65 | 6 |
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| 238846_at | TNFRSF11A | 172 | 3 | 107 | 26 | 22 | 6 | 15 | 3 |
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| 219736_at | TRIM36 | 307 | 25 | 273 | 53 | 48 | 6 | 30 | 11 |
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| 204822_at | TTK | 1809 | 45 | 1476 | 109 | 263 | 39 | 106 | 84 |
| 214023_x_at | TUBB2B | 5101 | 357 | 4535 | 475 | 2294 | 125 | 55 | 12 |
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| 239552_at | VWDE | 400 | 122 | 87 | 45 | 24 | 9 | 12 | 3 |

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| 204728_s_at | WDHD1 | 737 | 37 | 579 | 47 | 120 | 12 | 48 | 25 |
| 1556429_a_at | WDR67 | 324 | 7 | 226 | 27 | 23 | 4 | 22 | 5 |
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| 206698_at | XK | 82 | 5 | 88 | 12 | 62 | 14 | 38 | 20 |
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| 214823_at | ZNF204 | 415 | 25 | 397 | 76 | 267 | 12 | 15 | 2 |
| 242919_at | ZNF253 | 614 | 25 | 416 | 39 | 136 | 9 | 60 | 10 |
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| 214761_at | ZNF423 | 1494 | 140 | 2038 | 269 | 672 | 189 | 66 | 26 |
| 1568873_at | ZNF519 | 367 | 30 | 290 | 29 | 50 | 7 | 30 | 7 |
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| 243229_at | | 189 | 13 | 147 | 20 | 39 | 2 | 26 | 4 |
| 241466_at | | 187 | 8 | 141 | 22 | 35 | 3 | 36 | 4 |
| 231470_at | | 164 | 92 | 136 | 44 | 22 | 5 | 10 | 0 |
| 232202_at | | 161 | 44 | 166 | 21 | 445 | 47 | 30 | 0 |
| 1563357_at | | 159 | 11 | 152 | 40 | 82 | 13 | 11 | 6 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 228440_at | | 158 | 17 | 83 | 17 | 81 | 15 | 21 | 0 |
| 231594_at | | 152 | 57 | 99 | 30 | 19 | 1 | 17 | 1 |
| 230951_at | | 152 | 13 | 106 | 15 | 116 | 18 | 345 | 194 |
| 244867_at | | 148 | 27 | 208 | 62 | 21 | 2 | 8 | 0 |
| 238175_at | | 145 | 18 | 45 | 18 | 9 | 1 | 9 | 1 |
| 231925_at | | 144 | 11 | 232 | 105 | 101 | 18 | 15 | 2 |
| 241776_at | | 131 | 57 | 201 | 34 | 28 | 4 | 16 | 1 |
| 241772_at | | 117 | 32 | 106 | 24 | 34 | 5 | 8 | 1 |
| 1564964_at | | 89 | 22 | 40 | 9 | 7 | 0 | 8 | 1 |

| PSC-FBs (5) | | Tissue-FB (8) | | PSC-NPC (6) | | Fetal-NPC (5) | |
|---|---|---|---|---|---|---|---|
| *avg* | *stderr* | *avg* | *stderr* | *avg* | *stderr* | *avg* | *stderr* |
| 113 | 18 | 101 | 18 | 300 | 59 | 197 | 24 |
| 8 | 0 | 8 | 0 | 8 | 0 | 8 | 1 |
| 50 | 15 | 30 | 3 | 33 | 4 | 31 | 5 |
| 1815 | 1118 | 1183 | 522 | 392 | 143 | 193 | 36 |
| 203 | 28 | 159 | 26 | 2901 | 209 | 711 | 187 |
| 81 | 28 | 33 | 3 | 118 | 36 | 35 | 4 |
| 73 | 13 | 66 | 6 | 379 | 86 | 560 | 126 |
| 25 | 3 | 35 | 4 | 115 | 17 | 192 | 25 |
| 84 | 21 | 91 | 43 | 777 | 174 | 142 | 61 |
| 54 | 3 | 35 | 2 | 1138 | 159 | 1639 | 207 |
| 69 | 32 | 22 | 3 | 43 | 7 | 39 | 10 |
| 76 | 10 | 69 | 26 | 86 | 6 | 31 | 6 |
| 170 | 42 | 12 | 2 | 1298 | 178 | 614 | 119 |
| 659 | 170 | 163 | 30 | 199 | 39 | 29 | 13 |
| 89 | 6 | 47 | 5 | 85 | 14 | 63 | 7 |
| 227 | 54 | 425 | 70 | 2418 | 630 | 1756 | 213 |
| 226 | 39 | 137 | 27 | 987 | 252 | 917 | 376 |
| 482 | 99 | 95 | 13 | 453 | 42 | 277 | 92 |
| 146 | 46 | 134 | 58 | 215 | 37 | 87 | 9 |
| 144 | 13 | 105 | 11 | 565 | 98 | 130 | 16 |
| 158 | 57 | 199 | 89 | 931 | 91 | 386 | 93 |
| 34 | 4 | 30 | 2 | 45 | 6 | 44 | 15 |
| 362 | 27 | 142 | 54 | 501 | 190 | 1235 | 817 |
| 132 | 6 | 118 | 13 | 336 | 59 | 199 | 23 |
| 16 | 0 | 13 | 1 | 103 | 19 | 81 | 45 |
| 1456 | 1267 | 513 | 202 | 4156 | 762 | 5305 | 1237 |
| 157 | 28 | 32 | 9 | 1214 | 164 | 973 | 309 |
| 156 | 31 | 118 | 26 | 85 | 12 | 214 | 17 |
| 55 | 11 | 66 | 22 | 302 | 29 | 170 | 24 |
| 51 | 7 | 61 | 9 | 112 | 20 | 41 | 5 |
| 145 | 47 | 233 | 93 | 829 | 62 | 559 | 99 |
| 337 | 116 | 459 | 173 | 1885 | 115 | 1174 | 226 |
| 113 | 23 | 155 | 52 | 353 | 16 | 281 | 29 |
| 234 | 53 | 237 | 37 | 483 | 31 | 301 | 56 |
| 60 | 9 | 66 | 19 | 122 | 8 | 61 | 6 |
| 49 | 2 | 43 | 11 | 133 | 33 | 55 | 21 |
| 96 | 3 | 91 | 3 | 115 | 19 | 82 | 4 |
| 188 | 53 | 210 | 20 | 1007 | 103 | 408 | 87 |
| 740 | 460 | 48 | 4 | 101 | 23 | 30 | 5 |
| 125 | 13 | 79 | 13 | 937 | 138 | 263 | 24 |
| 116 | 18 | 111 | 31 | 244 | 50 | 139 | 9 |
| 15 | 2 | 13 | 1 | 314 | 64 | 476 | 50 |
| 273 | 44 | 273 | 71 | 859 | 41 | 467 | 47 |
| 94 | 24 | 56 | 11 | 310 | 55 | 242 | 47 |
| 136 | 14 | 172 | 33 | 421 | 84 | 234 | 38 |
| 14 | 1 | 15 | 2 | 145 | 25 | 108 | 55 |
| 139 | 18 | 84 | 15 | 284 | 44 | 158 | 23 |
| 48 | 6 | 44 | 6 | 730 | 127 | 634 | 385 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 326 | 29 | 367 | 50 | 1355 | 64 | 646 | 93 |
| 68 | 7 | 54 | 5 | 444 | 106 | 116 | 14 |
| 16 | 0 | 14 | 1 | 283 | 173 | 612 | 282 |
| 14 | 1 | 13 | 1 | 46 | 11 | 18 | 2 |
| 91 | 12 | 95 | 20 | 1115 | 138 | 387 | 85 |
| 8 | 1 | 10 | 1 | 32 | 7 | 15 | 4 |
| 34 | 4 | 24 | 2 | 277 | 34 | 262 | 69 |
| 44 | 1 | 36 | 2 | 54 | 6 | 44 | 7 |
| 296 | 85 | 368 | 105 | 1484 | 126 | 762 | 124 |
| 485 | 171 | 582 | 185 | 2441 | 161 | 1386 | 360 |
| 226 | 98 | 45 | 14 | 1285 | 231 | 885 | 266 |
| 4014 | 2385 | 109 | 36 | 9649 | 794 | 6567 | 1176 |
| 140 | 34 | 135 | 45 | 653 | 99 | 260 | 56 |
| 137 | 49 | 182 | 85 | 252 | 18 | 179 | 16 |
| 166 | 38 | 196 | 51 | 1364 | 131 | 757 | 169 |
| 180 | 39 | 319 | 68 | 1263 | 107 | 955 | 211 |
| 31 | 8 | 20 | 8 | 710 | 166 | 132 | 68 |
| 331 | 127 | 68 | 3 | 610 | 117 | 198 | 21 |
| 84 | 24 | 29 | 2 | 153 | 24 | 90 | 16 |
| 70 | 25 | 101 | 31 | 183 | 44 | 109 | 12 |
| 104 | 36 | 142 | 28 | 797 | 258 | 473 | 87 |
| 90 | 9 | 93 | 10 | 244 | 26 | 170 | 27 |
| 62 | 15 | 124 | 25 | 481 | 61 | 269 | 52 |
| 32 | 3 | 34 | 2 | 31 | 4 | 27 | 3 |
| 184 | 46 | 74 | 12 | 280 | 33 | 266 | 113 |
| 163 | 38 | 36 | 13 | 1964 | 207 | 987 | 203 |
| 155 | 25 | 206 | 49 | 381 | 57 | 260 | 29 |
| 116 | 28 | 22 | 1 | 84 | 18 | 32 | 6 |
| 24 | 4 | 22 | 4 | 132 | 22 | 82 | 16 |
| 10 | 1 | 10 | 1 | 25 | 2 | 40 | 11 |
| 48 | 2 | 44 | 3 | 111 | 15 | 89 | 22 |
| 30 | 2 | 29 | 4 | 85 | 15 | 98 | 31 |
| 36 | 1 | 23 | 1 | 284 | 97 | 25 | 3 |
| 108 | 11 | 88 | 7 | 243 | 45 | 78 | 8 |
| 77 | 9 | 78 | 5 | 650 | 171 | 270 | 95 |
| 31 | 8 | 29 | 8 | 122 | 18 | 132 | 26 |
| 25 | 2 | 23 | 2 | 83 | 9 | 228 | 39 |
| 53 | 12 | 17 | 1 | 307 | 61 | 231 | 50 |
| 66 | 9 | 43 | 5 | 5100 | 1013 | 155 | 23 |
| 86 | 15 | 76 | 50 | 992 | 181 | 454 | 173 |
| 158 | 32 | 28 | 5 | 82 | 12 | 25 | 4 |
| 69 | 20 | 44 | 2 | 145 | 96 | 52 | 7 |
| 81 | 15 | 14 | 1 | 242 | 44 | 148 | 28 |
| 10 | 1 | 10 | 1 | 10 | 1 | 10 | 1 |
| 15 | 1 | 13 | 1 | 14 | 0 | 13 | 1 |
| 93 | 9 | 75 | 5 | 7308 | 1098 | 303 | 114 |
| 371 | 58 | 25 | 12 | 1438 | 148 | 1485 | 354 |
| 22 | 2 | 21 | 1 | 34 | 6 | 22 | 4 |
| 165 | 19 | 133 | 8 | 355 | 59 | 227 | 27 |
| 338 | 119 | 57 | 13 | 2570 | 398 | 1969 | 341 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 29 | 4 | 15 | 1 | 87 | 13 | 70 | 13 |
| 43 | 19 | 146 | 86 | 53 | 18 | 124 | 73 |
| 46 | 5 | 32 | 2 | 237 | 95 | 35 | 4 |
| 201 | 106 | 40 | 4 | 51 | 9 | 32 | 3 |
| 31 | 13 | 20 | 5 | 419 | 33 | 112 | 20 |
| 10 | 1 | 8 | 1 | 8 | 2 | 8 | 1 |
| 97 | 43 | 129 | 42 | 1025 | 284 | 1809 | 291 |
| 77 | 7 | 58 | 10 | 115 | 17 | 58 | 8 |
| 36 | 3 | 35 | 4 | 96 | 10 | 168 | 49 |
| 55 | 18 | 73 | 29 | 707 | 63 | 331 | 77 |
| 152 | 27 | 200 | 23 | 119 | 22 | 87 | 11 |
| 216 | 71 | 354 | 124 | 1367 | 116 | 862 | 169 |
| 368 | 274 | 31 | 14 | 7327 | 1798 | 229 | 87 |
| 70 | 11 | 83 | 21 | 108 | 23 | 36 | 8 |
| 270 | 22 | 184 | 18 | 1032 | 66 | 485 | 55 |
| 143 | 17 | 112 | 13 | 1129 | 368 | 204 | 19 |
| 28 | 1 | 26 | 2 | 40 | 5 | 25 | 3 |
| 11 | 1 | 11 | 1 | 13 | 2 | 12 | 1 |
| 73 | 16 | 20 | 1 | 1353 | 180 | 83 | 32 |
| 177 | 54 | 25 | 4 | 601 | 106 | 77 | 30 |
| 131 | 21 | 188 | 56 | 373 | 28 | 288 | 26 |
| 1758 | 141 | 61 | 23 | 1564 | 176 | 409 | 162 |
| 279 | 111 | 447 | 203 | 1649 | 87 | 948 | 189 |
| 226 | 58 | 107 | 24 | 1188 | 161 | 853 | 87 |
| 53 | 13 | 115 | 80 | 2750 | 620 | 1173 | 167 |
| 52 | 14 | 32 | 6 | 71 | 19 | 73 | 16 |
| 14 | 2 | 13 | 1 | 45 | 15 | 34 | 16 |
| 554 | 144 | 251 | 65 | 299 | 40 | 65 | 15 |
| 72 | 8 | 48 | 3 | 390 | 58 | 65 | 4 |
| 100 | 18 | 83 | 14 | 154 | 19 | 109 | 56 |
| 39 | 13 | 23 | 2 | 42 | 12 | 25 | 7 |
| 27 | 4 | 25 | 3 | 792 | 230 | 67 | 21 |
| 44 | 4 | 47 | 7 | 66 | 9 | 39 | 6 |
| 156 | 80 | 26 | 3 | 644 | 103 | 423 | 132 |
| 41 | 3 | 46 | 2 | 60 | 5 | 49 | 5 |
| 132 | 28 | 28 | 5 | 161 | 32 | 30 | 6 |
| 52 | 8 | 40 | 6 | 556 | 220 | 198 | 131 |
| 76 | 11 | 112 | 38 | 177 | 23 | 99 | 9 |
| 25 | 1 | 20 | 1 | 215 | 83 | 20 | 3 |
| 56 | 7 | 44 | 4 | 108 | 23 | 40 | 10 |
| 306 | 102 | 28 | 4 | 1145 | 268 | 276 | 73 |
| 1402 | 343 | 212 | 118 | 902 | 221 | 1598 | 993 |
| 27 | 3 | 23 | 1 | 32 | 4 | 50 | 9 |
| 2233 | 283 | 1097 | 345 | 4994 | 459 | 11312 | 1191 |
| 396 | 73 | 262 | 33 | 456 | 45 | 272 | 31 |
| 37 | 3 | 36 | 5 | 119 | 31 | 200 | 56 |
| 66 | 27 | 13 | 2 | 248 | 117 | 59 | 17 |
| 42 | 10 | 24 | 4 | 248 | 32 | 265 | 18 |
| 10 | 1 | 10 | 1 | 15 | 2 | 10 | 1 |
| 208 | 54 | 144 | 26 | 172 | 23 | 127 | 23 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 482 | 46 | 347 | 78 | 2520 | 178 | 564 | 36 |
| 220 | 67 | 402 | 210 | 855 | 47 | 542 | 104 |
| 40 | 2 | 37 | 3 | 41 | 5 | 38 | 2 |
| 189 | 56 | 83 | 6 | 153 | 44 | 38 | 10 |
| 1055 | 277 | 37 | 18 | 1096 | 162 | 1438 | 336 |
| 181 | 56 | 191 | 61 | 986 | 195 | 571 | 111 |
| 386 | 154 | 138 | 34 | 157 | 19 | 134 | 47 |
| 186 | 24 | 157 | 38 | 150 | 25 | 136 | 42 |
| 45 | 16 | 110 | 29 | 1875 | 274 | 1381 | 277 |
| 35 | 1 | 31 | 2 | 250 | 56 | 37 | 5 |
| 140 | 69 | 52 | 14 | 838 | 113 | 608 | 166 |
| 41 | 19 | 13 | 2 | 53 | 10 | 33 | 13 |
| 72 | 2 | 92 | 13 | 92 | 8 | 84 | 8 |
| 48 | 6 | 46 | 2 | 51 | 4 | 46 | 3 |
| 31 | 9 | 14 | 2 | 11 | 1 | 10 | 1 |
| 214 | 53 | 140 | 15 | 496 | 72 | 279 | 56 |
| 33 | 2 | 29 | 2 | 43 | 6 | 32 | 3 |
| 10 | 1 | 11 | 0 | 83 | 45 | 94 | 78 |
| 40 | 1 | 37 | 1 | 61 | 13 | 42 | 2 |
| 287 | 94 | 124 | 35 | 328 | 28 | 150 | 54 |
| 268 | 43 | 467 | 129 | 445 | 75 | 250 | 194 |
| 149 | 12 | 121 | 8 | 233 | 27 | 103 | 8 |
| 9 | 2 | 7 | 0 | 702 | 153 | 332 | 98 |
| 441 | 314 | 44 | 24 | 1191 | 257 | 80 | 13 |
| 60 | 24 | 61 | 14 | 1554 | 726 | 237 | 67 |
| 40 | 3 | 50 | 4 | 195 | 41 | 379 | 114 |
| 134 | 39 | 412 | 339 | 150 | 19 | 111 | 68 |
| 77 | 35 | 43 | 15 | 70 | 12 | 30 | 13 |
| 93 | 19 | 102 | 18 | 144 | 35 | 103 | 21 |
| 92 | 33 | 48 | 27 | 386 | 84 | 200 | 48 |
| 64 | 2 | 61 | 3 | 177 | 41 | 81 | 24 |
| 281 | 78 | 345 | 141 | 1823 | 259 | 1206 | 218 |
| 217 | 65 | 276 | 113 | 1439 | 155 | 986 | 245 |
| 6 | 0 | 6 | 0 | 8 | 1 | 6 | 0 |
| 73 | 4 | 76 | 12 | 1363 | 261 | 1550 | 146 |
| 177 | 105 | 55 | 11 | 39 | 4 | 111 | 19 |
| 20 | 6 | 17 | 4 | 185 | 37 | 126 | 28 |
| 319 | 182 | 35 | 4 | 2953 | 558 | 387 | 244 |
| 625 | 263 | 141 | 28 | 1166 | 168 | 912 | 206 |
| 48 | 9 | 26 | 1 | 76 | 11 | 29 | 3 |
| 32 | 7 | 21 | 3 | 31 | 5 | 44 | 22 |
| 21 | 2 | 28 | 2 | 419 | 71 | 171 | 35 |
| 16 | 2 | 19 | 2 | 38 | 10 | 31 | 6 |
| 566 | 223 | 95 | 27 | 884 | 104 | 2070 | 405 |
| 34 | 8 | 13 | 2 | 48 | 8 | 112 | 8 |
| 23 | 7 | 12 | 0 | 900 | 122 | 44 | 11 |
| 32 | 3 | 25 | 2 | 54 | 9 | 28 | 2 |
| 14 | 1 | 51 | 10 | 99 | 35 | 82 | 16 |
| 59 | 18 | 25 | 3 | 146 | 33 | 94 | 23 |
| 1146 | 186 | 933 | 170 | 2464 | 187 | 1351 | 94 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 164 | 43 | 92 | 46 | 82 | 23 | 296 | 169 |
| 68 | 11 | 44 | 4 | 43 | 8 | 35 | 5 |
| 195 | 50 | 303 | 108 | 1028 | 154 | 488 | 88 |
| 32 | 5 | 53 | 18 | 91 | 11 | 105 | 20 |
| 15 | 1 | 14 | 1 | 518 | 375 | 16 | 3 |
| 36 | 1 | 43 | 4 | 100 | 42 | 46 | 6 |
| 40 | 14 | 22 | 1 | 187 | 46 | 631 | 168 |
| 202 | 33 | 133 | 17 | 1181 | 183 | 188 | 34 |
| 9 | 1 | 8 | 0 | 37 | 16 | 12 | 2 |
| 2362 | 277 | 663 | 146 | 5391 | 721 | 619 | 247 |
| 242 | 45 | 315 | 31 | 958 | 91 | 598 | 111 |
| 337 | 129 | 569 | 201 | 1612 | 188 | 564 | 90 |
| 27 | 7 | 11 | 1 | 151 | 25 | 19 | 2 |
| 40 | 10 | 22 | 2 | 61 | 13 | 28 | 3 |
| 298 | 95 | 17 | 2 | 322 | 66 | 232 | 22 |
| 517 | 155 | 14 | 1 | 1053 | 99 | 716 | 117 |
| 18 | 2 | 16 | 1 | 35 | 3 | 20 | 2 |
| 58 | 4 | 52 | 2 | 54 | 9 | 210 | 126 |
| 38 | 4 | 30 | 3 | 4790 | 382 | 250 | 78 |
| 558 | 72 | 93 | 13 | 1454 | 175 | 175 | 14 |
| 1429 | 149 | 660 | 167 | 2909 | 450 | 1474 | 343 |
| 96 | 12 | 122 | 24 | 323 | 62 | 154 | 31 |
| 45 | 11 | 27 | 3 | 1641 | 239 | 1280 | 811 |
| 124 | 23 | 184 | 34 | 3145 | 394 | 1923 | 354 |
| 336 | 98 | 318 | 43 | 444 | 56 | 176 | 25 |
| 606 | 43 | 402 | 90 | 846 | 75 | 680 | 77 |
| 724 | 427 | 19 | 6 | 483 | 46 | 538 | 107 |
| 25 | 11 | 17 | 3 | 380 | 74 | 73 | 18 |
| 50 | 2 | 47 | 3 | 86 | 25 | 47 | 7 |
| 93 | 18 | 44 | 5 | 186 | 39 | 307 | 126 |
| 330 | 41 | 271 | 29 | 1380 | 224 | 604 | 29 |
| 337 | 147 | 25 | 2 | 79 | 21 | 241 | 91 |
| 18 | 2 | 13 | 1 | 159 | 41 | 168 | 62 |
| 1603 | 550 | 34 | 4 | 696 | 125 | 1410 | 319 |
| 97 | 16 | 54 | 24 | 79 | 19 | 62 | 12 |
| 20 | 3 | 12 | 1 | 38 | 12 | 230 | 78 |
| 12 | 1 | 11 | 0 | 22 | 7 | 11 | 1 |
| 40 | 3 | 44 | 5 | 132 | 19 | 38 | 6 |
| 623 | 363 | 32 | 12 | 143 | 42 | 76 | 27 |
| 36 | 3 | 53 | 9 | 53 | 9 | 56 | 4 |
| 623 | 309 | 31 | 3 | 342 | 142 | 380 | 171 |
| 84 | 19 | 44 | 4 | 247 | 26 | 239 | 12 |
| 162 | 22 | 105 | 34 | 427 | 46 | 82 | 11 |
| 72 | 15 | 35 | 3 | 57 | 6 | 47 | 2 |
| 72 | 16 | 44 | 5 | 365 | 53 | 100 | 24 |
| 47 | 4 | 33 | 3 | 210 | 65 | 42 | 3 |
| 91 | 26 | 137 | 36 | 1374 | 131 | 922 | 224 |
| 255 | 32 | 280 | 52 | 337 | 30 | 379 | 25 |
| 47 | 3 | 45 | 3 | 52 | 5 | 46 | 6 |
| 39 | 2 | 36 | 3 | 52 | 9 | 42 | 6 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 13634 | 2598 | 674 | 346 | 2487 | 429 | 163 | 44 |
| 1951 | 1005 | 410 | 283 | 693 | 204 | 16 | 3 |
| 5076 | 2073 | 135 | 10 | 1344 | 293 | 95 | 7 |
| 48 | 10 | 51 | 7 | 701 | 354 | 52 | 16 |
| 78 | 22 | 82 | 26 | 441 | 60 | 224 | 25 |
| 629 | 92 | 564 | 85 | 905 | 159 | 1141 | 333 |
| 37 | 3 | 30 | 2 | 46 | 5 | 35 | 4 |
| 27 | 2 | 24 | 1 | 53 | 8 | 143 | 72 |
| 27 | 1 | 25 | 3 | 34 | 4 | 29 | 2 |
| 58 | 5 | 42 | 3 | 84 | 16 | 140 | 67 |
| 19 | 3 | 15 | 1 | 3502 | 1223 | 20 | 7 |
| 230 | 122 | 12 | 1 | 2952 | 460 | 23 | 5 |
| 102 | 13 | 82 | 8 | 746 | 157 | 482 | 179 |
| 15 | 2 | 13 | 1 | 36 | 14 | 18 | 5 |
| 140 | 39 | 32 | 3 | 289 | 75 | 133 | 28 |
| 51 | 12 | 30 | 3 | 49 | 4 | 72 | 19 |
| 26 | 2 | 23 | 1 | 63 | 12 | 143 | 82 |
| 47 | 24 | 48 | 15 | 124 | 40 | 191 | 40 |
| 24 | 2 | 23 | 1 | 31 | 4 | 35 | 6 |
| 246 | 84 | 10 | 0 | 224 | 30 | 14 | 3 |
| 49 | 4 | 42 | 3 | 129 | 38 | 88 | 9 |
| 39 | 6 | 20 | 1 | 76 | 14 | 39 | 7 |
| 1679 | 192 | 82 | 27 | 2601 | 233 | 81 | 44 |
| 50 | 17 | 9 | 1 | 84 | 22 | 6 | 1 |
| 50 | 6 | 81 | 23 | 187 | 19 | 133 | 28 |
| 22 | 1 | 17 | 1 | 19 | 2 | 18 | 2 |
| 57 | 6 | 33 | 2 | 82 | 9 | 33 | 5 |
| 35 | 5 | 27 | 2 | 81 | 19 | 118 | 25 |
| 45 | 10 | 33 | 1 | 160 | 29 | 128 | 46 |
| 33 | 6 | 63 | 15 | 179 | 16 | 117 | 28 |
| 9 | 0 | 9 | 0 | 13 | 2 | 10 | 0 |
| 152 | 37 | 18 | 10 | 1994 | 274 | 1551 | 194 |
| 52 | 9 | 86 | 8 | 221 | 68 | 41 | 10 |
| 304 | 40 | 388 | 69 | 447 | 67 | 224 | 14 |
| 21 | 1 | 18 | 1 | 26 | 4 | 19 | 2 |
| 631 | 176 | 733 | 242 | 2673 | 147 | 1419 | 183 |
| 476 | 265 | 14 | 2 | 279 | 72 | 79 | 32 |
| 121 | 34 | 22 | 2 | 157 | 40 | 123 | 52 |
| 19 | 4 | 86 | 20 | 180 | 29 | 160 | 35 |
| 199 | 23 | 147 | 14 | 248 | 71 | 153 | 17 |
| 56 | 3 | 54 | 3 | 63 | 5 | 56 | 6 |
| 47 | 7 | 83 | 10 | 53 | 7 | 30 | 5 |
| 13 | 1 | 16 | 1 | 365 | 78 | 115 | 51 |
| 131 | 43 | 252 | 153 | 529 | 23 | 441 | 80 |
| 445 | 94 | 538 | 132 | 1822 | 211 | 1004 | 220 |
| 89 | 9 | 140 | 46 | 275 | 31 | 265 | 30 |
| 244 | 85 | 339 | 104 | 809 | 88 | 597 | 133 |
| 36 | 13 | 53 | 8 | 58 | 16 | 21 | 3 |
| 289 | 16 | 354 | 33 | 401 | 55 | 249 | 39 |
| 93 | 45 | 13 | 3 | 1343 | 302 | 2568 | 751 |

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|---|---|---|---|---|---|---|---|
| 28 | 3 | 59 | 12 | 783 | 325 | 459 | 219 |
| 36 | 4 | 62 | 13 | 151 | 48 | 32 | 8 |
| 416 | 153 | 619 | 267 | 2158 | 257 | 1404 | 486 |
| 50 | 17 | 79 | 35 | 468 | 64 | 201 | 41 |
| 92 | 27 | 19 | 5 | 44 | 11 | 12 | 2 |
| 61 | 24 | 67 | 17 | 501 | 107 | 329 | 62 |
| 314 | 40 | 227 | 20 | 440 | 32 | 145 | 26 |
| 314 | 56 | 339 | 67 | 1137 | 69 | 825 | 127 |
| 368 | 13 | 331 | 22 | 512 | 31 | 193 | 18 |
| 524 | 42 | 396 | 37 | 1614 | 215 | 560 | 80 |
| 45 | 22 | 24 | 7 | 2338 | 69 | 1039 | 431 |
| 138 | 21 | 95 | 23 | 801 | 107 | 602 | 131 |
| 55 | 8 | 40 | 2 | 276 | 95 | 192 | 42 |
| 289 | 39 | 174 | 22 | 748 | 39 | 404 | 73 |
| 124 | 58 | 19 | 2 | 238 | 34 | 31 | 9 |
| 26 | 3 | 29 | 2 | 146 | 66 | 49 | 6 |
| 45 | 13 | 45 | 13 | 206 | 60 | 252 | 68 |
| 1138 | 251 | 336 | 92 | 1092 | 135 | 1460 | 168 |
| 554 | 73 | 845 | 96 | 4059 | 282 | 1735 | 289 |
| 18 | 1 | 18 | 1 | 133 | 27 | 99 | 21 |
| 71 | 28 | 22 | 2 | 430 | 104 | 223 | 68 |
| 54 | 4 | 51 | 7 | 2986 | 712 | 1960 | 806 |
| 102 | 34 | 86 | 29 | 40 | 3 | 98 | 42 |
| 18 | 1 | 18 | 1 | 468 | 142 | 99 | 76 |
| 87 | 40 | 102 | 43 | 1077 | 170 | 2232 | 209 |
| 61 | 27 | 20 | 2 | 150 | 79 | 37 | 6 |
| 89 | 45 | 22 | 2 | 652 | 67 | 243 | 34 |
| 90 | 6 | 78 | 2 | 76 | 12 | 73 | 4 |
| 940 | 505 | 48 | 3 | 470 | 291 | 35 | 3 |
| 28 | 10 | 7 | 1 | 100 | 62 | 14 | 1 |
| 84 | 4 | 63 | 6 | 320 | 42 | 70 | 5 |
| 128 | 4 | 109 | 5 | 412 | 109 | 105 | 3 |
| 18 | 1 | 15 | 2 | 353 | 113 | 38 | 14 |
| 87 | 8 | 63 | 5 | 433 | 71 | 227 | 48 |
| 16 | 5 | 9 | 1 | 132 | 21 | 21 | 2 |
| 21 | 3 | 22 | 4 | 28 | 5 | 25 | 4 |
| 173 | 52 | 211 | 91 | 936 | 41 | 666 | 133 |
| 41 | 4 | 46 | 10 | 111 | 11 | 62 | 10 |
| 791 | 248 | 222 | 50 | 162 | 47 | 81 | 34 |
| 10 | 1 | 10 | 1 | 1175 | 567 | 176 | 114 |
| 24 | 1 | 24 | 2 | 34 | 4 | 20 | 2 |
| 35 | 9 | 40 | 8 | 833 | 167 | 522 | 335 |
| 36 | 3 | 31 | 3 | 166 | 19 | 85 | 13 |
| 10 | 1 | 8 | 0 | 13 | 2 | 11 | 1 |
| 95 | 47 | 18 | 4 | 190 | 22 | 86 | 20 |
| 1258 | 105 | 32 | 4 | 929 | 142 | 702 | 87 |
| 749 | 89 | 86 | 8 | 732 | 111 | 125 | 35 |
| 298 | 60 | 43 | 11 | 512 | 158 | 277 | 39 |
| 34 | 11 | 21 | 2 | 88 | 18 | 215 | 30 |
| 146 | 15 | 163 | 21 | 872 | 110 | 412 | 93 |

| 1302 | 686 | 97 | 27 | 400 | 65 | 752 | 188 |
|---|---|---|---|---|---|---|---|
| 26 | 6 | 19 | 3 | 127 | 69 | 150 | 60 |
| 379 | 57 | 357 | 76 | 1119 | 186 | 611 | 18 |
| 254 | 18 | 289 | 27 | 729 | 52 | 649 | 84 |
| 310 | 21 | 337 | 42 | 752 | 56 | 601 | 52 |
| 168 | 19 | 130 | 15 | 207 | 24 | 155 | 15 |
| 106 | 9 | 92 | 6 | 123 | 15 | 113 | 10 |
| 37 | 12 | 17 | 2 | 248 | 49 | 321 | 150 |
| 1079 | 146 | 20 | 4 | 1273 | 240 | 414 | 211 |
| 836 | 370 | 10 | 2 | 193 | 30 | 28 | 5 |
| 310 | 105 | 319 | 50 | 353 | 69 | 925 | 194 |
| 59 | 9 | 40 | 5 | 144 | 16 | 86 | 27 |
| 28 | 0 | 42 | 11 | 507 | 115 | 183 | 47 |
| 49 | 8 | 29 | 5 | 183 | 46 | 123 | 25 |
| 103 | 27 | 49 | 6 | 341 | 71 | 236 | 31 |
| 33 | 14 | 17 | 1 | 1010 | 349 | 532 | 277 |
| 66 | 8 | 36 | 13 | 146 | 15 | 104 | 24 |
| 1126 | 369 | 862 | 331 | 825 | 172 | 568 | 231 |
| 254 | 101 | 761 | 470 | 1153 | 145 | 1003 | 152 |
| 126 | 35 | 161 | 69 | 460 | 20 | 288 | 33 |
| 151 | 39 | 107 | 27 | 94 | 17 | 47 | 11 |
| 151 | 14 | 127 | 4 | 398 | 194 | 123 | 14 |
| 176 | 11 | 144 | 5 | 399 | 181 | 131 | 7 |
| 42 | 5 | 40 | 2 | 134 | 71 | 36 | 4 |
| 61 | 8 | 44 | 2 | 140 | 63 | 51 | 4 |
| 67 | 6 | 67 | 13 | 45 | 5 | 56 | 9 |
| 264 | 25 | 196 | 35 | 561 | 77 | 468 | 101 |
| 97 | 10 | 34 | 6 | 137 | 32 | 41 | 8 |
| 22 | 6 | 34 | 13 | 492 | 92 | 524 | 87 |
| 206 | 122 | 32 | 9 | 306 | 89 | 1833 | 490 |
| 48 | 21 | 26 | 3 | 80 | 31 | 114 | 34 |
| 17 | 4 | 37 | 16 | 96 | 28 | 23 | 6 |
| 70 | 3 | 68 | 4 | 89 | 16 | 66 | 7 |
| 722 | 100 | 227 | 40 | 303 | 73 | 208 | 103 |
| 153 | 40 | 222 | 83 | 706 | 91 | 467 | 100 |
| 63 | 7 | 46 | 3 | 97 | 12 | 62 | 3 |
| 37 | 20 | 14 | 2 | 1560 | 192 | 1606 | 445 |
| 26 | 6 | 14 | 1 | 43 | 15 | 12 | 1 |
| 49 | 3 | 37 | 3 | 174 | 56 | 34 | 3 |
| 25 | 3 | 24 | 3 | 3405 | 733 | 50 | 14 |
| 179 | 22 | 234 | 25 | 671 | 48 | 579 | 104 |
| 14 | 4 | 8 | 1 | 2576 | 570 | 7628 | 757 |
| 24 | 3 | 24 | 4 | 144 | 43 | 21 | 1 |
| 216 | 9 | 221 | 32 | 430 | 46 | 464 | 35 |
| 300 | 120 | 421 | 170 | 1377 | 197 | 763 | 154 |
| 49 | 11 | 23 | 5 | 40 | 3 | 36 | 2 |
| 33 | 10 | 47 | 15 | 51 | 12 | 52 | 9 |
| 102 | 18 | 98 | 23 | 452 | 73 | 407 | 122 |
| 379 | 184 | 55 | 14 | 193 | 23 | 110 | 11 |
| 917 | 422 | 169 | 42 | 1718 | 131 | 397 | 68 |

| 1305 | 86 | 1000 | 76 | 2526 | 100 | 1222 | 49 |
|---|---|---|---|---|---|---|---|
| 325 | 93 | 416 | 127 | 839 | 64 | 682 | 60 |
| 890 | 453 | 117 | 61 | 1247 | 501 | 142 | 45 |
| 12 | 1 | 11 | 0 | 51 | 27 | 18 | 2 |
| 11 | 1 | 10 | 1 | 942 | 251 | 59 | 10 |
| 78 | 10 | 65 | 6 | 545 | 48 | 264 | 16 |
| 23 | 2 | 13 | 1 | 27 | 7 | 32 | 18 |
| 61 | 4 | 71 | 8 | 137 | 17 | 106 | 25 |
| 782 | 226 | 399 | 92 | 453 | 187 | 178 | 93 |
| 170 | 88 | 38 | 8 | 495 | 132 | 443 | 140 |
| 1261 | 260 | 174 | 47 | 2130 | 126 | 182 | 62 |
| 123 | 15 | 57 | 5 | 374 | 36 | 222 | 43 |
| 312 | 62 | 402 | 53 | 54 | 7 | 37 | 14 |
| 336 | 20 | 241 | 46 | 2702 | 421 | 1308 | 103 |
| 19 | 1 | 15 | 1 | 127 | 44 | 1077 | 224 |
| 135 | 21 | 105 | 8 | 592 | 90 | 120 | 11 |
| 48 | 2 | 39 | 2 | 1166 | 199 | 833 | 326 |
| 9 | 1 | 8 | 0 | 100 | 30 | 150 | 90 |
| 31 | 3 | 32 | 3 | 38 | 8 | 32 | 5 |
| 52 | 13 | 35 | 2 | 91 | 26 | 27 | 2 |
| 52 | 17 | 37 | 12 | 196 | 35 | 134 | 38 |
| 229 | 90 | 56 | 8 | 1031 | 192 | 1387 | 266 |
| 10 | 1 | 11 | 0 | 11 | 0 | 18 | 8 |
| 531 | 53 | 412 | 49 | 1543 | 67 | 915 | 148 |
| 852 | 353 | 157 | 46 | 437 | 211 | 51 | 27 |
| 484 | 458 | 420 | 191 | 3508 | 716 | 573 | 202 |
| 563 | 293 | 58 | 6 | 310 | 46 | 296 | 105 |
| 520 | 380 | 11 | 2 | 649 | 192 | 222 | 86 |
| 64 | 8 | 60 | 5 | 447 | 105 | 67 | 7 |
| 194 | 92 | 92 | 28 | 276 | 66 | 63 | 12 |
| 556 | 24 | 368 | 46 | 536 | 116 | 483 | 76 |
| 28 | 4 | 24 | 3 | 117 | 17 | 56 | 8 |
| 79 | 5 | 39 | 5 | 500 | 21 | 612 | 90 |
| 9 | 0 | 8 | 1 | 309 | 87 | 96 | 54 |
| 358 | 21 | 214 | 46 | 1062 | 105 | 340 | 45 |
| 10 | 1 | 12 | 3 | 18 | 2 | 11 | 1 |
| 694 | 133 | 670 | 143 | 1953 | 456 | 421 | 44 |
| 1097 | 202 | 954 | 260 | 3038 | 545 | 574 | 48 |
| 712 | 317 | 243 | 179 | 267 | 31 | 349 | 171 |
| 83 | 11 | 109 | 10 | 71 | 8 | 56 | 8 |
| 45 | 22 | 48 | 12 | 73 | 10 | 47 | 10 |
| 41 | 5 | 33 | 4 | 349 | 62 | 98 | 20 |
| 16 | 1 | 12 | 1 | 25 | 6 | 27 | 6 |
| 15 | 3 | 14 | 2 | 18 | 2 | 11 | 1 |
| 29 | 3 | 19 | 2 | 24 | 4 | 18 | 1 |
| 535 | 79 | 282 | 71 | 808 | 111 | 631 | 121 |
| 1163 | 105 | 912 | 171 | 1785 | 138 | 1517 | 139 |
| 38 | 17 | 23 | 3 | 393 | 146 | 55 | 8 |
| 53 | 12 | 55 | 29 | 502 | 59 | 471 | 138 |
| 13 | 3 | 17 | 6 | 6639 | 629 | 7734 | 949 |

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|---|---|---|---|---|---|---|---|
| 70 | 22 | 119 | 67 | 347 | 37 | 227 | 41 |
| 46 | 11 | 33 | 3 | 169 | 60 | 34 | 2 |
| 134 | 33 | 43 | 3 | 149 | 29 | 42 | 5 |
| 708 | 311 | 107 | 26 | 716 | 118 | 138 | 41 |
| 124 | 20 | 65 | 8 | 452 | 37 | 199 | 50 |
| 494 | 432 | 8 | 0 | 311 | 135 | 201 | 78 |
| 165 | 62 | 25 | 9 | 634 | 102 | 173 | 25 |
| 25 | 3 | 39 | 10 | 391 | 71 | 393 | 32 |
| 162 | 68 | 118 | 97 | 777 | 125 | 779 | 209 |
| 136 | 19 | 129 | 18 | 687 | 59 | 416 | 78 |
| 202 | 31 | 104 | 22 | 420 | 78 | 494 | 44 |
| 43 | 3 | 43 | 4 | 60 | 3 | 97 | 15 |
| 7 | 0 | 7 | 0 | 548 | 446 | 563 | 539 |
| 90 | 16 | 110 | 27 | 136 | 9 | 61 | 15 |
| 9 | 1 | 10 | 1 | 10 | 1 | 19 | 5 |
| 72 | 22 | 40 | 2 | 468 | 308 | 41 | 2 |
| 40 | 2 | 23 | 2 | 187 | 26 | 172 | 27 |
| 314 | 53 | 168 | 24 | 131 | 21 | 69 | 17 |
| 997 | 231 | 539 | 20 | 1062 | 111 | 716 | 23 |
| 145 | 20 | 143 | 22 | 1003 | 141 | 255 | 59 |
| 22 | 1 | 18 | 1 | 20 | 1 | 19 | 1 |
| 10 | 1 | 8 | 1 | 44 | 12 | 61 | 30 |
| 260 | 32 | 252 | 38 | 546 | 60 | 341 | 40 |
| 79 | 9 | 57 | 5 | 239 | 23 | 172 | 22 |
| 194 | 43 | 317 | 43 | 2442 | 152 | 1326 | 539 |
| 60 | 3 | 52 | 3 | 99 | 19 | 63 | 7 |
| 18 | 3 | 21 | 2 | 40 | 15 | 18 | 5 |
| 12 | 2 | 14 | 5 | 9 | 0 | 9 | 1 |
| 14 | 1 | 12 | 1 | 20 | 5 | 344 | 321 |
| 16 | 0 | 15 | 1 | 23 | 5 | 14 | 1 |
| 35 | 2 | 25 | 2 | 72 | 23 | 22 | 1 |
| 267 | 130 | 82 | 23 | 153 | 34 | 40 | 5 |
| 739 | 408 | 73 | 25 | 647 | 107 | 355 | 139 |
| 1006 | 299 | 488 | 90 | 675 | 90 | 230 | 72 |
| 71 | 40 | 46 | 15 | 29 | 3 | 62 | 39 |
| 405 | 35 | 191 | 26 | 1270 | 102 | 1196 | 200 |
| 26 | 3 | 17 | 2 | 317 | 115 | 409 | 187 |
| 252 | 15 | 205 | 31 | 197 | 44 | 51 | 8 |
| 33 | 10 | 8 | 1 | 91 | 26 | 208 | 72 |
| 180 | 67 | 267 | 111 | 918 | 55 | 571 | 120 |
| 363 | 225 | 175 | 70 | 5115 | 356 | 6165 | 532 |
| 812 | 292 | 923 | 385 | 3873 | 255 | 1952 | 440 |
| 7 | 0 | 8 | 0 | 94 | 11 | 247 | 50 |
| 19 | 1 | 24 | 3 | 247 | 67 | 333 | 61 |
| 25 | 4 | 107 | 53 | 215 | 71 | 39 | 6 |
| 326 | 170 | 33 | 6 | 308 | 93 | 69 | 29 |
| 230 | 53 | 49 | 18 | 2144 | 228 | 1236 | 232 |
| 56 | 19 | 59 | 37 | 214 | 52 | 204 | 63 |
| 171 | 24 | 49 | 11 | 157 | 37 | 272 | 41 |
| 8 | 0 | 9 | 0 | 18 | 5 | 8 | 0 |

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|---|---|---|---|---|---|---|---|
| 69 | 7 | 107 | 21 | 275 | 31 | 182 | 28 |
| 37 | 5 | 39 | 8 | 97 | 17 | 51 | 3 |
| 17 | 1 | 17 | 1 | 76 | 33 | 66 | 26 |
| 55 | 6 | 55 | 6 | 220 | 59 | 602 | 256 |
| 52 | 6 | 20 | 2 | 349 | 64 | 240 | 46 |
| 19 | 5 | 10 | 1 | 31 | 7 | 20 | 4 |
| 94 | 64 | 14 | 1 | 109 | 64 | 15 | 2 |
| 59 | 6 | 52 | 5 | 294 | 69 | 115 | 10 |
| 176 | 100 | 12 | 1 | 2671 | 509 | 1536 | 302 |
| 34 | 9 | 23 | 2 | 484 | 157 | 191 | 53 |
| 83 | 26 | 110 | 16 | 217 | 16 | 295 | 47 |
| 25 | 2 | 15 | 2 | 93 | 3 | 59 | 15 |
| 12 | 1 | 16 | 1 | 29 | 7 | 17 | 1 |
| 37 | 15 | 26 | 9 | 167 | 37 | 55 | 21 |
| 45 | 5 | 53 | 8 | 212 | 43 | 180 | 37 |
| 237 | 26 | 71 | 17 | 256 | 68 | 169 | 20 |
| 155 | 48 | 92 | 30 | 3108 | 256 | 2023 | 396 |
| 30 | 5 | 47 | 6 | 179 | 41 | 82 | 24 |
| 146 | 11 | 153 | 12 | 235 | 25 | 172 | 12 |
| 47 | 5 | 29 | 6 | 76 | 6 | 53 | 9 |
| 48 | 4 | 62 | 5 | 71 | 10 | 70 | 7 |
| 79 | 14 | 78 | 16 | 380 | 31 | 253 | 17 |
| 43 | 3 | 53 | 8 | 173 | 36 | 107 | 16 |
| 350 | 49 | 341 | 49 | 2264 | 343 | 1057 | 240 |
| 52 | 4 | 98 | 11 | 87 | 14 | 105 | 11 |
| 36 | 3 | 41 | 5 | 57 | 12 | 50 | 8 |
| 29 | 2 | 46 | 9 | 178 | 42 | 49 | 18 |
| 112 | 24 | 58 | 9 | 139 | 29 | 79 | 11 |
| 178 | 31 | 171 | 23 | 554 | 31 | 456 | 85 |
| 73 | 2 | 65 | 4 | 62 | 8 | 65 | 5 |
| 115 | 12 | 145 | 12 | 764 | 381 | 132 | 27 |
| 24 | 3 | 22 | 4 | 2299 | 578 | 39 | 8 |
| 11 | 0 | 62 | 44 | 201 | 34 | 12 | 1 |
| 68 | 10 | 99 | 31 | 537 | 67 | 458 | 53 |
| 27 | 5 | 20 | 2 | 1245 | 181 | 616 | 182 |
| 177 | 58 | 29 | 8 | 1465 | 212 | 1171 | 235 |
| 54 | 23 | 38 | 2 | 588 | 552 | 25 | 3 |
| 9 | 1 | 10 | 1 | 54 | 29 | 10 | 1 |
| 510 | 125 | 266 | 32 | 1134 | 132 | 744 | 148 |
| 192 | 5 | 185 | 23 | 226 | 36 | 190 | 14 |
| 236 | 55 | 200 | 47 | 1349 | 320 | 721 | 188 |
| 129 | 46 | 133 | 37 | 342 | 75 | 200 | 40 |
| 33 | 8 | 57 | 23 | 148 | 48 | 42 | 10 |
| 55 | 6 | 60 | 13 | 215 | 42 | 171 | 23 |
| 183 | 33 | 81 | 5 | 272 | 53 | 147 | 48 |
| 49 | 2 | 37 | 3 | 127 | 32 | 716 | 115 |
| 61 | 5 | 60 | 7 | 335 | 81 | 698 | 170 |
| 9 | 0 | 8 | 0 | 22 | 6 | 9 | 0 |
| 71 | 14 | 93 | 26 | 388 | 39 | 361 | 59 |
| 147 | 6 | 173 | 17 | 243 | 65 | 170 | 21 |

| 8 | 0 | 7 | 0 | 8 | 1 | 8 | 0 |
|---|---|---|---|---|---|---|---|
| 44 | 4 | 40 | 2 | 103 | 33 | 53 | 17 |
| 110 | 36 | 121 | 71 | 355 | 24 | 186 | 30 |
| 42 | 10 | 16 | 2 | 634 | 136 | 593 | 319 |
| 1036 | 455 | 92 | 45 | 58 | 21 | 60 | 36 |
| 79 | 17 | 75 | 15 | 363 | 108 | 182 | 24 |
| 125 | 7 | 46 | 9 | 715 | 89 | 539 | 93 |
| 18 | 2 | 16 | 1 | 27 | 5 | 15 | 1 |
| 18 | 8 | 9 | 0 | 499 | 317 | 9 | 1 |
| 17 | 1 | 19 | 2 | 22 | 4 | 15 | 2 |
| 283 | 48 | 128 | 18 | 175 | 26 | 65 | 10 |
| 609 | 269 | 74 | 31 | 48 | 11 | 48 | 22 |
| 15 | 1 | 25 | 6 | 43 | 19 | 19 | 4 |
| 47 | 2 | 41 | 2 | 49 | 6 | 37 | 3 |
| 74 | 3 | 63 | 4 | 89 | 9 | 80 | 7 |
| 67 | 16 | 93 | 12 | 90 | 11 | 73 | 12 |
| 41 | 3 | 33 | 3 | 111 | 10 | 171 | 30 |
| 27 | 3 | 25 | 1 | 45 | 4 | 27 | 3 |
| 24 | 1 | 22 | 1 | 27 | 2 | 34 | 2 |
| 11 | 1 | 11 | 1 | 139 | 54 | 21 | 3 |
| 35 | 5 | 31 | 8 | 75 | 17 | 45 | 8 |
| 47 | 9 | 47 | 7 | 42 | 10 | 29 | 4 |
| 209 | 88 | 80 | 28 | 45 | 4 | 40 | 12 |
| 45 | 3 | 45 | 4 | 56 | 14 | 38 | 3 |
| 9 | 0 | 11 | 1 | 23 | 6 | 18 | 8 |
| 22 | 3 | 51 | 8 | 53 | 7 | 67 | 13 |
| 60 | 8 | 42 | 3 | 169 | 44 | 33 | 3 |
| 12 | 1 | 11 | 0 | 16 | 2 | 10 | 0 |
| 11 | 0 | 11 | 0 | 11 | 1 | 11 | 1 |
| 16 | 1 | 16 | 1 | 71 | 17 | 91 | 17 |
| 31 | 1 | 26 | 2 | 38 | 11 | 28 | 2 |
| 111 | 41 | 48 | 13 | 53 | 13 | 35 | 6 |
| 16 | 1 | 26 | 4 | 27 | 4 | 16 | 0 |
| 31 | 4 | 29 | 2 | 275 | 80 | 130 | 37 |
| 9 | 1 | 9 | 1 | 538 | 176 | 50 | 12 |
| 33 | 1 | 34 | 2 | 40 | 3 | 40 | 3 |
| 201 | 79 | 20 | 2 | 177 | 29 | 79 | 19 |
| 17 | 1 | 17 | 1 | 18 | 1 | 14 | 1 |
| 19 | 1 | 20 | 5 | 24 | 3 | 30 | 3 |
| 16 | 1 | 14 | 0 | 21 | 2 | 16 | 1 |
| 9 | 1 | 9 | 0 | 8 | 0 | 8 | 1 |
| 17 | 3 | 10 | 1 | 29 | 15 | 20 | 6 |
| 21 | 2 | 17 | 1 | 19 | 2 | 14 | 1 |
| 12 | 1 | 10 | 0 | 37 | 5 | 12 | 1 |
| 50 | 6 | 65 | 11 | 28 | 3 | 23 | 3 |
| 28 | 2 | 27 | 3 | 44 | 8 | 27 | 3 |
| 35 | 2 | 31 | 2 | 44 | 5 | 25 | 1 |
| 9 | 0 | 11 | 1 | 29 | 9 | 39 | 12 |
| 60 | 17 | 20 | 1 | 63 | 14 | 24 | 2 |
| 23 | 8 | 11 | 2 | 18 | 8 | 6 | 0 |

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|---|---|---|---|---|---|---|---|
| 12 | 1 | 11 | 1 | 13 | 1 | 10 | 1 |
| 21 | 1 | 18 | 1 | 23 | 6 | 15 | 1 |
| 18 | 4 | 11 | 1 | 19 | 4 | 13 | 2 |
| 9 | 0 | 9 | 0 | 12 | 2 | 9 | 0 |
| 9 | 0 | 9 | 0 | 9 | 0 | 9 | 0 |
| 12 | 3 | 12 | 1 | 170 | 80 | 138 | 20 |
| 27 | 3 | 22 | 2 | 57 | 12 | 38 | 8 |
| 8 | 0 | 9 | 1 | 56 | 13 | 67 | 31 |
| 8 | 0 | 8 | 1 | 9 | 1 | 9 | 2 |

**Supplemental Table 1** Expression of pluripotency genes across samples**.** Average normalized expression values across groups of similar samples for unique genes and unannotated probe sets identified as being highly expressed in pluripotent cells over specified somatic cells (Figure 3B). Note: in the event that more than one probe set for a single gene was identified, the probe set with the highest overall expression was selected.

# CHAPTER 4:

## Functional role of *LIN28*/let-7 in the developmental maturity of pluripotent stem cell derived neural progenitors

## Introduction

Human pluripotent stem cells (hPSCs), derived from the developing blastocyst[1] or from transcription factor-induced reprogramming[2], have the capacity to generate all cell types of the human body making them quite attractive for clinical therapeutics. However, in order for these cells to reach the clinic we must have a better understanding of the cell types we are generating and how similar they are to tissue-derived counterparts. Recently, we performed large scale transcriptome profiling comparing neural, hepatic and mesenchymal derivatives of hPSCs to their respective tissue-derived counterparts in order to determine how similar *in vitro* differentiation is to normal, *in vivo* development[3]. We determined that, by both gene expression and *in vitro* function, the derivatives of hPSCs more closely resembled cell types found prior to 6 weeks of gestation than later timepoints. In fact, this appears to be an emerging theme. For example, Zambidis *et al.* reported that hematopoietic differentiation from human embryonic stem cells (hESCs) resembled that of the first wave of hematopoiesis found in the developing yolk sac[4], while Chang *et al.* found that erythrocytes derived from hESCs expressed ε-globin and γ-globin (embryonic and fetal globins respectively), but little to no adult β-globin[5]. This suggests that hPSC derivatives are developmentally young, which could stem from inadequate culturing methods or alternatively could suggest that developmental timing is somewhat conserved *in vitro*.

In our own transcriptome comparison of hPSC derivatives, we identified a list of genes that were differentially expressed between the derivatives and their respective natural counterparts regardless of the cell type generated[3]. Many of these genes, including homologues *LIN28A* and *LIN28B*, are known to be expressed during early embryonic development and were not completely silenced upon *in vitro* differentiation from either hPSC source. *LIN28A* and

*LIN28B* are RNA binding proteins that selectively block the post-transcriptional processing of let-7 miRNAs to their mature functional state[6]. *LIN28B* seems to function primarily in the nucleus by sequestering the pri-let-7s and preventing maturation by Microprocessor, while *LIN28A* functions in the cytoplasm by recruiting an uridylyl transferase to poly-uridylate the pre-let-7s and prevent their further processing by Dicer[7]. The let-7 miRNAs, which target such genes as *HMGA2, MYC, LIN28A,* and *LIN28B*, are highly conserved and strongly correlated with the differentiation status and self-renewing capacity of cells throughout development (Figure 1C)[8]. However, there is little evidence for the role of this pathway in human development specifically. Many groups have demonstrated that *LIN28* is re-expressed in a variety of human cancers and is highly correlated with prognosis and disease progression[9-11]. Furthermore, it has also been used to reprogram somatic cells to the pluripotent state[12]. All of these known roles are linked to developmental immaturity and make *LIN28* an attractive candidate for manipulating the developmental maturity of PSC-derived cells.

We decided to explore the role of the *LIN28*/let-7 pathway in the developmental maturity of PSC-derived neural progenitor cells (PSC-NPCs). During normal development, it is known that neurogenesis precedes gliogenesis[13]. In our original analysis, PSC-NPCs displayed a higher propensity to generate neurons, while 16 week fetal tissue derived NPCs had a higher propensity to generate glia, suggesting that PSC-NPCs were developmentally less mature than their tissue-derived counterpart. Here we demonstrate that this developmental immaturity correlates with high *LIN28A* and *LIN28B* expression along with low let-7 processing. Addition of shRNAs or siRNAs against the *LIN28* homologues was sufficient to induce let-7 maturity only modestly and corrected just a small number of transcriptional differences observed between PSC-NPCs and tissue derived-NPCs. However, addition of let-7 mimics not only corrected many of the

transcriptional discrepancies, but also had an effect on the functional maturity of PSC-NPCs. These results suggest that the let-7 pathway may help to regulate to the "gestational age" of PSC-derivatives.

**Results**

*PSC-NPCs are functionally and transcriptionally less mature than tissue-derived counterparts*

NPCs have the capacity to generate all cell types of the central nervous system, including neurons and glia. During mammalian development, NPCs progress through three phases: an early expansion phase, characterized by symmetric divisions; a neurogenic phase, characterized by asymmetric divisions and resulting in new neurons; and finally a gliogenic phase, another asymmetric phase where glia are primarily produced[13]. The result is that neurogenesis precedes gliogenesis on a developmental timescale. In a comparison of the functional maturity of NPCs derived from either hPSCs or from fetal tissue sources (7-19 weeks of gestation), we determined that PSC-NPCs had a higher propensity to differentiate into Tuj1+ neurons (>50%) over GFAP+ glia (<10%) (Figure 1A and [3]). Meanwhile, tissue-derived NPCs isolated from fetal brain and spinal cord samples at 7-19 weeks of gestation were more apt to differentiate into glia (>70%) over neurons (<20%) (Figure 1B and [3]). These findings suggest that PSC-NPCs are functionally less mature than tissue-derived counterparts from 6-19 weeks of gestation.

Gene expression microarrays on PSC-derivatives and tissue-derived counterparts, were used to identify the molecular differences underlying this functional discrepancy[3]. Among the most differentially expressed genes conserved in all three lineages profiled were *LIN28A* and *LIN28B*. These homologues are known to negatively regulate the highly conserved let-7 family of miRNAs (Figure 1C), making them a candidate regulator of developmental maturity. By

microarray expression, early passage PSC-NPCs expressed both homologues at a level more than 100-fold higher than their fetal-derived counterparts. While continued passaging, does reduce the levels of *LIN28A* and *LIN28B* in PSC-NPCs it does not bring it to a level found in the fetal tissue NPCs (Figure 1D and[3]).

To determine if let-7 was also differentially active as a result of *LIN28* altered expression, we asked if let-7 targets were among the differentially expressed genes separating PSC-NPCs from their tissue-derived counterparts (Figure 1E). Two lists of let-7 targets were generated: one of published let-7 targets that have been experimentally confirmed (77 genes) and one of predicted let-7 targets generated by TargetScan 5.2 (751 genes). Of the 77 published let-7 targets, 20 were differentially expressed between PSC-NPCs and their tissue derived counterpart, while 134 of the 751 TargetScan predicted let-7 targets were differentially expressed. Notably, 95% and 63% respectively were upregulated in the PSC-NPCs, suggesting let-7 is less active in PSC-NPCs. Targets of unrelated control miRNA families (generated by TargetScan) did demonstrate overlap with the genes differentially expressed, however they did not show a directional trend like the let-7 family. This observation also held true for the two other lineages profiled.

*shRNAs against LIN28A and LIN28B knock down their targets and cooperatively induce let-7 maturation*

After demonstrating that the *LIN28*/let-7 pathway is differentially regulated in PSC-NPCs compared to tissue derived cells, we sought to determine if genetic manipulation of the pathway could correct the transcriptional and functional differences observed between the two types of NPCs. shRNAs against *LIN28A* (IRES-GFP) and *LIN28B* (IRES-RFP) were transfected into

PSC-NPCs and FACS sorted for double positive cells. shRNAs could cooperatively knock down *LIN28A* (to a level 20% of the negative control) and *LIN28B* (to a level 50% of the negative control) (Figure 2A). In addition, by RT-PCR for mature let-7 miRNAs, many of the let-7 family members were upregulated as a result of this knockdown (Figure 2B).

In order to determine if the shRNA knockdown corrected the transcriptional differences observed between the two sources of NPCs, microarray profiling was performed on two separate knockdown experiments. 1611 probe sets were found to be differentially expressed (≥2.0 fold difference) in the same direction between double positive (sh*LIN28A* and sh*LIN28B*) cells and their respective negative controls in both experiments. Of the original 2,625 probesets differentially expressed between PSC-NPCs and their tissue derived counterpart[3], 140 were also differentially expressed in the anticipated direction in the two knockdown experiments, suggesting their misexpression had been "corrected" by the manipulation (Figure 2C). Of the 1140 unique genes (represented by 1611 probe sets) that were affected by sh*LIN28A/B* dual knock downs, only a modest number were let-7 downstream targets (Figure 2D), and even fewer were specifically suppressed, as would be expected if let-7 family members were induced.

*siRNAs against LIN28A and LIN28B induce let-7 maturation, but cannot correct gene expression differences*

In an attempt to improve the knockdown of *LIN28* homologues and thereby more strongly induce the let-7 family members, siRNA based knockdown strategy was used. Indeed, when si*LIN28A* and si*LIN28B* were introduced by transfection we saw a 70-75% knockdown of both homologues by RT-PCR (Figure 3A). This was also confirmed by western blot (Figure 3B). The let-7 family members were induced, as measured by RT-PCR, at 2 and 4 days post-

transfection, however by 7 days their levels were not above that of the non-target control siRNA (siNT) (Figure 3C). Also of note, siRNAs against both homologues were necessary to observe an induction of the let-7 family members. This was not surprising given their known semi-redundant roles in let-7 maturation[6].

Microarray profiling of day 2 and 4 double knockdowns identified 403 probe sets differentially expressed (1.54 fold) at each time point over their respective siNT control. These differentially expressed probe sets did not significantly overlap with the original list of probe sets differentially expressed between PSC-NPCs and their natural counterparts (Figure 3D), nor were let-7 downstream targets, besides *LIN28A* and *LIN28B*, selectively knocked down (data not show). This suggested that siRNA based knockdowns can induce let-7s, however the resulting let-7 induction exerted little effect on the system, similar to what was observed using shRNA against Lin28.

There are several possible explanations for these results using RNA interference to silence *LIN28*. First, a two- to ten-fold increase in let-7 maturation is not sufficient to affect the downstream targets. Second, the RISC complex may be overburdened with exogenous siRNAs such that the endogenous miRNAs cannot bind and function. Or third, *LIN28*, being a downstream target of let-7, may not be the driver of let-7 levels, but rather simply a result of altered transcriptional expression of the family members. To determine if the first or second explanation were possible, an alternative approach was chosen to more directly induce let-7 activity.

*Addition of let-7 mimics knocks down let-7 targets and corrects transcriptional discrepancies between PSC-NPCs and tissue-derived NPCs*

After two attempts to manipulate the let-7 pathway through knockdown of the upstream regulators, *LIN28A* and *LIN28B*, we instead directly increased mature let-7s in the system. Using mature oligonucleotides (mimics) for let-7b and let-7g transfected into PSC-NPCs, we were able to successfully knockdown the tested let-7 targets (*LIN28A, LIN28B, HMGA2, IGF2BP1,* and *PLAGL2)* by RT-PCR (Figure 4a). These results were also confirmed by gene expression microarray profiling (Figure 4b).

Microarray profiling of let-7 mimic treated NPCs identified 938, 1044 and 2184 probe sets differentially expressed between let-7 mimic treated NPCs and mimic controls (miCtrl) at 3, 5 and 7 days post-transfection respectively. Many, but not all, of the differences were shared across the three time points (Figure 4C). Furthermore, the transcriptional differences overlapped significantly with the original list of differentially expressed genes (Figure 4D), suggesting those differences had been corrected by the addition of let-7. Interestingly, when we asked how many direct let-7 downstream targets were affected by the addition of the mimics, we determined that not only were let-7 target genes overrepresented in the data, but they were specifically downregulated in let-7b/g transfected NPCs over miCtrls (Figure 4E).

*Let-7 mimics contribute to the functional maturity of PSC-NPCs*

To determine if the correction of differentially expressed genes by addition of let-7 mimics also affected their propensity to differentiate into neurons or glia, differentiation potential was assayed following let-7 manipulation. Following three weeks of differentiation induced by growth factor withdrawal, miCtrl transfected NPCs overwhelmingly produced Tuj+

neurons, while only rare GFAP+ cells were found (Figure 1A-C; $p<0.005$). When let-7b/g mimics were instead transfected, NPCs were slightly less neurogenic and many more GFAP+ glia were produced (Figure 1A-C; $p<0.005$). These findings suggest that the let-7 pathway has at least modest effect on the developmental maturity of PSC-NPCs.

**Discussion**

This work demonstrates that PSC-NPCs functionally represent early stages of gestation. Using gene expression microarray, we previously reported that this functional immaturity corresponded with a differential gene expression signature, including genes known to be expressed during embryonic development. This analysis allowed us to date-stamp PSC-NPCs to be most similar to an NPC found <6 weeks of gestation[3]. Many other groups, deriving various different cell lineages, have also reported that hPSCs generate embryonic or primitive cell types[4,5,14]. Furthermore, when we looked at PSC-derivatives generated and profiled independently by other groups, we found that a significant number of the genes differentially expressed in our system also appeared in their data[3]. These findings taken together suggest that *in vitro* differentiation from hPSC generates immature cell types with a transcriptional signature akin to early embryonic development. It remains unknown, however, if this is a failure of *in vitro* differentiation protocols to fully recapitulate *in vivo* development or if instead developmental timing is innately controlled and 4-6 week differentiation protocols simply produce cells representing that gestational age.

If the transcriptional and functional discrepancies we observe between hPSC-derivatives and tissue-derived counterparts are at least partially the result of an intrinsic mechanism, the *LIN28*/let-7 pathway is a strong candidate. It is evolutionarily conserved and known to control

developmental timing in *C. elegans* and mice[8]. In humans, it has been linked to reprogramming[12] and transformation[9-11], both of which are related to developmental maturity. We report here that the use of RNA interference to knock down of *LIN28* homologues in PSC-NPCs is insufficient to correct the transcriptional discrepancies observed between PSC-NPCs and tissue-derived NPCs. Conversely, direct addition of let-7 family members can not only correct a significant portion of the transcriptional profile, but can also modestly affect their functional maturity. There are several possible reasons to explain this apparent discrepancy. First, it is possible that *LIN28* expression in this system is not the relevant mechanism controlling let-7, but instead let-7 is controlled transcriptionally and *LIN28* differential expression is simply a downstream effect of increased let-7 expression. In other words, let-7 is controlling developmental maturity through a *LIN28* independent mechanism. Alternatively, manipulating the pathway by knock down of *LIN28* may be technically more difficult. It is possible that a 2- to 10-fold induction of let-7 family members, observed when *LIN28* homologues are knocked down, is insufficient to then affect let-7 downstream targets. In fact, if we consider our previous finding that many let-7 family members are 100-fold higher in tissue-derived NPCs over PSC-NPCs[3], it may not be surprising that a 2- to 10-fold induction has little effect on the system. Furthermore, it is possible that overexpression of siRNA or shRNAs may bombard the RISC complex, such that the endogenous let-7s are outcompeted for binding and any induction observed may be functionally blocked.

To better address whether let-7 or *LIN28* is the regulator of the system and avoid any technical issues, it may instead be necessary to manipulate the pathway in fetal-derived NPCs. In this case, overexpression of *LIN28* homologues or introduction of antagomirs (siRNAs against miRNA specifically) against let-7 could be used to ask if either manipulation can force tissue-

derived NPCs to be more neurogenic rather than gliogenic. Yuan and colleagues recently reported that *LIN28B* was differentially expressed between human adult and fetal hematopoetic stem/progenitor cells (HS/PCs) and that overexpression of *LIN28B* reprogrammed adult HS/PCs into fetal-like HS/PCs based on their lymphogenic potential[15]. It is possible that a similar manipulation in our system may also be sufficient to reprogram NPCs.

Finally, we cannot rule out the possibility that another pathway or multiple pathways are responsible for the observed functional and transcriptional differences. Manipulation of let-7 corrected a significant, but modest number of genes differentially expressed between tissue-derived NPCs and PSC-NPCs. Furthermore, it only increased gliogenesis by 2%. Many other gene pathways were identified that could also be controlling the developmental maturity of PSC-derivatives. It will be interesting to see if any other pathways, singularly or in concert with let-7, can have a more profound effect on developmental timing in this system.

These data suggest that PSC-derivatives are functionally and transcriptionally primitive. From a clinical application viewpoint, it will be important to demonstrate that PSC-derivatives, being most similar to embryonic cell types, can incorporate and function within an adult tissue. It will be necessary to explore what consequence the continued expression of embryonic genes, such as *LIN28*, might have in adult tissues or if the expression of those genes can be manipulated to functionally age the cells prior to transplantation.

## Materials and Methods

### *Cell culture*

Undifferentiated hPSC lines, HSF1, H9, xiPSC2 were maintained as previously described[16]. Neural rosettes were derived, maintained and further differentiated as described[17].

Lipofectamine 2000 (Invitrogen) was used to transfect shRNA plasmids (Genecoepoia) at a ratio of 8ul lipofectamine:8ug of DNA/well of a 6 well plate. shLIN28A contained an IRES-GFP transgene while shLIN28B contained an IRES-RFP allowing for sorting of transfected cells 5 days post-transfection with a FACS ARIA (BD Biosciences). siRNAs and let-7 mimics (Dharmacon) were transfected with Lipofectamine RNAiMAX (Invitrogen) at a ratio of 5 ul lipofectamine:20nM siRNA or 5 ul lipofectamine:40nM mimic/well of a 6 well plate. All transfections were performed overnight in Optimem (Gibco).

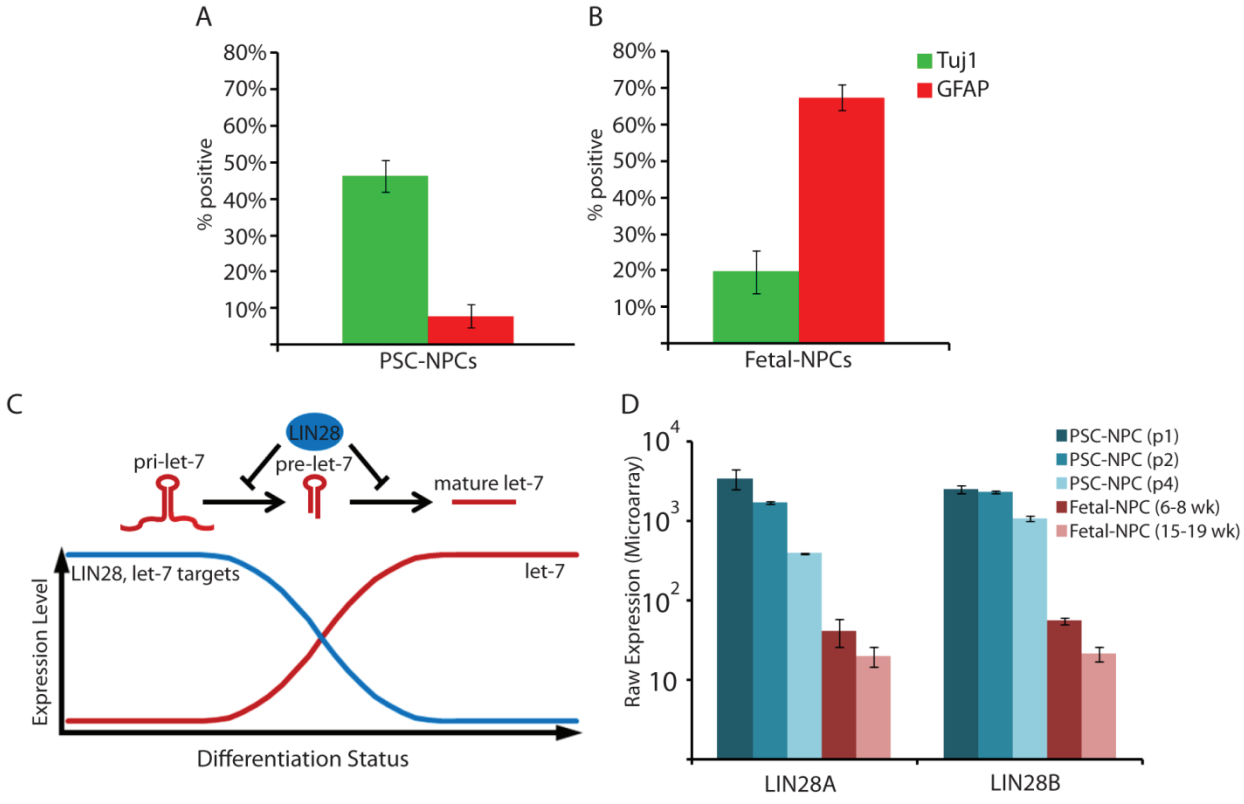
*Immunostaining*

Differentiated PSC-NPCs were passaged with TrypLE (Gibco) to glass coverslips 2 days prior to fixing. Coverslips were fixed and stained using standard protocols as described[3]. Primary antibodies included rabbit x Tuj1 (1:2000; Covance), rabbit x GFAP (1:1000; Dako), and chicken x GFAP (1:2000, Abcam). Western blot analysis was performed using standard procedures as described[18]. Primary western blot antibodies include rabbit x Lin28A (1:300; Cell Signaling), rabbit x Lin28B (1:300; Cell Signaling), and mouse x actin (1:1000).

*Expression Analysis*

RNA isolation, mRNA reverse transcription, and real-time PCR were performed as described[16]. miRNA reverse transcription was performed with miScript reverse transcription kit (Qiagen) and real-time PCR was performed using miScript Sybr Green PCR kit and miScript Primer Assay (Qiagen). Microarray profiling was performed with Affymetrix Human HG-U133 2.0 Plus arrays as described[16,19]. Data were normalized with Robust Multichip Algorithm in Genespring separately from previous analyses performed[3]. Probe sets that were not expressed at

a raw value of > 50 in at least 10% of samples were eliminated from further analysis. Gene expression differences for the genetic manipulations were identified by fold change of ≥2.0 (shRNA experiments) or ≥1.54 (siRNA and mimic analysis). Gene expression differences for the original list of genes differentially expressed between PSC-NPCs and Natural-NPCs were determined as previously described[3]. Only those probe sets that made it past the filtering after both normalization s were included in this analysis. Further statistical analysis for hypergeometric distribution and three-way simulation was performed with R, package 2.9.2 as described[19]. Overlap with let-7 target genes was performed in Excel using lists generated either from the literature of from TargetScan 5.2.

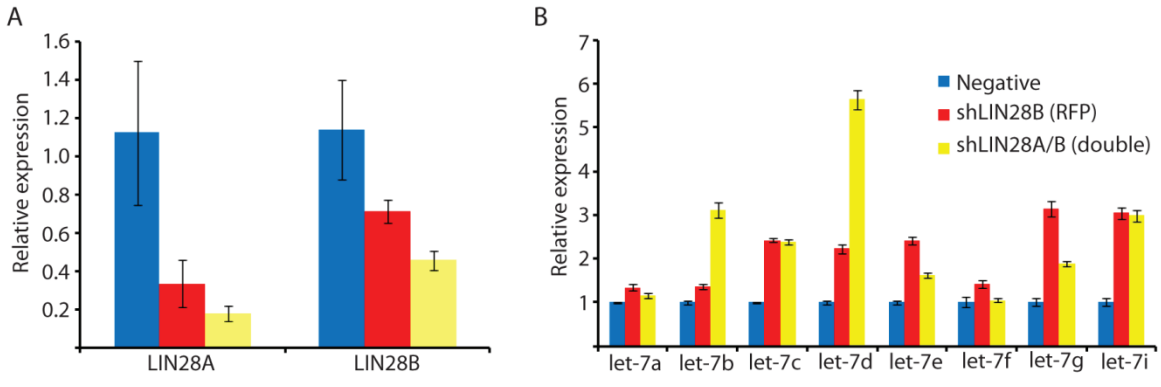


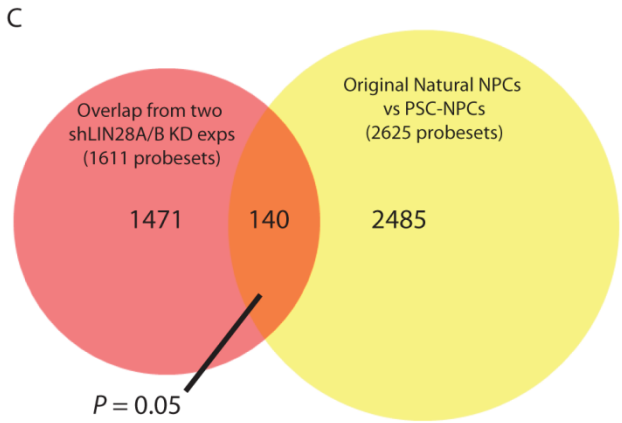

E

| | Total | | let-7 published (77 genes) | | TargetScan 5.2 | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | let-7 (751 genes) | | miR-15 (883 genes) | | miR-17 (898 genes) | | miR-130 (667 genes) | |
| | genes | % up in PSCD | overlap | % up in PSCD | overlap | % up in PSCD | overlap | % up in PSCD | overlap | % up in PSCD | overlap | % up in PSCD |
| PSC-NPC vs Natural NPC | 1885 | 44.4% | 20 | 95.0% | 134 | 62.7% | 133 | 50.4% | 127 | 25.2% | 135 | 27.4% |
| PSC-Hep vs Natural Hep | 3050 | 56.6% | 18 | 88.9% | 211 | 74.9% | 210 | 69.5% | 198 | 63.6% | 164 | 66.5% |
| PSC-FB vs Natural FB | 1961 | 52.2% | 9 | 55.6% | 142 | 60.6% | 141 | 48.9% | 147 | 46.9% | 119 | 40.3% |

**Figure 4.1 PSC-NPCs are functionally and transcriptionally less mature than tissue-derived NPCs.**

**(A)** Percent positive PSC-NPCs undergoing neuronal (Tuj1) vs. glial (GFAP) differentiation following 3 weeks growth factor withdrawal (n=7). **(B)** Percent positive fetal tissue-NPCs (7-19 weeks of gestation; n=7) undergoing neuronal vs. glial differentiation. Error bars represent standard error over biological replicates. **(C)** Schematic of *LIN28*'s role in the maturity of let-7 microRNAs and the pathway's link to development. **(D)** Average raw expression of *LIN28A* and *LIN28B* probe sets from the Patterson and Chan et al. microarray[3]. PSC-NPCs p1 (n=6), PSC-NPCs p2 (n=2), PSC-NPCs p4 (n=2), 6-8 week fetal-NPCs (n=6), 15-19 week fetal NPCs (n=5). Error bars represent standard error over biological replicates. **(E)** Genes differentially expressed between PSC-derivatives and their respective natural counterpart as identified[3] and the number of let-7 targets represented in the data. Let-7 target lists were generated from published articles and TargetScan 5.2. Unrelated control miRNA lists were generated from TargetScan 5.2.




D

| | | | shA/B (2 exps) |
|---|---|---|---|
| | Total | genes | 1140 |
| | | % KD | 57% |
| TargetScan 5.2 | let-7 published (77 genes) | Overlap | 6 |
| | | % KD | 66% |
| | let-7 (751 genes) | Overlap | 86 |
| | | % KD | 63% |
| | miR-15 (883 genes) | Overlap | 80 |
| | | % KD | 58% |
| | miR-17 (898 genes) | Overlap | 96 |
| | | % KD | 53% |
| | miR-130 (667 genes) | Overlap | 78 |
| | | % KD | 65% |

**Figure 4.2 shRNAs against *LIN28A* and *LIN28B* knock down their targets and cooperatively induce let-7 maturation.**
**(A)** Real time-PCR for *LIN28A* and *LIN28B* following shRNA knock downs. mRNA expression was normalized to *GAPDH*. **(B)** Real time-PCR for let-7 family members following shRNA knockdowns. miRNA expression was normalized to U6. Error bars represent standard error across three technical replicates. **(C)** Venn diagram demonstrating the original differences between PSC-NPCs and tissue derived-NPCs (yellow)[3] and the overlap with gene expression differences between sh*LIN28A/B* double knock downs and negative controls (n=2 biological replicates; red). Direction of differential expression was taken into account. Statistical analysis performed by hypergeometric distribution. **(D)** Genes differentially expressed between sh*LIN28A/B* double knock downs and negative controls and the number of let-7 targets represented in the data.

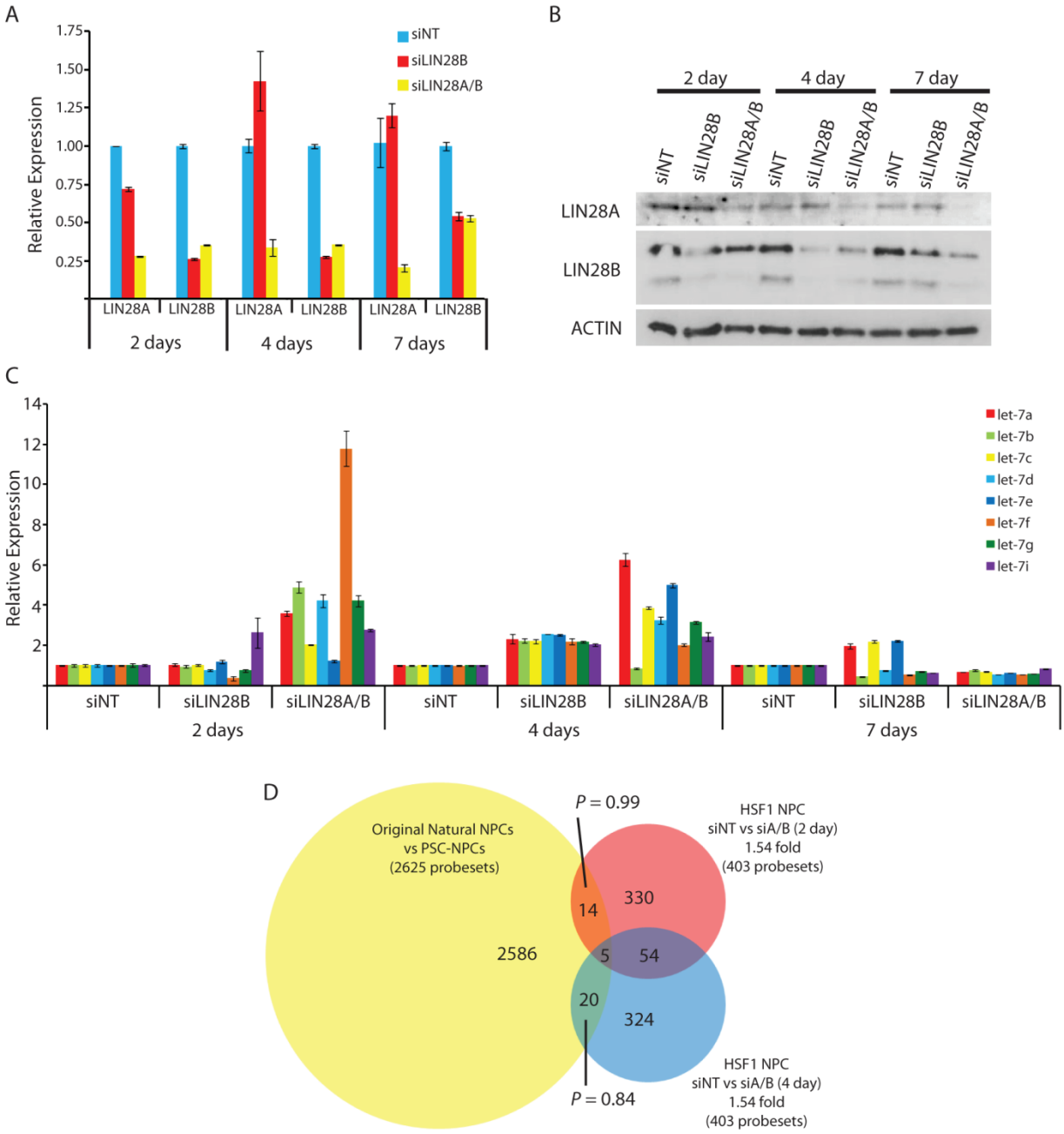
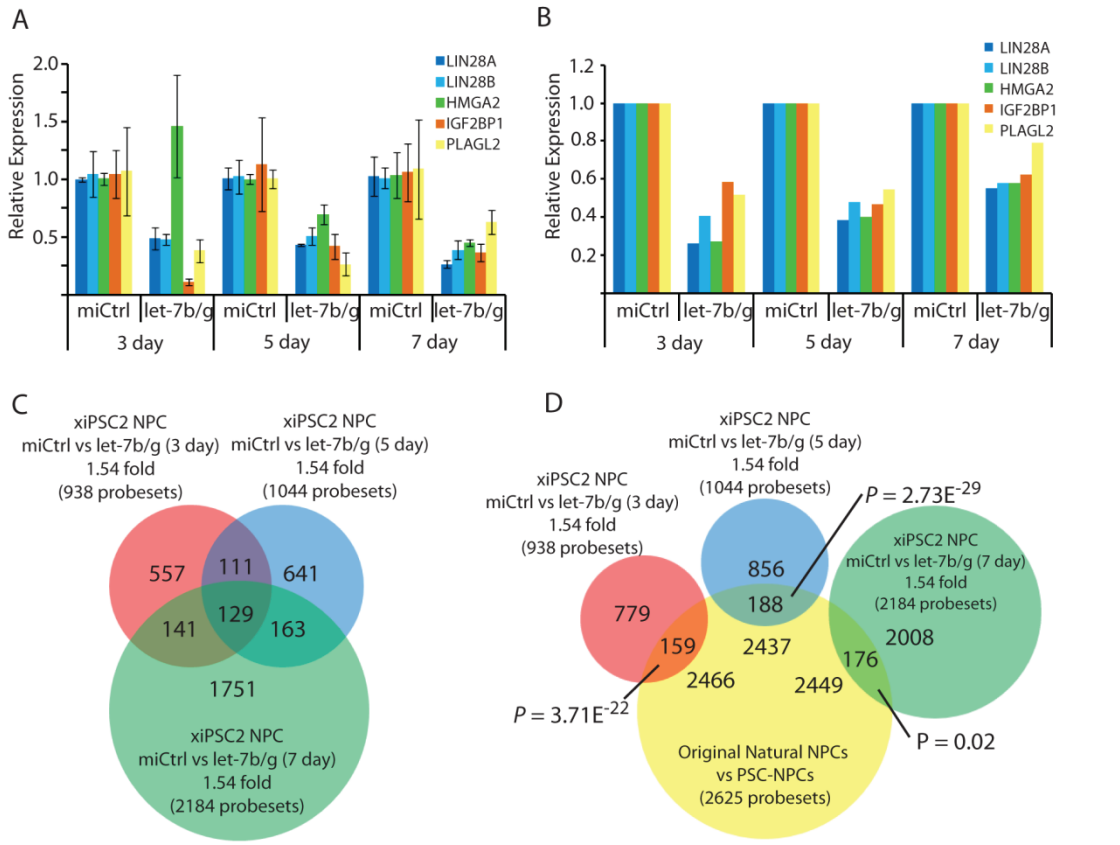



**Figure 4.3 siRNAs against *LIN28A* and *LIN28B* induce let-7 maturation, but cannot correct gene expression differences.**
**(A)** Real time-PCR for *LIN28A* and *LIN28B* following siRNA knock downs. mRNA expression was normalized to *GAPDH*. **(B)** Western blots for LIN28A, LIN28B, and ACTIN control following siRNA knock downs. **(C)** Real time-PCR for let-7 family members following siRNA knockdowns. miRNA expression was normalized to U6. Error bars represent standard error across three technical replicates. **(C)** Venn diagram demonstrating the original differences between PSC-NPCs and tissue derived-NPCs (yellow)[3] and the overlap with gene expression differences between si*LIN28A/B* double knock downs and non target controls (siNT) at day 2 (red) and day 4 (blue) post transfection.




E

| | Total | | let-7 published (77 genes) | | TargetScan 5.2 let-7 (751 genes) | | miR-15 (883 genes) | | miR-17 (898 genes) | | miR-130 (667 genes) | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | genes | % KD | overlap | % KD | overlap | % KD | overlap | % KD | overlap | % KD | overlap | % KD |
| let-7b/g (3 day) | 750 | 46% | 19 | 100% | 100 | 91% | 53 | 53% | 67 | 48% | 52 | 46% |
| let-7b/g (3 day) vs orig | 125 | 49% | 11 | 100% | 33 | 85% | 11 | 82% | 10 | 50% | 13 | 46% |
| let-7b/g (5 day) | 745 | 41% | 12 | 83% | 77 | 75% | 54 | 41% | 47 | 43% | 34 | 26% |
| let-7b/g (5day) vs orig | 150 | 37% | 8 | 100% | 25 | 80% | 11 | 45% | 10 | 40% | 11 | 36% |
| let-7b/g (7 day) | 1663 | 72% | 14 | 93% | 132 | 89% | 141 | 85% | 137 | 79% | 115 | 80% |
| let-7b/g (7 day) vs orig | 141 | 38% | 6 | 100% | 19 | 84% | 17 | 59% | 11 | 36% | 9 | 56% |

**Figure 4.4 Addition of let-7 mimics knocks down let-7 targets and corrects transcriptional discrepancies between PSC-NPCs and tissue-derived NPCs.**
**(A)** Real time-PCR for let-7 target genes following addition of let-7 mimics. mRNA expression was normalized to *GAPDH*. **(B)** Relative expression of let-7 target genes as measured by microarray probe set expression. Expression was normalized by RMA normalization in Genespring. **(C)** Venn diagram depicting the overlap of genes differentially expressed between let-7b/g and mimic controls (miCtrl) at 3 (red), 5 (blue), and 7 days (green) post transfection. **(D)** Venn diagram demonstrating the original differences between PSC-NPCs and tissue derived-NPCs (yellow)[3] and the overlap with gene expression differences between let-7b/g and miCtrl 3 (red), 5 (blue), and 7 days (green) post transfection. Note: Overlap between days is not depicted here. **(E)** Genes differentially expressed between let-7b/g and miCtrl and the number of let-7 targets represented in the data.

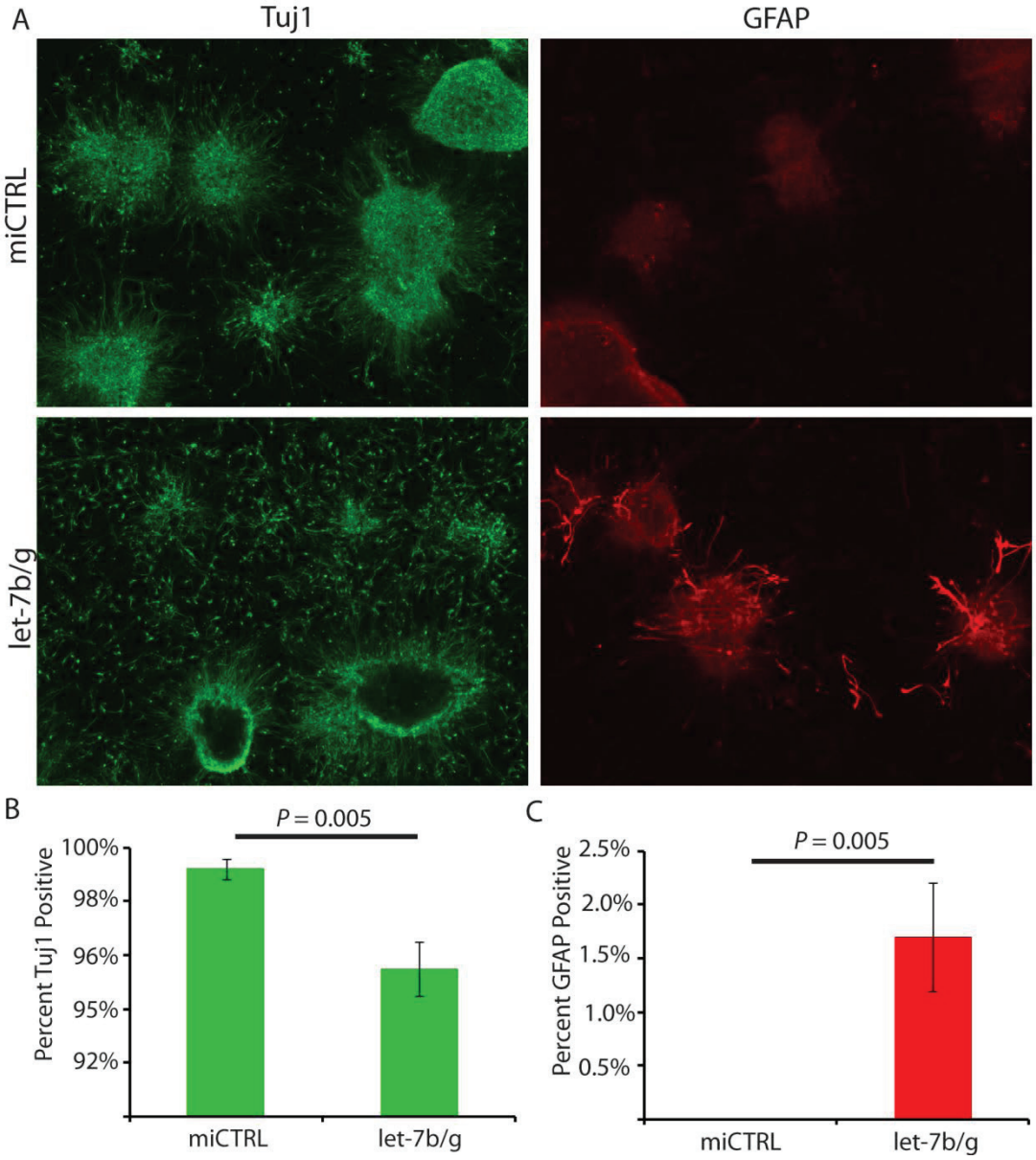



**Figure 4.5 Let-7 mimics contribute to the functional maturity of PSC-NPCs.**
**(A)** Immunofluorescence of Tuj1+ neurons and GFAP+ glia in PSC-NPCs transfected with let-7b/g or miCtrl following 3 weeks of growth factor withdrawal. **(B)** Percent Tuj1 positive neurons in let-7b/g or miCtrl transfected cells. **(C)** Percent GFAP positive glia in let-7b/g or miCtrl transfected cells. Error bars represent standard error over technical replicates (n=8).

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# CHAPTER 5:

# Discussion

Human PSCs have many proposed uses from clinical transplantation to *in vitro* modeling of development and disease. Our work, presented in this dissertation, provides several important insights into the differentiation of hPSCs and how they can best be exploited. We demonstrate that hPSCs are an excellent and tractable model for human development, but raise possible issues regarding advancement of this technology to a clinical setting or its application for disease modeling.

**Modeling development**

One proposed application of hPSCs is to model human development in an accessible system amenable to characterization and manipulation and various stages. The premise is that hPSCs have the capacity to differentiate into all cell types of the human body. Therefore, it should be possible to follow their differentiation to a specific lineage *in vitro* in order to determine what molecular mechanisms are required to arrive at that final fate. The issue with this proposed application is the lack of understanding whether *in vitro* differentiation accurately recapitulates *in vivo* development or if hPSCs generate artificial cell types not found in the human body. While aspects of this complex question remain widely unexplored, we have established that hPSC differentiation to the neural lineage is seemingly faithful to *in vivo* development.

In chapter 2, we show that both hESCs and hiPSCs can generate electrophysiologically active motor neurons. Furthermore, through immunocytochemistry analysis we demonstrate that hPSCs differentiate to this mature state through a progressive and predictable pattern. They first undergo neuralization, displaying markers of NPCs. This is followed by motor neuron specification, marked by expression of motor neuron progenitors, and finally motor neuron

maturation, marked by expression of mature motor neuron markers and electrophysiological activity. This preliminary analysis of the differentiation route suggests that hPSCs appear to obey normal developmental progression.

In chapter 3, we take a more comprehensive approach to directly compare hPSC derivatives to a counterpart isolated from human tissue sources to determine if *in vitro* differentiation recapitulates *in vivo* development. In this thorough analysis, we first determined that the derivatives of hESCs and hiPSCs are seemingly indistinguishable, suggesting that the transcriptional differences that discern hiPSC from hESCs in the undifferentiated state[1] seem to dissipate upon differentiation. Furthermore, it implies that despite widely different derivation methods, these hPSCs undergo differentiation via similar mechanisms.

With this detailed analysis we were also able to demonstrate hPSC derivatives are most similar to tissue-derived cells isolated prior to 6 weeks of gestation. While we are not the first to report that hPSCs generate primitive cell types[2-4], to our knowledge, this is the first report to provide such a narrow "date stamp" to hPSC differentiation. More importantly, it is perhaps not coincidental that a 3-6 week differentiation protocol generates cell types found prior to 6 weeks of gestation. These findings suggest that hPSC differentiation is a faithful model of human development and, more specifically, that developmental timing during *in vitro* differentiation is conserved and may be innate to the cell. It will be interesting to determine what mechanisms control this timing and if those mechanisms can be experimentally manipulated.

We have begun to explore the role of the evolutionarily conserved *LIN28*/let-7 pathway in this developmental timing. In chapter 4, we report that let-7 manipulations facilitate the functional maturation of hPSC derived NPCs; however not to a level comparable to 16-week fetal tissue NPCs. It remains unknown why the manipulation did not have a more profound

effect. It is possible that multiple gene pathways contribute to developmental timing and that manipulation of more than one pathway will be necessary. On the other hand, we cannot rule out the possibility that *in vitro* differentiation protocols do not fully recapitulate the niche and that hPSC derivatives cannot progress past a specific developmental age when supportive cells become necessary. Therefore, it remains possible that hPSCs will continue to mature only if transplanted into an appropriate environment.

**Regenerative medicine and transplantation**

From a regenerative medicine view point, it is relatively easy to theorize the issues that need to be addressed before the technology can advance to the clinic. Some of these issues include immune rejection, tumor formation, graft survivability and functional incorporation into the host tissue/organ system. Our work presented in the chapters of this dissertation raises an additional caveat.

From a development standpoint it is exciting to think that cells develop at the same pace *in vitro* as they do *in vivo*, however from a cell transplantation point of view, this could be problematic. If hPSC derivatives behave similar to counterparts isolated from fetal tissue prior to 6 weeks of gestation, can those cells incorporate and function properly in an adult patient? In the field of Parkinson's disease, fetal ventral mesencephalic tissue is transplanted into patients with varying success[5-6], so it is possible that fetal-like cells may be sufficient to cure adult patients; however each organ system or cell type is likely to have different tolerance. In a study performed in a mouse model of liver failure, it was determined that transplanted adult hepatocytes were significantly better at surviving, proliferating and rescuing the animal when compared to fetal liver or mESC-derived endoderm transplants[7]. Preclinical and clinical trials will be necessary to

demonstrate if complete maturity is necessary, if fetal-like cells will suffice, or if hPSCs will simply continue to mature once transplanted. Furthermore, one of the characteristics that allowed us to determine the gestational age of hPSCs derivatives was the continued expression of embryonic genes. Some of these genes, including *LIN28B*, are also re-expressed in human cancers and correlate with poor prognosis[8-10]. Continued expression of an oncogene in the grafted cell may incur undesirable proliferation or other cancer traits. Therefore, it will be necessary to determine, if these genes eventually silence upon transplantation or what consequence their continued expression might have on the grafted cells.

**Modeling human disease with disease-specific hiPSC**

Lastly, hiPSC specifically have been proposed to serve as an *in vitro* model of human disease. Similar to the idea of using hPSCs to tractably model development, hiPSCs can be derived from patients harboring a genetic disease and their differentiation to disease relevant cell types can be followed in order to determine the mechanisms underlying that disease. Furthermore, small molecule screening could be performed in order to identify drugs that may rescue the phenotype. Some of the diseases that have been proposed to benefit from this technology do not display symptoms until late into adulthood (ie ALS). Our finding that hPSC derivatives represent a developmentally young cell type raises the question whether we can model late-onset human disease in a petri dish? Will we capture the disease state or will the cells appear healthy until they reach the developmental age of onset? Our data suggests that it may be difficult to model a human genetic disease with these cells, unless that disease is characterized by early onset.

With this dissertation, we have demonstrated that *in vitro* differentiation of hPSCs is seemingly faithful to *in vivo* development. Previously, the study of human development has been limited to the use of animal models. These models, while extremely powerful, have their limitations. Some developmental mechanisms are not conserved from one species to another and it can be hard to capture transient events when using whole organisms. Our work presented here suggests that hPSCs could serve as a tractable model for understanding the mechanisms that govern human development and could help to overcome some of the limitations of animal models.

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