The neural stem cell lineage reveals novel relationships among spermatogonial germ stem cells and other pluripotent stem cells

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Footnote

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Abbreviations

BMP4, bone morphogenetic protein 4; dNSC, definitive neural stem cell; E, embryonic day; EB embryoid body; Epi-SC, epiblast-derived stem cell; ESC, embryonic stem cell; FAB-SC, b-FGF, Activin and BIO-derived stem cell; iPS, induced pluripotent cells; ICM, inner cell mass; LIF, leukemia inhibitory factor; MEF, mitotically-inactivated fibroblast; PC, pluripotent cell; pGC, primordial germ cells; Pin1, prolyl isomerase; pNSC, primitive neural stem cell; SFM, serum-free media; aSGSC, adult pluripotent spermatogonial germ stem cell; TE, Trypsin/EDTA; YFP, yellow fluorescent protein

Abstract

The embryonic stem cell (ESC) derived from the inner cell mass is viewed as the core pluripotent cell (PC) type from which all other cell types emanate. This familiar perspective derives from an embryological time line in which PCs are ordered according to their time of appearance. However, this schema does not take into account their potential for interconversion and thereby excludes this critical quality of PCs. The persistence of *bona fide* pluripotent adult stem cells has garnered increasing attention in recent years. Adult pluripotent spermatogonial germ stem cells (aSGSCs) arise from primordial germ cells (pGCs) that emerge from the epiblast during gastrulation. Adult definitive neural stem cells (dNSCs) arise clonally from pluripotent embryonic primitive neural stem cells (pNSCs), which can also be derived clonally from ESCs. To test for stem cell type convertibility, we employed differentiation in the clonal lineage from ESCs to pNSCs to dNSCs, and revealed the relationships and lineage positioning among various pluripotent cell populations, including spermatogonial germ cells (aSGSCs), the epiblast-derived stem cell (Epi-SCs) and the bFGF, Activin and BIO-derived stem cell (FAB-SC). Adult, murine aSGSCs assumed a 'pseudo-embryonic stem cell' state *in vitro*, and then differentiated into dNSCs, but not pNSCs. Similarly, Epi-SCs and FAB-SCs only gave rise to dNSCs and not pNSCs. The results of these experiments suggest a new pluripotency lineage model describing the relationship(s) among pluripotent cells that better reflects the transitions between these cell types *in vitro*.

Introduction

The cells of the inner cell mass and their *in vitro* counterpart, the embryonic stem (ESC) cell, are the consummate pluripotent cells, from which all other embryonic pluripotent, multipotent and progenitor cells arise [1-4]. If one considers the age of origin during development of all pluripotent, multipotent stem cell types and eventually somatic cell types[5], this conventional model suggests a serial timeline, with the ESC at the beginning from which all other pluri-/multi-potent cells extend (**Figure 1**). Yet, if one takes into account the high degree of interconversion that exists between various stem cell types, particularly in the era of induced pluripotent cells (iPS), the barriers between these cell populations begin to break down and new relationships may emerge among pluripotent cell types. The present work examines some of the more recently described pluripotent cell types, and through various (non-genetically manipulated) cell transitions, attempts to reveal previously unknown associations among these cell populations. Understanding and defining these interconversions could lead to previously unforeseen sources and applications of different pluripotent cell types.

In vivo, spermatogonial germ stem cells (aSGSCs) originate from the epiblast, through a primordial germ cell (PGC) intermediate. In the murine embryo, PGCs are specified within the epiblast at embryonic day (E) 6.25 [6]. These founder cells then move through the primitive streak and by E7.0-E7.5, a cluster of PGCs becomes apparent [7,8]. PGCs subsequently migrate through the embryo proper and colonize genital ridges, where, in the case of males, they give rise to the definitive aSGSC. The commitment of PGCs to form aSGSCs occurs at ED 12.5-14.5 [9]. These aSGSCs can self-renew at the basal membrane of the seminiferous tubules [10], and continue to differentiate into mature sperm [11,12]. aSGSCs reversibly express germ, some pluripotency, and early lineage cell markers and exhibit a high degree of plasticity [13-15].

aSGSCs were first isolated from the adult mouse by Guan *et al*. [15]. Previously, aSGSCs had been isolated only from murine neonatal testes [16]. Hence, an adult source of pluripotent cells was identified that, under specific conditions, could differentiate into cell types belonging to all three germinal lineages, form teratomas [17] and contribute to the formation of blastocyst-injected chimeras [15].

Epiblast stem cells are derived from the epiblast layer of post-implantation (murine) embryos [18,19]. They are distinguished from murine ESCs by their epigenetic and transcript profiles, their signaling requirements for differentiation and self-renewal, as well as by their impediment with respect to chimera formation [19]. However, their culturing requirements are more similar to those used for human ESCs [18]. By the same token, modifying the cell culture requirements of the Epi-SC cultures led to the identification of another epiblast-derived pluripotent cell type, the FAB-SC (b-FGF, Activin and BIO-derived) [20]. FAB-SCs are morphologically distinct in appearance from Epi-SCs and can generate chimeras (including germline contributions) after the transient addition of leukemia inhibitory factor (LIF) and bone morphogenetic protein 4 (BMP4) [20]. Recent work has suggested that epiblast cells maintained in standard or neurogenic culture conditions correspond to the murine *in vivo* states of the ~E6.5 epiblast or the ~E7.5 anterior neural plate, respectively [21]

Previously, we have demonstrated that single, pNSCs default from single, murine embryonic stem cells and proliferate in a LIF-dependent manner to form free-floating colonies of neural stem cells termed (primitive) neurospheres [22]. These clonally derived, primitive neurospheres [23] under appropriate conditions, have the ability to differentiate into all of the neural lineages. Blastocyst aggregation studies have shown that pNSCs derived from mouse ESCs can form chimeric mice [22,24]. The primitive neural stem cell (pNSC) is thought to arise

in vivo at approximately E5.5 during murine development and rapidly differentiates into the definitive neural stem cell (dNSC) [25]. This transition is presumed to occur during the course of 36 hours (E7.0-E8.5), all other neural lineages arise at~ E8.5 [25].

We postulated that ESCs, pNSCs, Epi-SCs, FAB-SCs and aSGSCs are more closely related than previously thought and may all be interconvertible *in vitro*. ESCs also form embryoid bodies (EBs), 3-dimensional, heterogeneous aggregations of cells, in suspension, in serum-contained media[5]. A defining feature of EBs is that they express genes from all three germ cell layers, namely the ectoderm, endoderm, and mesoderm [26], and in this manner EBs can model early embryonic development [27] . The results of the present study of gene expression analyses and the *in vitro* conversions between ESCs and each of the epiblast, neural, and spermatogonial germ stem cells, suggest potential relationships among the lineages of each of these stem cell types. We used the *in vitro* clonal neural lineage assay to test the hypothesis that these specific cell populations are interconvertible and exhibit comparable gene expression profiles, in order to develop a lineage model more reflective of such transitional similarities.

Materials and Methods

Formation of Embryoid Bodies (EB)s

Single mouse ESCs were aggregated in 'hanging drops' in media containing 15% FCS (Hyclone), 100 mM β-mercaptoethanol (Gibco), 100 U/ml penicillin, 100 mg/ml streptomycin (Invitrogen), 20% high glucose DMEM (Invitrogen), 1 mM L-glutamine (Invitrogen), 10mM hepes (Sigma), 0.6% glucose (Sigma), 0.1% sodium bicarbonate ($NaHCO_3$) (Sigma); 30 µl drops containing approximately 3000 cells were plated onto the lid of a 10 cm Petri dish, inverted and

cultured as hanging drops for 2 days before being rinsed onto uncoated plates (Phoenix Biomedical) and cultured for an additional 3 to 5 days [28].

aSGSC Isolation and Cell Culture:

Primary testicular dissections were performed on mice from multiple genetic backgrounds (CD1 (Charles River), C57/B6 (Charles River), Wnt1/Cre (gift from F. Miller, Toronto, ON, Canada), and Oct4/GFP (gift from A. Nagy, Toronto, ON Canada) and ranging in age from 6 weeks to over a year. Animals were housed and cared for in accordance with the standards set by the Canadian Animal Heath Care Committee. The seminiferous tubules were removed and microdissected. The tubules were then subjected to a 5-minute collagenase IV digestion (LS004188, Worthington), followed by a 5-minute trypsin digestion (Sigma), both at 37˚C. After straining through a 70-μm filter, primary cells were plated at high density (100-200 cells/ml) onto 60 mm gelatin-coated plates (0.1% Gelatin/Millipore) in standard ESC media (no LIF) in the presence or absence of GDNF (Sigma). After 5-7 days, ESC-like colonies were identified and individual colonies were picked for passaging. The cells were dissociated with Trypsin/EDTA (TE) and re-plated onto new 35 mm gelatin-coated plates in ESC media (with LIF, 1000 U/mL) and allowed to grow for 2 weeks. After 2 weeks ESC-like-colonies were again identified, individually isolated, disaggregated with TE, and passaged onto mitotically-inactivated fibroblasts (MEF)s. These aSGSCs were then grown in ESC media (with LIF 1000 U/ml). aSGSC cultures were passaged onto fresh MEFs every 5-7 days. Epiblast and FAB stem cells were kindly provided by M. Tesar (Cleveland, OH, U.S.A.) and N. Geijsen (Boston, MA, U.S.A.), respectively, and cultured as previously described [19,20].

Neurosphere Assay

The neural colony-forming assay was performed on all cells, as described previously [29]. In brief, cells were harvested, washed in serum-free media (SFM) and then plated in SFM containing LIF (1,000 U/ml), or for the FGF cultures: a combination of EGF (20 ng/ml), FGF2 (10 ng/ml), heparin (2 mg/ml) and B-27 supplement 50X (Invitrogen). Cells were allowed to grow for 1-3 weeks, with 50% of the media volume replenished every 7 days.

Cell viability and quantification was done by trypan blue exclusion, with cells seeded at a concentration of 10 cells/μl [23]. When spheres were ≥50 μm, they were counted or disaggregated and re-plated to assess clonality. To examine the differentiation potential, neurospheres were individually isolated and plated on a Matrigel® substrate in DMEM/F12 media containing 1% FCS from Hyclone and incubated for 7 days.

Immunocytochemistry

Samples were fixed with 4% paraformaldehyde/phosphate-buffered saline (PBS),then blocked for 2 hours at with a PBS solution containing 2% normal goat serum (Jackson Immunoresearch Laboratories) and 0.2% Triton-X (Sigma Aldrich). Mouse monoclonal anti-NES (Nestin) (IgG) (1:200, Chemicon), mouse monoclonal anti-O4 (IgM) (1:200, Chemicon), anti-beta III-tubulin (1:500, Sigma-Aldrich), anti-GFAP (1:500, Sigma-Aldrich), anti-Brachyury (ABCAM, ab20680, concentration 1:100) were used as primary antibodies. Secondary goat anti-mouse (1:400, Alexa secondary antibodies, Invitrogen) and donkey anti-rat (1:250, Jackson Immunolabs) antibodies were used for detection. Nuclei were counterstained with Hoechst (Invitrogen) for 10 minutes. Each step was followed by three 5-minute washes with PBS. Images were obtained using a Zeiss Axiovert inverted fluorescence microscope with AxioVision v4.6 imaging software and an AxioCam MRm camera with a monochrome CCD sensor (Carl Zeiss, Jena, Germany).

Morula Aggregation Studies

aSGSCs from reporter mice constitutively expressing the YFP reporter (Jackson: 129-Tg(CAG-EYFP)7AC5Nagy/J Stock Number: 005483) were grown on gelatin plates for 7 days. The zona pellucida was removed from the embryos with Tyrode's solution (Sigma). Individual colonies were then picked and put into wells with E2.5 embryos (CD1 mice) in KSOM media (Millipore) overnight. On the following day aggregation was assessed by examination of fluorescence. ESCs expressing YFP, under the transcriptional regulation of the Actin promoter, were grown and aggregated as a positive control [5].

Standard and Quantitative Polymerase Chain Reaction (qPCR)

Standard PCR was performed according to methods and using primers that have been previously described [29]. For qPCR, total RNA was extracted and purified with the Qiagen RNeasy extraction kit (Hilden, Germany) with inclusion of a 20-minute DNase treatment to remove genomic DNA contamination. RNA was quantified using the Nanodrop Spectrophotometer (ND1-100, Thermo Scientific Wilmington, DE); 6 ng of RNA (per qPCR reaction) was reverse transcribed to cDNA using Superscript III (Invitrogen). Real-time, quantitative PCR was performed on the ABI 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA) using Taqman Gene Expression Assays® for Dazl, Rnh2, Scp 1 and 3, Tex14, Vasa, Oct4, Sox1 and 2, Oct4, and Brachyury. Quantification of the aforementioned genes was achieved by normalizing data to Hprt for the aSGSC samples and Gapdh for the epiblast samples using the ΔΔCt (Ct) method and reported as the fold-change relative to ESCs for the aSGSC studies or Epi-SCs for the FAB-SCs gene expression analyses. The minimum number of experiments was n=3, and all sample/gene combinations were run in triplicate.

Statistical analyses

Statistical comparisons were made using Student's t-test or one-way ANOVA for binary comparisons or multiple groups, as appropriate. Calculations were made using GraphPad Prism (Version 4.0c, GraphPad Software, Inc, La Jolla CA).

Results

Embryoid bodies are irreversibly derived from pNSCs

The formation of EBs is one of the primary *in vitro* assays used to reveal the pluripotency of ESCs. We used this assay to test the relationship of cells from the neural lineage to EBs formed from embryonic stem cells. Single ESCs formed pNSCs when incubated under the conditions of the clonal neurosphere assay [22]. *In vitro* pNSCs are derived from ESCs in differentiation media (DMEM/F12, see methods) with the inclusion of LIF; dNSCs are derived in the presence of bFGF (with heparin), EGF and B27. These pNSCs were capable of producing EBs, which were similar to those formed by aggregates of ESCs, and expressed early mesodermal, ectodermal and endodermal markers, as assessed by RT-PCR (**Figure 2A, B**). Clonally-derived pNSCs also reverted to ESC cells when they were dissociated and returned to standard ESC media (see methods) and plated on MEFs (**Figure 2C**). Dissociation of these EBs subsequently gave rise to clonal dNSC colonies, but not to clonal pNSC colonies (i.e., when cultured with LIF alone) (**Figure 2D**). Furthermore, dNSCs derived from EBs expressed neural markers, i.e., Nestin and Sox1, but not endodermal or mesodermal markers (**Figure 2E**). The dNSCs formed from EBs were capable of differentiating into the neuronal and glial lineages (**Figure 2F-H**). Consistent with the default hypothesis noted above [22,29], these data highlight

the close link between the clonal neural lineage and the inherent differentiation program of ES cells *in vitro*. In summary, ESCs form EBs and reversibly (to ESCs) develop into pNSCs (**Figure 2A, 2C**). EBs irreversibly developed into dNSCs (**Figure 2D**), and pNSCS irreversibly produced EBs and dNSCs (**Figure 2A, 2D, 2I**).

aSGSCs give rise to definitive, but not primitive, clonal neurosphere colonies

To determine whether adult murine aSGSCs could form NSC colonies, similar to ESCs, we isolated adult-derived aSGSCs from mice from four independent genetic backgrounds and at ages ranging from 6 weeks to >1 year (**Figure 3A**). Under both primitive and definitive NSC forming conditions, aSGSCs generated from primary dissections and throughout the multi-week culturing period (i.e., primary dissections through multiple passages on mouse embryonic fibroblasts (MEFs) feeders, with ES media, see methods), were tested for their ability to form ESC, pNSC or dNSC colonies (**Figure 3A**). Colonies with an 'ESC-like' morphology appeared 5-7 days after the primary dissection of the seminiferous tubules (**Figure 3B**). After they first appeared on heavily-seeded gelatin plates (100-200 cells/μl), individual colonies were isolated manually, the cells were disaggregated and re-plated onto new gelatin or MEF feeder 24-well plates for second round gelatin colony isolation. Previous reports detailing the isolation and culture of aSGSCs have indicated that the addition of glial-derived neurotrophic factor (GDNF) to the initial media was requisite for subsequent derivation of aSGSCs [16,30,31]. In our studies, colonies that exhibited an 'ESC-like' appearance developed at comparable numbers in the presence or absence of GDNF; specifically, approximately 13% of plates of the primary dissected cultures produced self-renewing aSGSC colonies from both GDNF and non-GDNF treated cultures, which suggested that exogenous GDNF was not essential for the development of aSGSCs (n=3 mice). Thereafter, aSGSC cultures grown on gelatin (after 14 days) or MEF feeder

cultures (after 7 days) were passaged onto fresh MEF feeders and these cultures were continually passaged every 5-7 days (**Figure 3C**). LIF was then added to (standard) ESC media used to grow aSGSCs. Multiple attempts were made throughout the aSGSC isolation and cultivation to generate primitive and definitive neural stem cells (**Figure 3A)**. Cells from the primary dissections, were also tested in the neurosphere assay, but no primitive or definitive NSCs were ever obtained. At each stage, aSGSC-derived, ESC-like colonies were tested for their ability to form primitive or definitive NSCs (**Figure 3A-C**). Only the latter (p6) MEF cultures were able to generate clonal NSC-derived spheres, and only under the conditions used to generate dNSCs (**Figure 3D)**. Clonal pNSC colonies, derived in LIF, were never obtained (**Figure 3A, 3E**). These aSGSC-derived dNSC spheres demonstrated self-renewal, as evidenced by single sphere and bulk passaging of spheres derived from three independent samples, regardless of age or genetic strain of the source mouse. Furthermore, the aSGSC-derived dNSC colonies were allowed to differentiate on Matrigel® substrate in differentiation media. After 7 days, immunocytochemistry revealed staining specific for beta III tubulin (**Figure 3F**) and GFAP (**Figure 3G**), thus, confirming the neural and glial potential of the aSGSC-derived, clonal, dNSC neurospheres. Quantitative PCR (qPCR) analysis of the aSGSC-derived (definitive) neurospheres indicated that *Brachyury* and *Gata4* expression were not detectable (with average Ct values of over 35 and 32 respectively), whilst expression of *Nestin* was higher and *Sox1* was comparable to that of R1-derived, clonal, dNSC neurospheres (n=3 independent samples, data not shown), further supporting the neural character of these aSGSC-derived (definitive) neurospheres. Thus, we demonstrated that adult murine-derived aSGSCs are capable of making definitive, but not pNSCs.

Since *Wnt1* is a marker of the neural crest (a neural subtype from the embryonic border of the neural plate and ectoderm that migrates throughout the body) lineage [32], we used Wnt1CRe X ZEG-reporter mice to trace the lineage of *Wnt1* expression (i.e., transgenic mice that express the GFP reporter under transcriptional control of the *Wnt1* promoter) [33]. aSGSCs were isolated from four WntCre/ZEG mice; and over the course of culturing (Figure 3A) and neurosphere formation (14 neurospheres examined), as well as differentiation, they were regularly examined for expression of GFP. At no time-point was GFP ever observed, thereby confirming the non-neural crest lineage of aSGSCs.

In vitro aSGSCs exhibit decreased expression of germ cell-specific genes and increased expression of pluripotency genes over time.

To determine whether aSGSCs are equivalent to ESCs at the outset, or whether they become less restricted as they are cultured, we assessed the expression of germ cell- and pluripotency-specific gene profiles using qPCR. As the aSGSCs progressed through culture, under ESC conditions, from the initial testicular dissection through the early gelatin cultures and to the final MEF stage, notable decreases were observed in the expression of the germ cell marker transcripts for Dazl, Rnh2, Scp1, Scp3, Tex14 and Vasa (**Figure 4**), compared with ES cells. Conversely, as aSGSCs moved through the same culture stages, increased expression of the pluripotency markers, *Sox2*, *Oct4*, as well as for the primitive germ layer markers, *Gata4* (endoderm), *Nestin* and *Sox1* (ectoderm), *Brachyury* (mesoderm) and *Fgf5* (epiblast), were observed (**Figure 5**). Interestingly, very low levels of Oct4 mRNA were observed initially in freshly isolated aSGSC cultures (**Figure 5**), which was confirmed using Oct4/GFP reporter mice; i.e., expression of GFP fluorescence was exceedingly low (<1 cell/well) in primary and early gelatin cultures and on rare occasions heterogeneous, small clusters of faint Oct4 positive cells were observed in an early

colony. Moreover, Oct4 reporter expression was clearly more evident at the later MEF stages (**Figure 5, inset**).

aSGSCs can form morula-like aggregates that integrate into the inner cell mass of blastocyst embryos

With the very low levels of Oct4 message RNA assessed by RT-PCR, Q-PCR and Oct4 reporter expression, we were further motivated to assess the pluripotentiality of aSGSCs, by comparing the ability of aSGSCs and murine ESCs to aggregate with the inner cell mass of blastocysts. aSGSCs have previously been reported to contribute to chimera formation [15]. To measure the ability of aSGSCs to integrate into the inner cell mass (ICM), which is key to chimera development, we compared the frequency at which aSGSCs that were harvested 7 days after primary dissection, integrated into the ICM of blastocysts. Both mouse ESCs and aSGSCs derived from transgenic yellow fluorescent protein (YFP) reporter mice were used to determine the capacity for integration into the blastocyst, based upon the presence or absence of YFP. After 24 hours, the integration of ECSs and early aSGSCs morula aggregates was 62.5% and 5.0%, respectively. The observed integration of aSGSCs was significantly lower than that observed previously [15], The differences in aggregation could be attributed to differences in protocols in the two studies (i.e., morula aggregation at E2.5 versus blastocyst injection at E3.5). This finding confirms that aSGSCs are sufficiently pluripotent to contribute to blastocyst chimera formation.

Epi and FAB-SCs only form definitive NSCs

Given that Epi-SCs and FAB-SCs have close ontogenies to both ESCs and aSGSCs, we wanted to compare gene expression and establish whether Epi-SCs and FAB-SCs have the potential to produce NSCs. Given that both Epi-SCs and FAB-SCs were able to arise from the epiblast, we

wanted to identify any differences in lineage or pluripotency gene expression between these two pluripotent stem cell types. We performed qPCR to assess these expression profiles in the FAB-SCs and Epi-SCs (**Figure 6A**). A dramatic difference in *Brachyury* mRNA expression was observed in the FAB-SCs (~ 300 fold greater mRNA expression relative to the Epi-SCs); expression of *Brachyury* protein was confirmed by immunocytochemistry; (**Figure 6A'**). *Nanog*, *Nestin*, and *Oct4* expression profiles were similar between the two populations; although *Nanog* was slightly, but significantly, increased 1.75-fold in the FAB-SCs ($P<0.05$). FAB-SCs expressed Sox1 and Sox2 to a much lower extent; i.e., to approximately one-half and one-quarter of the expression in Epi-SCs, respectively ($P<0.05$). Expression of *Gata4* was also appreciably lower in FAB-SCs compared with Epi-SCs (with a 95% reduction, $P<0.05$). Therefore, FAB-SCs are considered distinct from Epi-SCs in terms of gene expression, particularly with respect to the expression of *Brachyury* (**Figure 6A, A'**). This is consistent with the findings of others who noted higher *Eomes* gene expression, also with qPCR, in FAB-SCs compared with Epi-SCs [20]. Dissociated cells were cultured under the conditions of the clonal neurosphere assay, which give rise to pNSCs or dNSCs, as described above for aSGSCs. Both Epi-SCs and FAB-SCs produced clonal, neural stem cell-derived spheres under definitive culture conditions, (**Figure 3E, 6B, 6D**) but not under primitive conditions, and the numbers of dNSC spheres produced by FAB-SCs were lower than those produced by Epi-SCs ($p<0.01$) (**Figure 3E**). Furthermore, both the FAB-SC- and Epi-SC-derived (definitive) neurospheres were able to give rise to neurons and glial cells under differentiation culturing conditions (**Figure 6 C, E-G**).

Discussion

The present work challenges conventional notions that all pluripotent cells descend from the fundamental pluripotent cell (the ESC) in a serial manner[5], and that they become increasingly restricted in their developmental potential as they differentiate along their respective lineages. We confirmed that adult aSGSCs can be derived from adult mouse testes (results and [15]) and discovered that they become increasingly pluripotent, more ESC-like and less germ cell-like, as they are cultured in ESC conditions in the absence of any direct, genetic manipulation. Adult aSGSCs can also aggregate into the inner cell mass. aSGSCs are easily converted into dNSCs at later stages in culture, but cannot produce primitive, LIF-dependent NSCs at any stage *in vitro*. Similarly, the Epi-SCs and FAB-SCs are only able to form dNSCs, rather than pNSCs. According to the traditional criteria for pluripotent cells (i.e., self-renewing, capable of multi-lineage differentiation, teratoma formation and chimera development) [34-36], as well as the (in)ability to interconvert along the neural line, we have arranged the cells utilized in this study (**Table 1),** and formulated a novel lineage model (**Figure 7**). *In vitro*, pNSCs appear to be the closest to ESCs, as principally evidenced by their efficient interconvertibility and ability to form chimeras. However, the inability of aSGSCs, Epi-SCs, and FAB-SCs pluripotent cell types to produce pNSCs allowed us to rank the these cell types below pNSCs in the lineage (**Table 1)**. The number of clonal dNSC spheres produced by Epi-SCs was appreciably more than by FAB-SCs (**Figure 3E**), which supported the existence of intrinsic differences in the proclivity towards definitive neural differentiation and was consistent with the order established in **Table 1**. This new lineage model (**Figure 7**) highlights non-genetic, cell-based *in vitro* interconvertibility, and differs from the *in vivo*-based serial chronological model (**Figure 1**) established based upon the embryological age of origin during development.

Pluripotency of aSGSCs: Reprogramming in Culture

The first spermatogonial stem cells/aSGSCs were isolated from neonatal mice [16,37,38] and subsequently from adult mice [15]. Adult aSGSCs can differentiate into somatic cell types of all three germ layers, and can be purified and used for successful chimera development shortly after isolation [15]. We were surprised by how little Oct4 mRNA and protein were produced by aSGSCs compared with ESCs. This led us to ask whether aSGSCs were equivalent to ESCs from the outset or whether they gradually lost their aSGSC/germ cell identity, thereby becoming more ESC-like. We addressed this question by quantifying the expression of germ cell, early lineage and pluripotency markers and by assessing the ability of aSGSCs to integrate into blastocyst chimeras after morula aggregation. In general, an inverse relationship was observed; i.e., as germ cell transcripts decreased over culturing passages, many early lineage and several key pluripotency markers, such as Sox2 and Oct4, were increased.

Our *in vitro* system is a reductionist attempt to emulate cellular interconvertibility, at a local level, as a function of changes in growth factor requirements. aSGSCs appear to undergo reprogramming over time in culture, causing them to express lower levels of germ cell markers and increase the expression of pluripotency markers, such as Oct4. Only when germ cell marker expression was lowest was it possible to derive dNSCs from cells grown on a feeder layer. However, it was not possible to derive pNSCs at any stage. Therefore, aSGSCs are ranked at the lineage step after pNSCs but just before FAB-SCs and Epi-SCs, because aSGSCs readily contribute to chimeras (without the application of LIF) (**Table 1**).

An interesting observation was the relatively high level of Nanog observed in the early gelatin cultures (**Figure 5**). This surge in transcription may represent a key turning point, in the transition from the germ to pluripotent state, yet the complexity of posttranslational modifications of Nanog, including the association with the prolyl isomerase (Pin1), suggest that

the effect of Nanog would not be immediate [39]. This is also supported by studies indicating the Nanog is required at late stage for acquisition of true "ground state" pluripotency, rather than being required for the early acquisition of intermediate multipotent stem cell like properties [40]. This further supports our conclusion that the full extent of aSGSC pluripotency is realized only gradually. One question that remains is why are only dNSCs and not pNSCs formed from aSGSCs? Perhaps the non-genetic reprogramming that occurs in culture is insufficient to bring about the full or true ESC state, hence the intrinsic elements that facilitate the swift and reversible transition from the full ESC state to pNSCs are not present. Thus, it may be that the microenvironment conferred by the inner cell mass at E3.5 is exquisitely suited to reprogramming the aSGSCs, as evidenced by the ability to form chimeras [15].

The pGCs give rise *in vivo,* exclusively and irreversibly to aSGSCs [41]. While previous reports have suggested that murine pGCs could express pluripotency markers and exhibited multipotentiality [42,43], the cultures of these earlier efforts were not clonally-derived, nor could they be maintained indefinitely [44]. The ability of pGCs to contribute to chimera formation is also an area of controversy [45,46] (**Table 1**). For these reasons, pGCs do not qualify as veritable pluripotent cells. These unipotent pGCs can undergo reprogramming or dedifferentiate, leading to pluripotent aSGSCs via mechanisms that remain not fully understood [47,48]. Recent lineage tracing experiments using *Blimp1,* an early transcriptional repressor of the somatic program, have provided some insight [49]. *Blimp1* in the inner cell mass of E3.5 embryos marks pGCs, which then go on to form ESCs; however, pGCs isolated at E7.5 failed to form ESCs, suggesting that pGCS go through a critical maturation step affecting their pluripotency. Thereafter, aSGSCs can either self-renew or progress through the traditional pathway of spermatogenesis [50].

The Spectrum of Pluripotency

The current polemic recognizes at least two distinct states of pluripotency, namely the "naïve" and "primed" states [51]. Simply stated, these two states differ at the molecular and epigenetic level, such that the naïve state represents a less complex condition or ground state [40] requiring more events to initiate differentiation. Our data suggests that epiblast-derived stem cells (i.e., Epi-SCs and FAB-SCs) have similar neuro-potential but may otherwise be primed differently based on the expression of early lineage gene markers, such as Gata4. Further, aSGSCs could represent a dedifferentiated intermediate that has not sufficiently transitioned to the ground state of pluripotency [40] and are rather in a poised state. Our body of work supports the concept that pluripotency represents a spectrum, with naïve versus primed at either extreme. This new paradigm also supports the idea that the transition of an Epi-SC from an ESC [20,52], may involve a 'FAB-SC-state' intermediary. It has been shown that there are innate differences in EpiSC lines with respect to their ability to revert to the ESC state or to undergo neural induction as related to their expression of mesoendodermal marker genes, such as Brachyury [53]. In our study, the probability of differentiation along the neural lineage with the Epi-SCs, but more so with the FAB-SCs (also derived from the epiblast), may be inhibited by induction of mesoderm genes. This is supported by the significantly elevated level of Brachyury mRNA observed with our qPCR experiments (**Figure 5A**) and the comparatively low numbers of definitive neurospheres that developed from the FAB-SC lines (**Figure 3E**). Conversely, it would appear that our Epi-SC lines are more apt to reversion or neural differentiation, given their low expression of Brachyury transcripts, (3 lines; average C_t >34, data not shown) as well as their relatively robust expression of Sox2 (3 lines; average C_t =25.5, data not shown) [21]. Based on their collective inability to make pNSCS, aSGSCs, Epi-SCs and FAB-SCs and their qPCR,

gene expression profiles represent intermediary pluripotent progenitors, and therefore are more restricted than ESCs.

pNSCs likely represent a more naïve state of pluripotency, given the ease with which they undergo interconversion between themselves and the ESC state *in vitro*[23,29]. The default hypothesis states that when an ESC is exposed to minimal external influences, it will swiftly transition into a (primitive) neural stem cell [29] implying that there is an underlying, intrinsic, mechanism in ESCs that directs its innate differentiation program towards the neural lineage. The pNSC is a self-renewing cell type that is capable of forming all of the neural cell types [22], yet can easily revert to ESCs when cultured under appropriate conditions (**Figure 2C**). *In vitro,* pNSCs that are derived from ESCs retain sufficient pluripotency to form EBs, and EBs can in turn form dNSCs, but not pNSCs. pNSCs can also form dNSCs; although this is not reversible [29]. Finally, the fact that pNSCs (but not dNSCs) aggregate in or adjacent to the ICM [54] also supports a more naïve potency state of pNSCs compared with other pluripotent cell types. Given that aSGSCs at any stage, along with Epi-SCs and FAB-SCs were not able to produce pNSCs, we conclude that Epi-SCs and FAB-SCs lie further towards the primed state on the pluripotency spectrum, which is consistent with previous findings from another group [55]. This holds true when one considers the ability of these cell types to contribute to chimeras, since both pNSCs [22,54] and aSGSCs [15] are able to integrate into the inner cell mass of the developing embryo, as is also the case for FAB-SCs in the presence of LIF [20] and Epi-SCs [52]. Therefore, in terms of the hierarchy of pluripotency, in which cells are ranked according to how many of the pluripotency criteria are met and how they fit into the neural lineage, the FAB-SCs and Epi-SCs would be ranked less pluripotent than aSGSCs (**Table 1, Figure 7**).

Model

With the demonstration of increasing numbers of both embryo and adult derived pluripotent cells (e.g. aSGSCs, Epi-SCs, FAB-SCs), the previous method of organizing them from ESCs in a linear model (based on earliest time of isolation from mice) only reflects their appearance during embryogenesis and is not representative of the true degree of their pluripotency. While the traditional model (**Figure 1**) is useful to understand the developmental ontogeny of these cells, the hallmark of a stem cell, namely the degree and scope of its pluripotency and/or interconvertibility, is not addressed with such a model. The inclusion of the neural lineage reflects the principle that the neural program of differentiation is the default lineage arising from the ESC [22], which is further supported by studies of induced pluripotent cells (IPSC)s where the least amount of genetic intervention is required to form an IPSC from a neural cell [56,57]. The positioning of cell lineages based on the lineage of neural differentiation also takes advantage of the finding that pNSCs and dNSCs are clonally-derived from the initial populations, thus avoiding the interpretive problems that can occur with the polyclonal derivation of lineages.

In summary, our findings suggest the application of a three-stream model that allows the representation of achievable transitions between cell types (**Figure 7**). In terms of pluripotency, adult-derived aSGSCs are slightly more restricted than pNSCs because: (1) they do not transition to ESCs with the same rapidity as pNSCs (days versus weeks), (2) they only form dNSCs, (3) in the ESC-like state, their expression of pluripotency markers is still lower than that of ESCs (**Figure 5**) and pNSCs [24]. Likewise, Epi and FAB-SCs are considered less pluripotent than pNSCs because: (1) they do not easily transition into ESCs (results and [20,52]), (2) they only form dNSCs (**Figure 3E, 6B, 6D)**, (3) their expression of pluripotency genes and lineage

markers is markedly different from ESCs [20,52], (4) the ease with which formation of chimeras is possible [58] and (5) the FAB-SCs have yet to be defined *in vivo*. However, in the new model (**Figure 7)** FAB-SCs are placed closer to ESCs than the Epi-SCs with respect to pluripotency because the possibility exists that when Epi-SCs transition to ESCs [20,52], they may go through a FAB-SC-like state. Finally, it may be no longer sufficient to simply use a one-dimensional, ontological (ancestral) approach, given the potential for interconversion among these stem cell populations. It is necessary to pursue a multifactorial approach that seeks to define various pluripotent cells and the relationships among them.

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Author Disclosure Statement

The authors declare that they have no conflicts of interest.

REFERENCES

1. Scholer HR, R Balling, AK Hatzopoulos, N Suzuki and P Gruss. (1989). Octamer binding proteins confer transcriptional activity in early mouse embryogenesis. EMBO J 8:2551-7.

2. Scholer HR, AK Hatzopoulos, R Balling, N Suzuki and P Gruss. (1989). A family of octamer-specific proteins present during mouse embryogenesis: evidence for germline-specific expression of an Oct factor. EMBO J 8:2543-50.

3. Kuci S, Z Kuci, H Latifi-Pupovci, D Niethammer, R Handgretinger, M Schumm, G Bruchelt, P Bader and T Klingebiel. (2009). Adult stem cells as an alternative source of multipotential (pluripotential) cells in regenerative medicine. Curr Stem Cell Res Ther 4:107-17.

4. McCulloch EA and JE Till. (2005). Perspectives on the properties of stem cells. Nat Med 11:1026-8.

5. Hogan B, F Costantini and E Lacy. *Manipulating the mouse embryo : a laboratory manual*. (1986). Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

6. McLaren A and KA Lawson. (2005). How is the mouse germ-cell lineage established? Differentiation 73:435-7.

7. Gomperts M, M Garcia-Castro, C Wylie and J Heasman. (1994). Interactions between primordial germ cells play a role in their migration in mouse embryos. Development 120:135-41.

8. Ginsburg M, MH Snow and A McLaren. (1990). Primordial germ cells in the mouse embryo during gastrulation. Development 110:521-8.

9. Ohta H, T Wakayama and Y Nishimune. (2004). Commitment of fetal male germ cells to spermatogonial stem cells during mouse embryonic development. Biol Reprod 70:1286-91.

10. Oulad-Abdelghani M, P Bouillet, D Decimo, A Gansmuller, S Heyberger, P Dolle, S Bronner, Y Lutz and P Chambon. (1996). Characterization of a premeiotic germ cell-specific cytoplasmic protein encoded by Stra8, a novel retinoic acid-responsive gene. J Cell Biol 135:469-77.

11. Chuma S, M Kanatsu-Shinohara, K Inoue, N Ogonuki, H Miki, S Toyokuni, M Hosokawa, N Nakatsuji, A Ogura and T Shinohara. (2005). Spermatogenesis from epiblast and primordial germ cells following transplantation into postnatal mouse testis. Development 132:117-22.

12. de Rooij DG and LD Russell. (2000). All you wanted to know about spermatogonia but were afraid to ask. J Androl 21:776-98.

13. Zovoilis A, J Nolte, N Drusenheimer, U Zechner, H Hada, K Guan, G Hasenfuss, K Nayernia and W Engel. (2008). Multipotent adult germline stem cells and embryonic stem cells have similar microRNA profiles. Mol Hum Reprod 14:521-9.

14. Guan K, S Wagner, B Unsold, LS Maier, D Kaiser, B Hemmerlein, K Nayernia, W Engel and G Hasenfuss. (2007). Generation of functional cardiomyocytes from adult mouse spermatogonial stem cells. Circ Res 100:1615-25.

15. Guan K, K Nayernia, LS Maier, S Wagner, R Dressel, JH Lee, J Nolte, F Wolf, M Li, W Engel and G Hasenfuss. (2006). Pluripotency of spermatogonial stem cells from adult mouse testis. Nature 440:1199-203.

16. Kanatsu-Shinohara M, N Ogonuki, K Inoue, H Miki, A Ogura, S Toyokuni and T Shinohara. (2003). Long-term proliferation in culture and germline transmission of mouse male germline stem cells. Biol Reprod 69:612-6.

17. Martin GR. (1980). Teratocarcinomas and mammalian embryogenesis. Science 209:768-76.

18. Brons IG, LE Smithers, MW Trotter, P Rugg-Gunn, B Sun, SM Chuva de Sousa Lopes, SK Howlett, A Clarkson, L Ahrlund-Richter, RA Pedersen and L Vallier. (2007). Derivation of pluripotent epiblast stem cells from mammalian embryos. Nature 448:191-5.

19. Tesar PJ, JG Chenoweth, FA Brook, TJ Davies, EP Evans, DL Mack, RL Gardner and RD McKay. (2007). New cell lines from mouse epiblast share defining features with human embryonic stem cells. Nature 448:196-9.

20. Chou YF, HH Chen, M Eijpe, A Yabuuchi, JG Chenoweth, P Tesar, J Lu, RD McKay and N Geijsen. (2008). The growth factor environment defines distinct pluripotent ground states in novel blastocyst-derived stem cells. Cell 135:449-61.

21. Iwafuchi-Doi M, K Matsuda, K Murakami, H Niwa, PJ Tesar, J Aruga, I Matsuo and H Kondoh. (2012). Transcriptional regulatory networks in epiblast cells and during anterior neural plate development as modeled in epiblast stem cells. Development 139:3926-37.

22. Tropepe V, S Hitoshi, C Sirard, TW Mak, J Rossant and D van der Kooy. (2001). Direct neural fate specification from embryonic stem cells: a primitive mammalian neural stem cell stage acquired through a default mechanism. Neuron 30:65-78.

23. Coles-Takabe BL, I Brain, KA Purpura, P Karpowicz, PW Zandstra, CM Morshead and D van der Kooy. (2008). Don'T look: growing clonal versus nonclonal neural stem cell colonies. Stem Cells 26:2938-44.

24. Akamatsu W, B DeVeale, H Okano, AJ Cooney and D van der Kooy. (2009). Suppression of Oct4 by germ cell nuclear factor restricts pluripotency and promotes neural stem cell development in the early neural lineage. J Neurosci 29:2113-24.

25. Hitoshi S, RM Seaberg, C Koscik, T Alexson, S Kusunoki, I Kanazawa, S Tsuji and D van der Kooy. (2004). Primitive neural stem cells from the mammalian epiblast differentiate to definitive neural stem cells under the control of Notch signaling. Genes Dev 18:1806-11.

26. Choi YY, BG Chung, DH Lee, A Khademhosseini, JH Kim and SH Lee. (2010). Controlled-size embryoid body formation in concave microwell arrays. Biomaterials 31:4296-303.

27. Martin GR and MJ Evans. (1975). Differentiation of clonal lines of teratocarcinoma cells: formation of embryoid bodies in vitro. Proc Natl Acad Sci U S A 72:1441-5.

28. Dang SM, M Kyba, R Perlingeiro, GQ Daley and PW Zandstra. (2002). Efficiency of embryoid body formation and hematopoietic development from embryonic stem cells in different culture systems. Biotechnol Bioeng 78:442-53.

29. Smukler SR, SB Runciman, S Xu and D van der Kooy. (2006). Embryonic stem cells assume a primitive neural stem cell fate in the absence of extrinsic influences. J Cell Biol 172:79-90.

30. Kanatsu-Shinohara M, K Inoue, N Ogonuki, H Morimoto, A Ogura and T Shinohara. (2011). Serum- and feeder-free culture of mouse germline stem cells. Biol Reprod 84:97-105.

31. Sariola H and T Immonen. (2008). GDNF maintains mouse spermatogonial stem cells in vivo and in vitro. Methods Mol Biol 450:127-35.

32. Dunn KJ, BO Williams, Y Li and WJ Pavan. (2000). Neural crest-directed gene transfer demonstrates Wnt1 role in melanocyte expansion and differentiation during mouse development. Proc Natl Acad Sci U S A 97:10050-5.

33. Jinno H, O Morozova, KL Jones, JA Biernaskie, M Paris, R Hosokawa, MA Rudnicki, Y Chai, F Rossi, MA Marra and FD Miller. (2010). Convergent genesis of an adult neural crest-like dermal stem cell from distinct developmental origins. Stem Cells 28:2027-40.

34. Pera MF and AO Trounson. (2004). Human embryonic stem cells: prospects for development. Development 131:5515-25.

35. Guasch G and E Fuchs. (2005). Mice in the world of stem cell biology. Nat Genet 37:1201-6.

36. Thomson JA, J Itskovitz-Eldor, SS Shapiro, MA Waknitz, JJ Swiergiel, VS Marshall and JM Jones. (1998). Embryonic stem cell lines derived from human blastocysts. Science 282:1145-7.

37. Kanatsu-Shinohara M, N Ogonuki, K Inoue, A Ogura, S Toyokuni and T Shinohara. (2003). Restoration of fertility in infertile mice by transplantation of cryopreserved male germline stem cells. Hum Reprod 18:2660-7.

38. Kanatsu-Shinohara M, S Toyokuni, T Morimoto, S Matsui, T Honjo and T Shinohara. (2003). Functional assessment of self-renewal activity of male germline stem cells following cytotoxic damage and serial transplantation. Biol Reprod 68:1801-7.

39. Moretto-Zita M, H Jin, Z Shen, T Zhao, SP Briggs and Y Xu. (2010). Phosphorylation stabilizes Nanog by promoting its interaction with Pin1. Proc Natl Acad Sci U S A 107:13312-7.

40. Silva J, J Nichols, TW Theunissen, G Guo, AL van Oosten, O Barrandon, J Wray, S Yamanaka, I Chambers and A Smith. (2009). Nanog is the gateway to the pluripotent ground state. Cell 138:722-37.

41. Sato T, K Katagiri, A Gohbara, K Inoue, N Ogonuki, A Ogura, Y Kubota and T Ogawa. (2011). In vitro production of functional sperm in cultured neonatal mouse testes. Nature 471:504-7.

42. Shamblott MJ, J Axelman, S Wang, EM Bugg, JW Littlefield, PJ Donovan, PD Blumenthal, GR Huggins and JD Gearhart. (1998). Derivation of pluripotent stem cells from cultured human primordial germ cells. Proc Natl Acad Sci U S A 95:13726-31.

43. Turnpenny L, S Brickwood, CM Spalluto, K Piper, IT Cameron, DI Wilson and NA Hanley. (2003). Derivation of human embryonic germ cells: an alternative source of pluripotent stem cells. Stem Cells 21:598-609.

44. Turnpenny L, CM Spalluto, RM Perrett, M O'Shea, KP Hanley, IT Cameron, DI Wilson and NA Hanley. (2006). Evaluating human embryonic germ cells: concord and conflict as pluripotent stem cells. Stem Cells 24:212-20.

45. Durcova-Hills G, IR Adams, SC Barton, MA Surani and A McLaren. (2006). The role of exogenous fibroblast growth factor-2 on the reprogramming of primordial germ cells into pluripotent stem cells. Stem Cells 24:1441-9.

46. Matsui Y, K Zsebo and BL Hogan. (1992). Derivation of pluripotential embryonic stem cells from murine primordial germ cells in culture. Cell 70:841-7.

47. Leitch HG, K Blair, W Mansfield, H Ayetey, P Humphreys, J Nichols, MA Surani and A Smith. (2010). Embryonic germ cells from mice and rats exhibit properties consistent with a generic pluripotent ground state. Development 137:2279-87.

48. Hayashi K and MA Surani. (2009). Self-renewing epiblast stem cells exhibit continual delineation of germ cells with epigenetic reprogramming in vitro. Development 136:3549-56.

49. Chu LF, MA Surani, R Jaenisch and TP Zwaka. (2011). Blimp1 expression predicts embryonic stem cell development in vitro. Curr Biol 21:1759-65.

50. Toshimori K. (2009). Dynamics of the mammalian sperm head: modifications and maturation events from spermatogenesis to egg activation. Adv Anat Embryol Cell Biol 204:5-94.

51. Nichols J and A Smith. (2009). Naive and primed pluripotent states. Cell Stem Cell 4:487-92.

52. Bao S, F Tang, X Li, K Hayashi, A Gillich, K Lao and MA Surani. (2009). Epigenetic reversion of post-implantation epiblast to pluripotent embryonic stem cells. Nature 461:1292-5.

53. Bernemann C, B Greber, K Ko, J Sterneckert, DW Han, MJ Arauzo-Bravo and HR Scholer. (2011). Distinct developmental ground states of epiblast stem cell lines determine different pluripotency features. Stem Cells 29:1496-503.

54. Karpowicz P, T Inoue, S Runciman, B Deveale, R Seaberg, M Gertsenstein, L Byers, Y Yamanaka, S Tondat, J Slevin, S Hitoshi, J Rossant and D van der Kooy. (2007). Adhesion is prerequisite, but alone insufficient, to elicit stem cell pluripotency. J Neurosci 27:5437-47.

55. Buecker C and N Geijsen. (2010). Different flavors of pluripotency, molecular mechanisms, and practical implications. Cell Stem Cell 7:559-64.

56. Kim JB, H Zaehres, G Wu, L Gentile, K Ko, V Sebastiano, MJ Arauzo-Bravo, D Ruau, DW Han, M Zenke and HR Scholer. (2008). Pluripotent stem cells induced from adult neural stem cells by reprogramming with two factors. Nature 454:646-50.

57. Han DW, N Tapia, A Hermann, K Hemmer, S Hoing, MJ Arauzo-Bravo, H Zaehres, G Wu, S Frank, S Moritz, B Greber, JH Yang, HT Lee, JC Schwamborn, A Storch and HR Scholer. (2012). Direct reprogramming of fibroblasts into neural stem cells by defined factors. Cell Stem Cell 10:465-72.

58. Han DW, N Tapia, JY Joo, B Greber, MJ Arauzo-Bravo, C Bernemann, K Ko, G Wu, M Stehling, JT Do and HR Scholer. (2010). Epiblast stem cell subpopulations represent mouse embryos of distinct pregastrulation stages. Cell 143:617-27.

FIGURE LEGENDS

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Figure 1

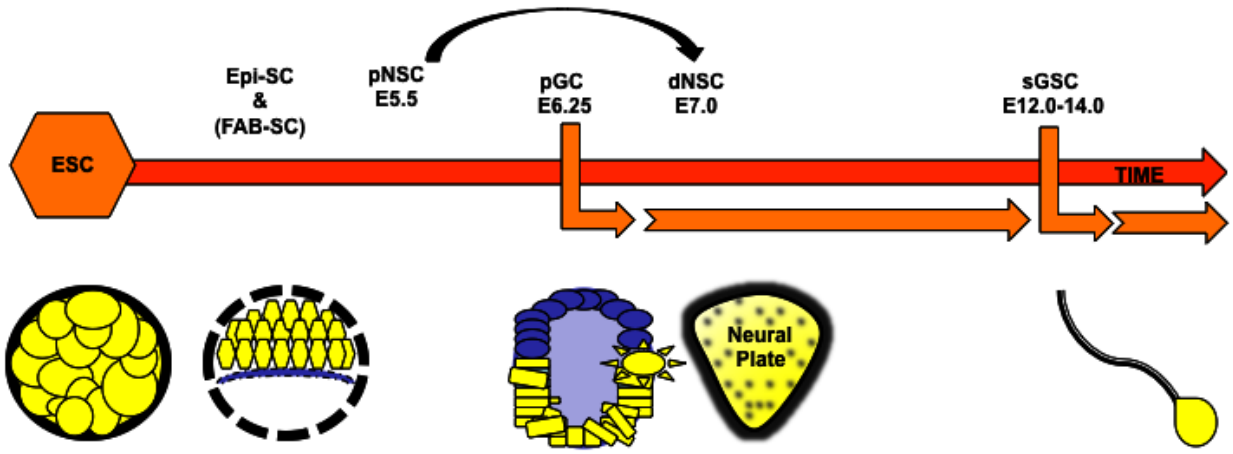

Figure 1

Conventional linear timeline of the origin of key pluripotent cell types arising from ESCs of the inner cell mass based on their earliest time of isolation from mouse embryos. The model implies that with lineage progression, cells become increasingly restricted in their potency. FAB-SCs are bracketed because an *in vivo* correlate is to date undefined.

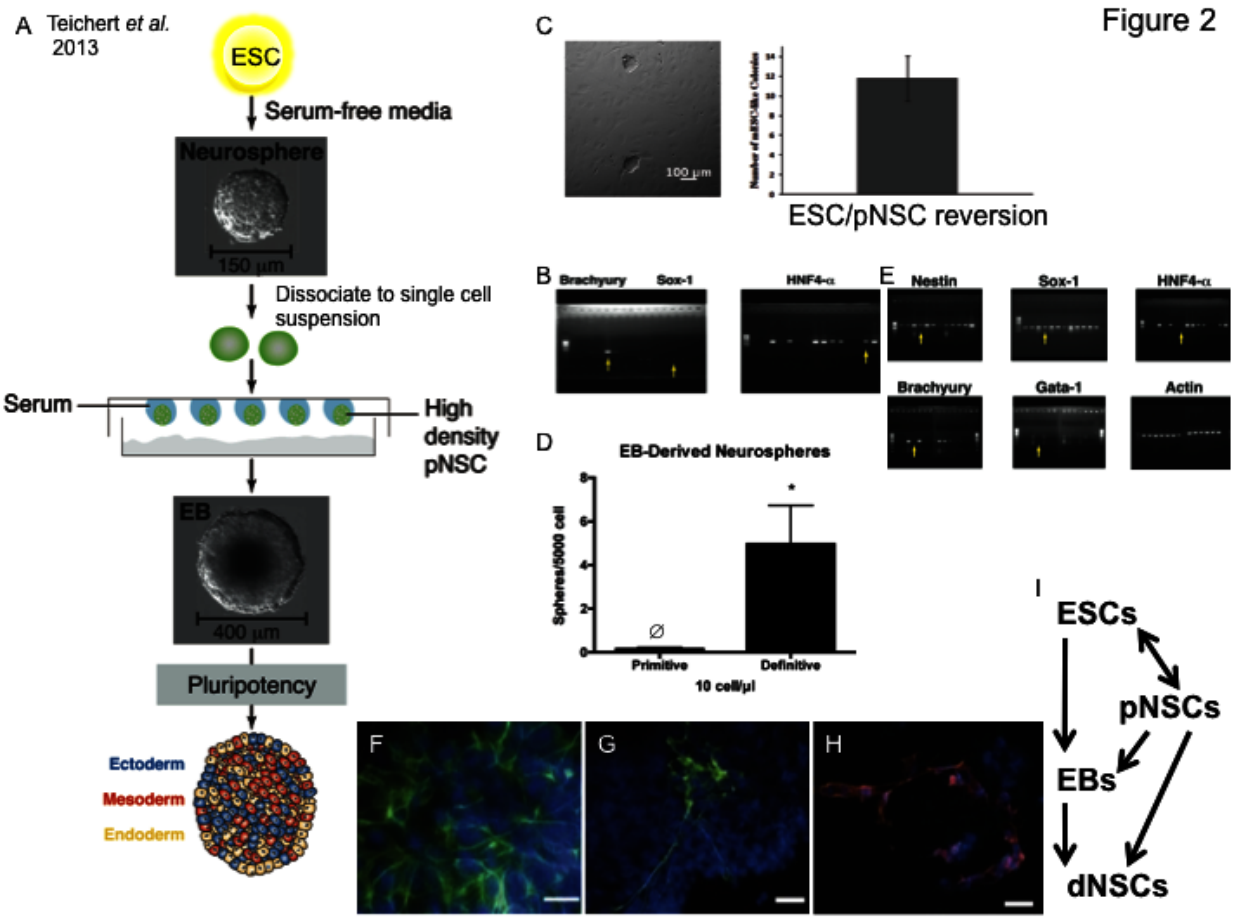

Figure 2.

Multiple interconversions are possible amongst ESCs, EBs and pNSCs. Schema illustrating the course of pNSC and EB formation; pNSCs were derived in serum-free media, dissociated and then plated under hanging drop conditions for EB formation (A). pNSC-derived EBs express mesodermal, ectodermal and endodermal markers (B). ESC reverted colonies were derived from pNSCs and quantified. Both shown are from a clonal single sphere passage of 7-day pNSCs (derived from R1 p18 mESC) under mouse ESC conditions (C). Only dNSCs can be derived from EBs (Ø) denotes zero, N=3 (D). EB-derived dNSCs express neural, but not endodermal or mesodermal markers (arrows) (E). Upon differentiation dNSCs derived from EBs form neurons and glial cells, and positive immunostaining was observed for nestin (F), β III tubulin (G), and Olig4 (H). Schema summarizing possible interconvertibilities of ESCs and EBs and the neural lineage (I). * $P<0.05$ to primitive neurospheres. N=3 Scale Bars:10 μm in F; 20 μm in G and H

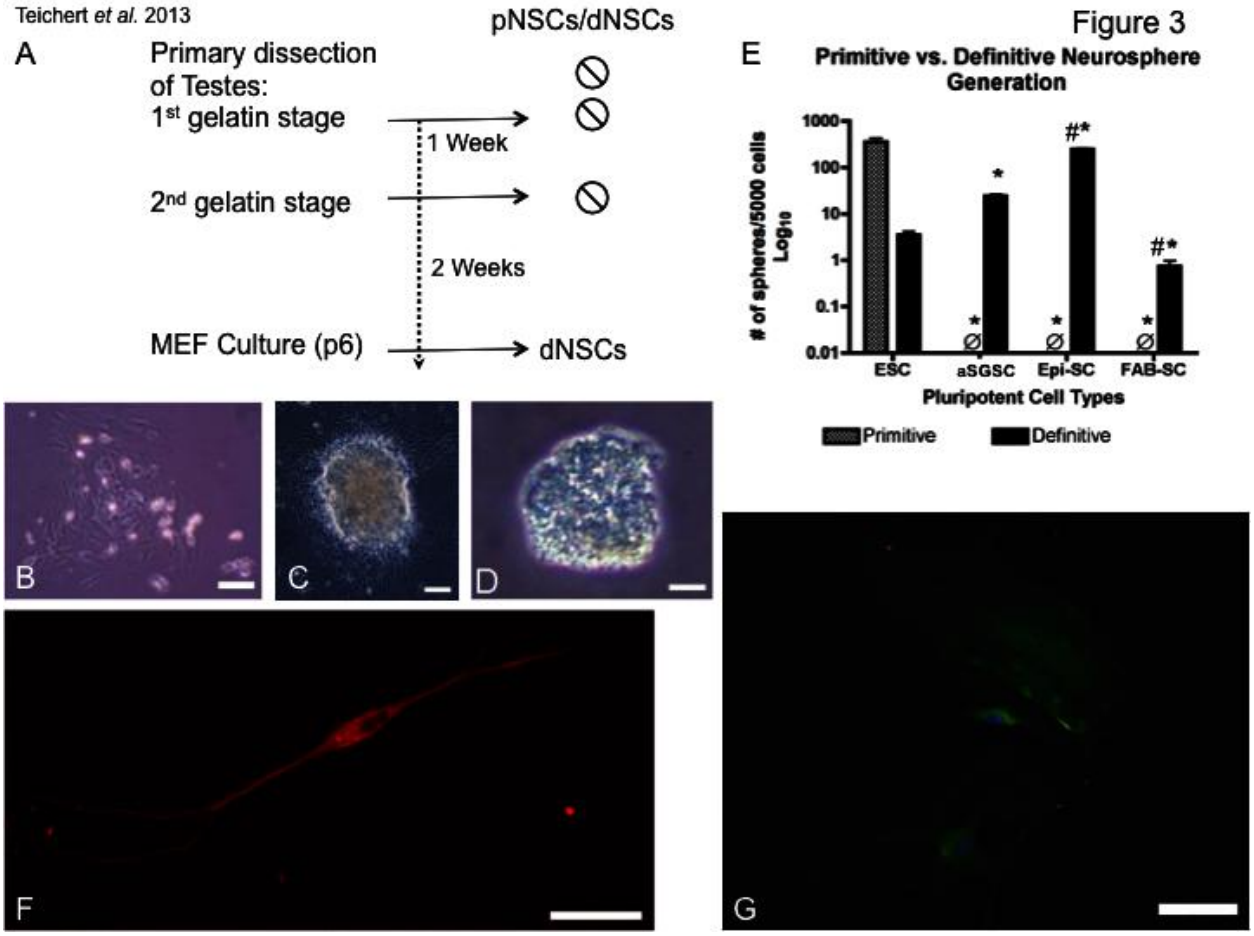

Figure 3.

Adult-derived aSGSCs regain pluripotency and neural potential in culture. Schema illustrating the course of aSGSC derivation, culturing and efforts to derive primitive and definitive neural stem cells (A). aSGSCs give rise to ESC-like colonies 5 days after primary dissection (B). An aSGSC-derived, ESC-like colony growing on MEF feeders at p4 (C). An aSGSC-derived definitive neurosphere (D). Quantification of primitive and definitive neurospheres produced by ESCs, aSGSCs, Epi-SCs, and FAB-SCs (E). Upon differentiation, aSGSC-derived dNSCs form neurons and glia. Positive immunostaining is shown for βIII tubulin (F), for GFAP (G) and DAPI: blue *$P<0.05$ to primitive neurospheres #$P<0.0001$ to FAB-derived dNSCs. N =10. Scale bars: 50 μm in B-D and F, 100 μm in G.

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Figure 4

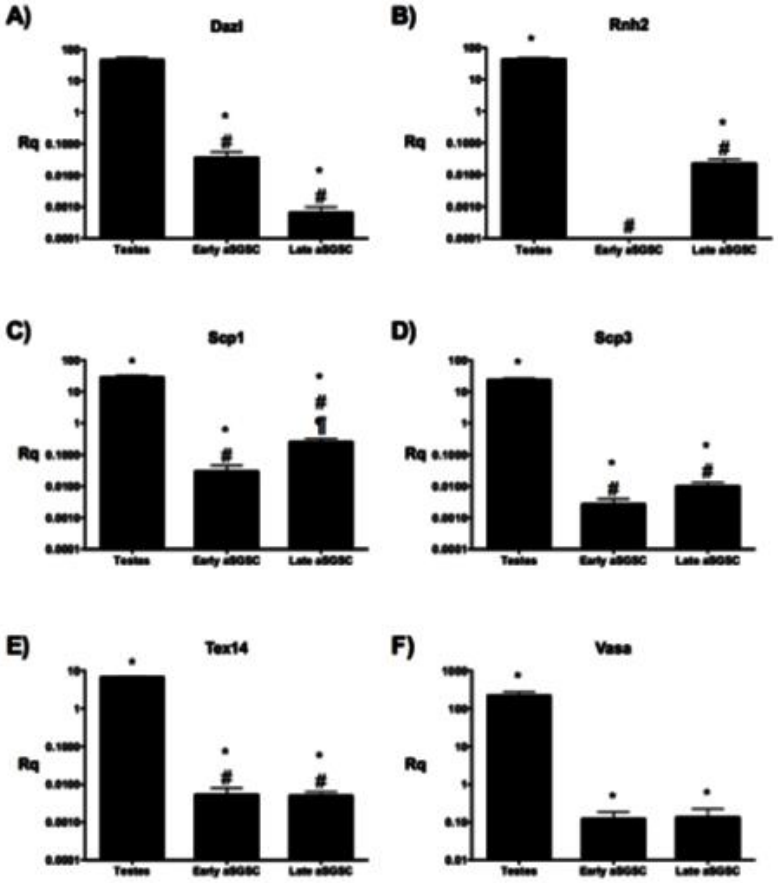

Figure 4.

As aSGSCs progress in culture, expression of germ cell markers decrease: Relative quantification of the expression of germ cell markers using qPCR shows decreased expression of germ cell markers: Dazl (A). Rnh2 (B), Scp1 (C), Scp3 (D), Tex14 (E), Vasa (F), over the cell culture progression; *P<0.05 to ESC cells.

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Figure 5

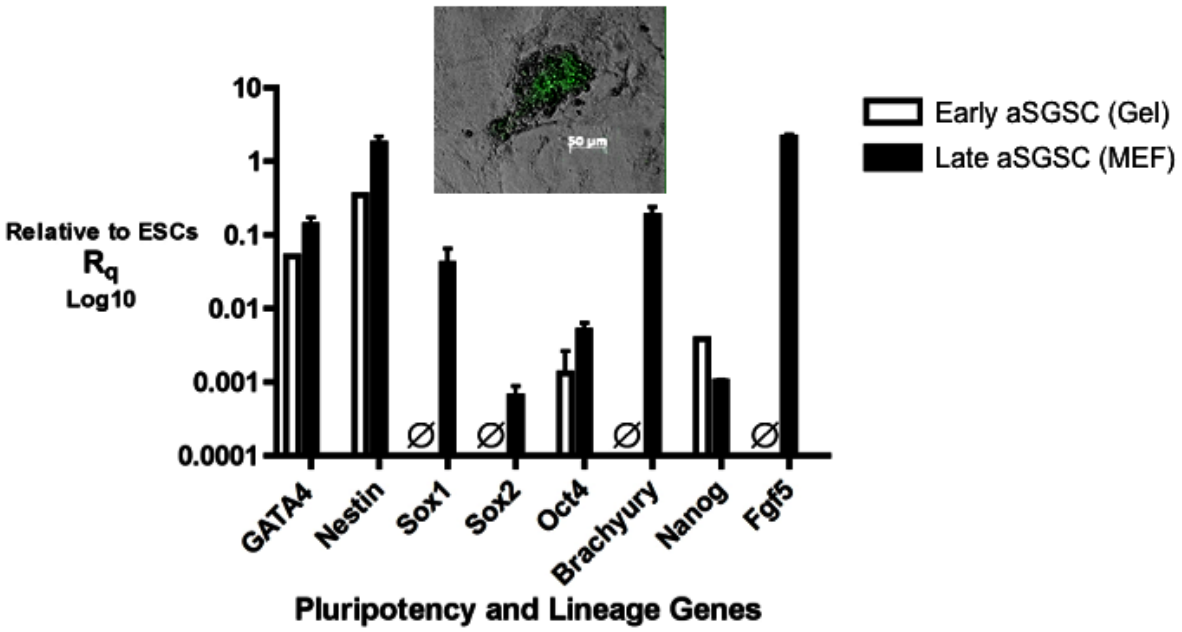

Figure 5.

Early gelatin cultures express very low levels of Oct4 mRNA relative to ESCs. Sox2 and Oct4 transcript levels increased as aSGSCs matured in culture. aSGSC colonies on MEF feeders derived from Oct4/GFP reporter mice show heterogonous expression of GFP fluorescence (inset) N=3.

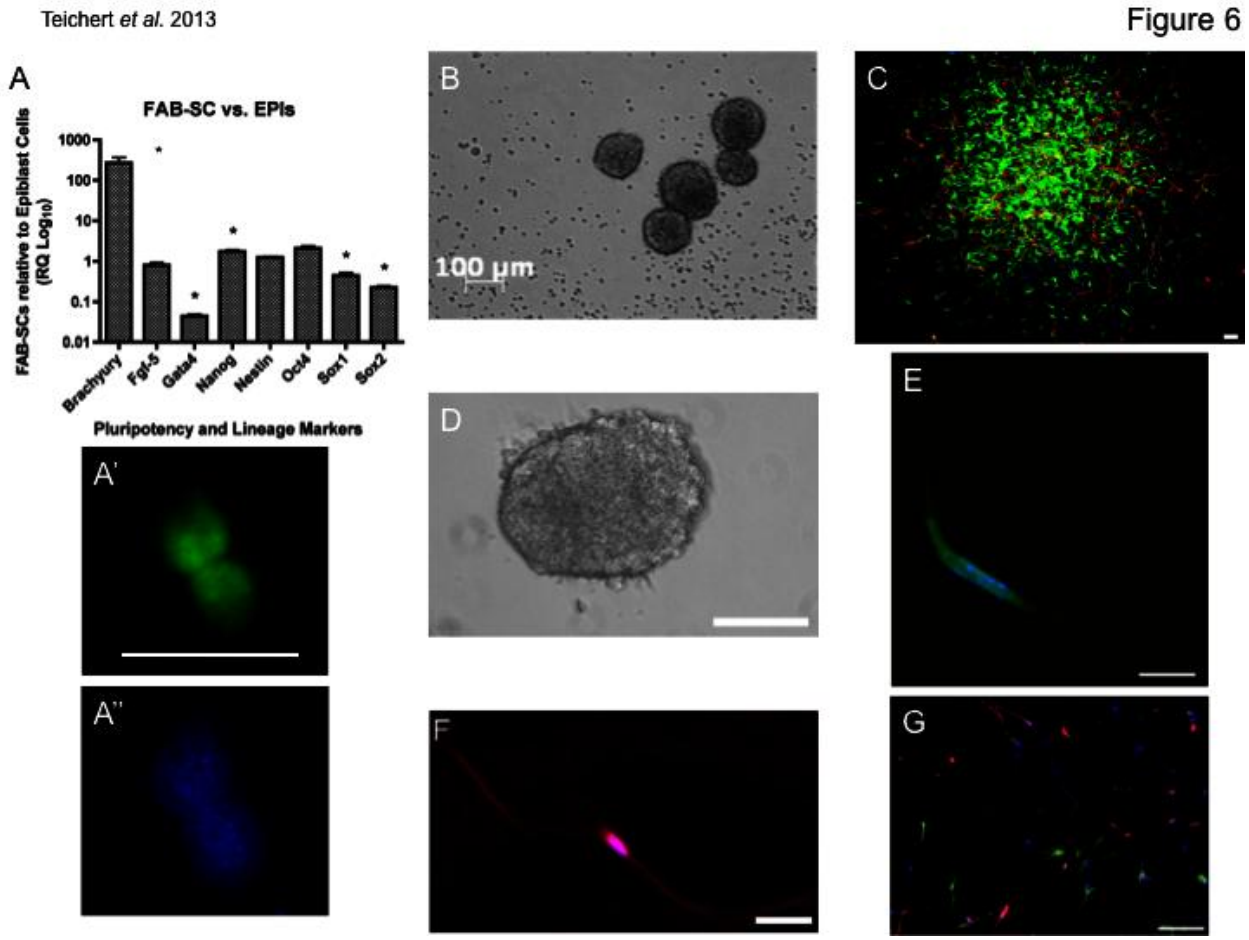

Figure 6.

FAB-SCs express greater levels of Brachyury and significantly lower levels of GATA4 than Epi-SCs. qPCR quantification of pluripotency and early lineage markers reveals higher expression levels of Brachyury and significantly lower GATA4 expression in FAB-SCs relative to Epi-SCs (* $P<0.05$ to epiblast cells; A N=3), and by immunohistochemistry of Brachyury in FAB-SCs (A'). Nuclei are stained with DAPI (blue) (A''). Scale Bars: 25 μm in A'/A''.

Epi- and FAB-SCs only produce clonal, dNSCs. Definitive Epi-SC-derived NSC spheres, 7 days post sphere-forming assay (B). Upon differentiation, astrocytes (green, GFAP) and neurons (red, βIII tubulin) develop (C), in a clonal, definitive, FAB-SC-derived NSC sphere (D), which produces astrocytes (green, GFAP) (E), and neurons (red, βIII tubulin) (F), higher magnification of D and E. (G), Nuclei in blue (DAPI). Scale Bars:100 μm in D, and 50 μm in F and G.

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Figure 7

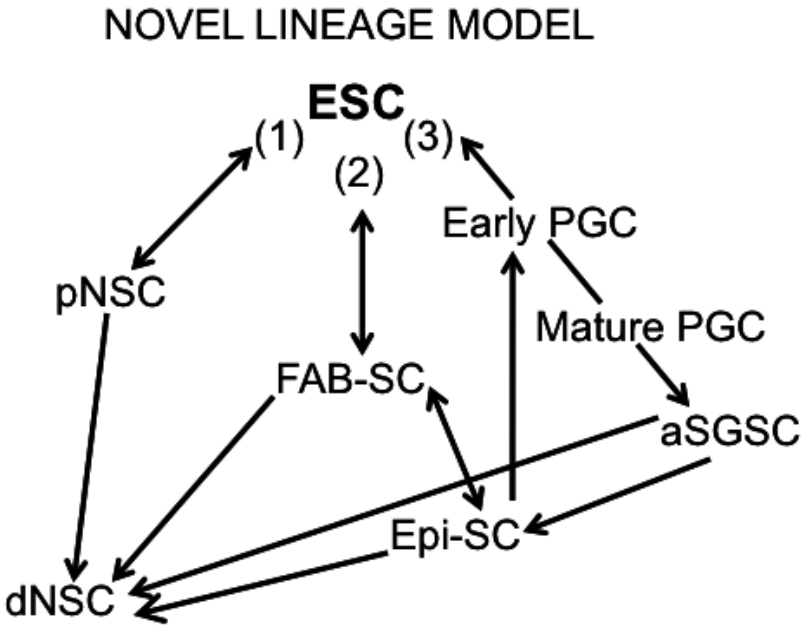

Figure 7.

Novel lineage model addressing pluripotency and interconvertibility between cell types. (1) ESCs give rise to pNSCs, reversibly, and pNSCs give rise to dNSCs, irreversibly. (2) ESCs are derived from the ICM, which gives rise to the epiblast, and hence the Epi-SC in a reversible manner. FAB-SCs may represent an intermediary PC, and both Epi-SC and FAB-SCs can give rise to dNSCs but not pNSCs. (3) aSGSCs are derived from ESCs (through a unipotent intermediate, the PGC) and can go back to an ESC state or differentiate into (adult) aSGSCs in males. Adult-derived cultured aSGSCs can only form dNSCs, but can go back to an ESC-like state and therefore lie slightly below the level of pNSCs.

Pluripotent Cell Types	Unlimited self-renewal	EB formation (Ability to differentiate into cell types of all 3 germinal layers)	Ability to form teratomas of all 3 germinal layers	Chimera formation	Ability to differentiate into primitive NSCs	Ability to diferentiate into definitive NSCs
ESC	No (*in vivo*) Yes (*in vitro*)	Yes	Yes	Yes	Yes	No
pNSC	No (*in vivo*) No (*in vitro*)	Yes[A]	Yes	Yes[A]	No	Yes
aSGSC	Yes (*in vivo*) Yes (*in vitro*)	Yes	Yes	Yes	No	Yes
FAB-SC	Yes (*in vitro*) No (*in vivo*)	Yes	No	Yes[B]	No	Yes
EpiSC	No (*in vivo*) Yes (*in vitro*)	Yes	Yes	No[C]	No	Yes
dNSC	Yes (*in vivo*) Yes (*in vitro*)	No	No	Yes	No	No

[A] *In vitro*-derived
[B] With transient application of LIF
[C] >0.2% chimera formation

Table 1.

Hierarchy of pluripotency. Cells are ranked according to how many of the hallmarks of pluripotency they attain and their (in)ability to transition into p(primitive) neural stem cells (NSCs) or d(definitive) NSCs. Abbreviations: embryonic stem cell (ESC), adult spermatogonial germ stem cells (aSGSCs), epiblast-derived stem cell (Epi-SCs), bFGF, Activin and Bio-derived stem cell (FAB-SC).

The neural stem cell lineage reveals novel relationships among spermatogonial germ stem cells and other pluripotent stem cells (doi: 10.1089/scd.2013.0245)