

Direct Reprogramming of Fibroblasts into Embryonic Sertoli-like Cells by Defined Factors

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<http://dx.doi.org/10.1016/j.stem.2012.07.019>

SUMMARY

Sertoli cells are considered the “supporting cells” of the testis that play an essential role in sex determination during embryogenesis and in spermatogenesis during adulthood. Their essential roles in male fertility along with their immunosuppressive and neurotrophic properties make them an attractive cell type for therapeutic applications. Here we demonstrate the generation of induced embryonic Sertoli-like cells (ieSCs) by ectopic expression of five transcription factors. We characterize the role of specific transcription factor combinations in the transition from fibroblasts to ieSCs and identify key steps in the process. Initially, transduced fibroblasts underwent a mesenchymal to epithelial transition and then acquired the ability to aggregate, formed tubular-like structures, and expressed embryonic Sertoli-specific markers. These Sertoli-like cells facilitated neuronal differentiation and self-renewal of neural progenitor cells (NPCs), supported the survival of germ cells in culture, and cooperated with endogenous embryonic Sertoli and primordial germ cells in the generation of testicular cords in the fetal gonad.

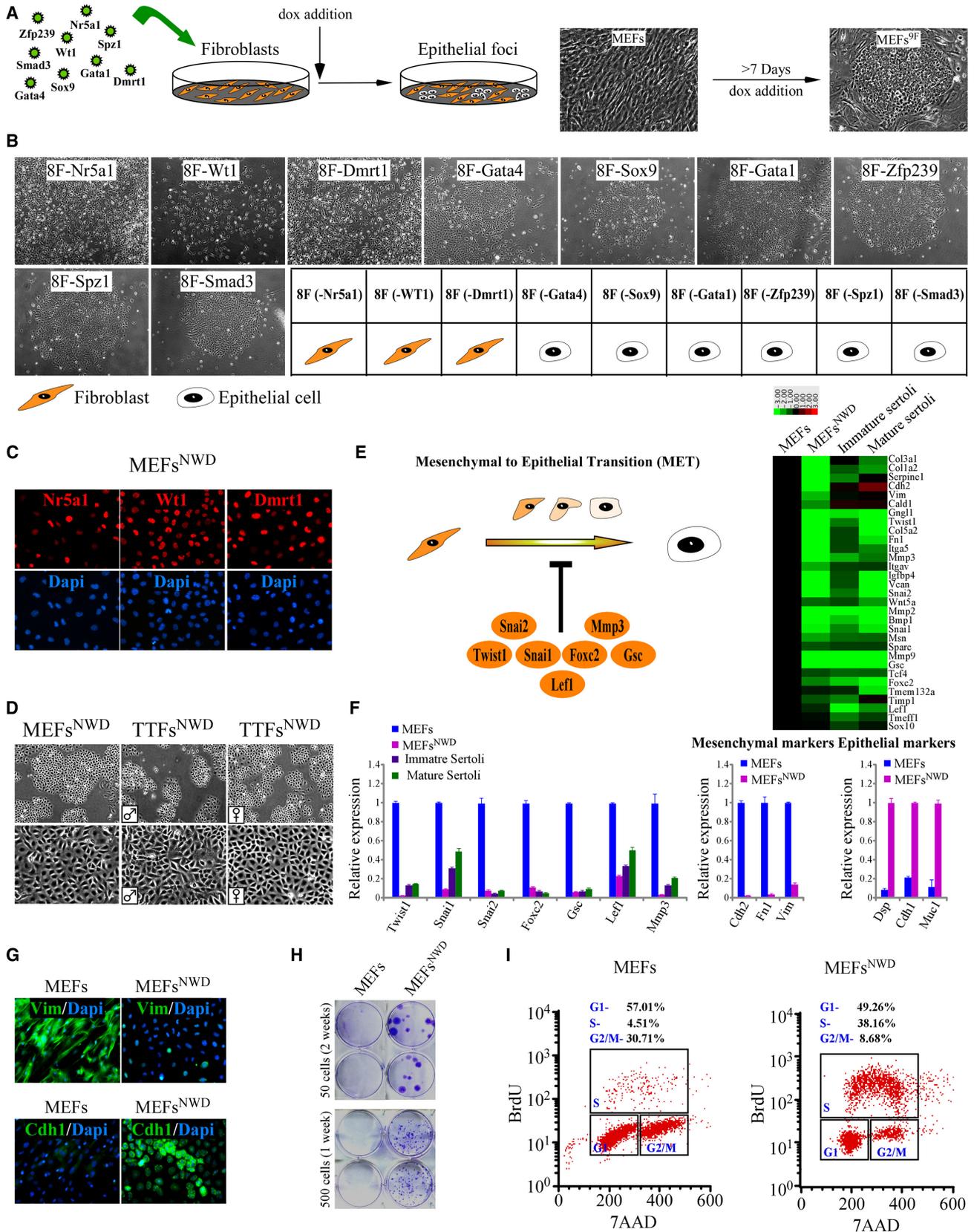
INTRODUCTION

Embryonic Sertoli cells (eSCs) play a pivotal role in testis morphogenesis because they are the first cell type to differentiate in the bipotential gonad, an event which enables testicular cord formation (Skinner and Griswold, 2005). In the mouse XY gonad, eSC differentiation is initiated by the expression of the testis-determining gene, *Sry*, within the genital ridge at 10.5 days postcoitum (dpc) (Koopman et al., 1990). Upon induction of *Sry*, a cascade of molecular signaling events and developmental processes occur to ensure proper testis development. Expression of *Sox9*, a target gene of *Sry*, in gonadal somatic cells is sufficient to induce the differentiation of eSCs even in the absence of *Sry* (Sekido and Lovell-Badge, 2008; Vidal et al., 2001). One morphologically distinct event in testis devel-

opment is the aggregation and rearrangement of the eSCs and primordial germ cells (PGCs) to form testicular cords. For cellular aggregation to occur, eSCs must differentiate and undergo mesenchymal to epithelial transition (MET) and polarization (Skinner and Griswold, 2005). Accumulation of various extracellular matrix (ECM) components, such as laminin and collagen, has been shown to induce eSC polarization (Skinner and Griswold, 2005). As the cords develop, eSCs attract cells from the coelomic epithelium and from the mesonephros such as endothelial cells. Endothelial cells migrate into the gonad in a male-specific manner and contribute to the characteristic male pattern of vasculature (Brennan et al., 2002). Although the basic events in Sertoli cell development have been defined, the key transcription factors that mediate each of these processes are still unknown.

In the adult, proper spermatogenesis is dependent on Sertoli cells, which are in constant and intimate interaction with all stages of the differentiating germ cells inside the seminiferous tubules. The main role of mature Sertoli cells is to provide support and nutrition to the developing sperm cells. Furthermore, Sertoli cells have been demonstrated to possess trophic properties, which have been utilized for the protection of nontesticular cellular grafts in transplantations (Mital et al., 2010). However, mature Sertoli cells are mitotically inactive, and the primary immature Sertoli cells during prolonged cultures lose their unique characteristics (Skinner and Griswold, 2005). Therefore, finding an alternative source of these cells independent of donor testis cells is of paramount interest both for basic research and clinical applications. Although it has been shown recently that Sertoli-like cells can be derived from embryonic stem cells (ESCs), the efficiency and purity of the differentiating Sertoli cells remain a barrier (Bucay et al., 2009).

Here we show that concomitant expression of *Nr5a1*, *Wt1*, *Dmrt1*, *Gata4*, and *Sox9* efficiently reprogrammed mouse fibroblasts into induced embryonic Sertoli-like cells (ieSCs). While the expression of *Nr5a1*, *Wt1*, and *Dmrt1* promoted proliferation and initiated MET, the combination of *Nr5a1*, *Wt1*, and *Sox9* stimulated cell aggregation. ieSCs exhibited migratory potential, formed tubular-like structures, recruited endothelial cells, facilitated embryonic cortical progenitor self-renewal and differentiation, expressed eSC-specific markers, facilitated germ cell survival in culture, and interacted with endogenous eSCs and PGCs in gonad cultures.



RESULTS

Nr5a1, Wt1, and Dmrt1 Initiate Proliferation and MET in Fibroblasts

Primary immature Sertoli cells were cultured for 30 days and examined for changes in morphology and expression of key immature Sertoli cell markers. During the first few days of culture, the cells kept epithelial morphology and displayed Sertoli cell characteristics as indicated by high expression of the Sertoli markers *Nr5a1*, *Dmrt1*, *Gata4*, *Sox9*, *Amh*, *Shbg*, *Ptgds*, *Clu*, *ErbB4*, and *Gdnf* and the epithelial marker *Krt18* (Figures S1A–S1D available online). With prolonged culture, however, loss of marker expression, loss of epithelial morphology, and acquisition of fibroblastic morphology with expression of *Thy1* and *Col5a2* occurred (Figures S1B and S1E), which is similar to the changes seen in the most studied immature Sertoli cell line TM4 (Figures S1B and S1F). In contrast to TM4, the primary Sertoli cells maintained relatively high levels of several other Sertoli markers like *Aldh1a1*, *Dhh*, *Kitl*, and *Wt1* for at least 30 days when compared to mouse embryonic fibroblasts (MEFs) (Figure S1G). These results suggest that primary Sertoli cells can retain their full properties in vitro only for several days.

Based on the enrichment of their binding sites within the promoters of several known markers of Sertoli cells using the MatInspector software (Cartharius et al., 2005), we screened nine transcription factors to reprogram fibroblasts into ieSCs: Nr5a1, Wt1, Dmrt1, Gata4, Sox9, Gata1, Spz1, Smad3, and Zfp239 (Figure S2A). One of the initial steps in eSC differentiation is the transformation from mesenchymal-like cells to epithelial-like cells (Nel-Themaat et al., 2011). The factors that control MET in eSCs are unknown, but are thought to be induced by Sry. To unravel which of the Sertoli cell factors can initiate MET in fibroblasts, we introduced the nine factors into MEFs using the doxycycline (dox)-inducible lentiviral system and monitored generation of epithelial foci-like morphology within the culture, which appeared within 1 week of dox treatment (Figure 1A). To determine which of the nine factors are essential for initiating MET, we removed individual genes from the pool of factors and found that the removal of Nr5a1 or Wt1 or Dmrt1 impaired significantly the ability to generate epithelial foci (Figure 1B). Accordingly, introduction of Nr5a1, Wt1, and Dmrt1 in MEFs (MEFs^{NWD}) (Figure 1C) or tail tip fibroblasts (TTFs^{NWD}) from both sexes (Figure 1D) rapidly initiated MET. Expression of an

individual factor (Nr5a1 or Wt1 or Dmrt1) was not sufficient to induce MET (Figure S2B). To assess whether genes known to affect MET were differentially expressed between MEFs and MEFs^{NWD}, we performed cDNA microarray on MEFs, MEFs^{NWD}, and immature Sertoli and mature Sertoli cells. We found that genes that block the MET process and induce epithelial to mesenchymal transition (EMT) such as *Twist1*, *Snai1*, *Snai2*, *Foxc2*, *Lef1*, *Gsc*, and *Mmp3* were significantly downregulated in MEFs^{NWD} and immature and mature Sertoli cells as compared to MEFs (Figure 1E). Also, three mesenchymal markers (*Cdh2*, *Vim*, and *) were inhibited and three epithelial markers (*Muc1*, *Dsp*, and *Cdh1*) were upregulated. We confirmed the microarray results by qRT-PCR and immunostaining (Figures 1F, 1G, and S2C). Nr5a1, Wt1, and Dmrt1 also promoted the proliferation of the induced epithelial cells as indicated by colony forming experiments and BrdU staining (Figures 1H, 1I, and S2D). Additionally, we observed an increase in the levels of endogenous Sox9 in the transduced cells (Figure S2E). These data suggest that Nr5a1, Wt1, and Dmrt1 promote proliferation, induce low Sox9 expression, and initiate MET, all characteristics of proliferating coelomic epithelium, one of the precursors of eSCs (Karl and Capel, 1998; Morais da Silva et al., 1996).*

Nr5a1, Wt1, and Sox9 Promote Cell Aggregation

We asked whether the nine factors can induce cell aggregation as is seen in vivo with eSCs in the gonad and in vitro with endogenous Sertoli cells (Gassei et al., 2008). Cell aggregates were observed in factor-transduced cells 3 weeks after dox addition (Figure 2A). To dissect which of the factors were responsible for generating cell aggregates, we removed individual genes from the pool and measured aggregate formation (Figure 2B). Removal of Nr5a1, Wt1, or Sox9 impaired significantly their ability to aggregate, and transduction of these factors into MEFs (MEFs^{NWS}) or TTFs (TTFs^{NWS}) resulted in the generation of cell aggregates 2 weeks postinfection (Figure 2C). When the three factors were introduced into keratinocytes (Krts^{NWS}), which lack the intrinsic capability to aggregate, aggregates were detected within 3 weeks (Figure 2C). Endogenous immature and mature Sertoli cells formed cell aggregates within 3 days of culture (Figure 2C). Because Sox9 is a key developmental regulator of chondrocytes and osteoblasts (Akiyama et al., 2005) that have the capability to aggregate in culture, we measured the

Figure 1. Nr5a1, Wt1, and Dmrt1 Promote Proliferation and Induce Mesenchymal to Epithelial Transition

- (A) Schematic representation of the strategy to test candidates that induce mesenchymal to epithelial transition (MET).
 (B) Systematic approach to discover the candidates that are responsible for the formation of epithelial foci. In each infection a different transcription factor was removed from the pool of nine factors. The formation of the epithelial foci was examined 1–2 weeks postinfection.
 (C) Immunostaining of Nr5a1, Wt1, and Dmrt1 (red) in MEFs (MEFs^{NWD}) 3 weeks postinfection.
 (D) Bright field images of MEFs and tail tip fibroblasts (TTFs) from both sexes that were infected with Nr5a1, Wt1, and Dmrt1 and were cultured for 3 weeks.
 (E) Left: schematic representation of the key factors that block the MET process. Right: a cluster of genes with known roles in MET inhibition that were downregulated in MEFs^{NWD} and immature and mature Sertoli cells. Log₂ relative gene expression is visualized as shades of red (higher than MEFs) and shades of green (lower than MEFs) in the indicated cells.
 (F) qRT-PCR of the indicated genes normalized to the *Hprt* housekeeping gene in MEFs, ieSCs, and immature and mature Sertoli cells.
 (G) Immunostaining of *Vim* and *Cdh1* (green) in MEFs and MEFs^{NWD}.
 (H) Colony formation assay of MEFs and MEFs^{NWD}. Cells (number as indicated) were cultured for the indicated time periods. The colonies were stained with crystal violet and imaged.
 (I) FACS analyses demonstrating the percentage of cells that are actively synthesizing DNA after 30 min of BrdU pulse. Error bars represent standard deviation of technical duplicates of the same experiment.

See also Figures S1 and S2.

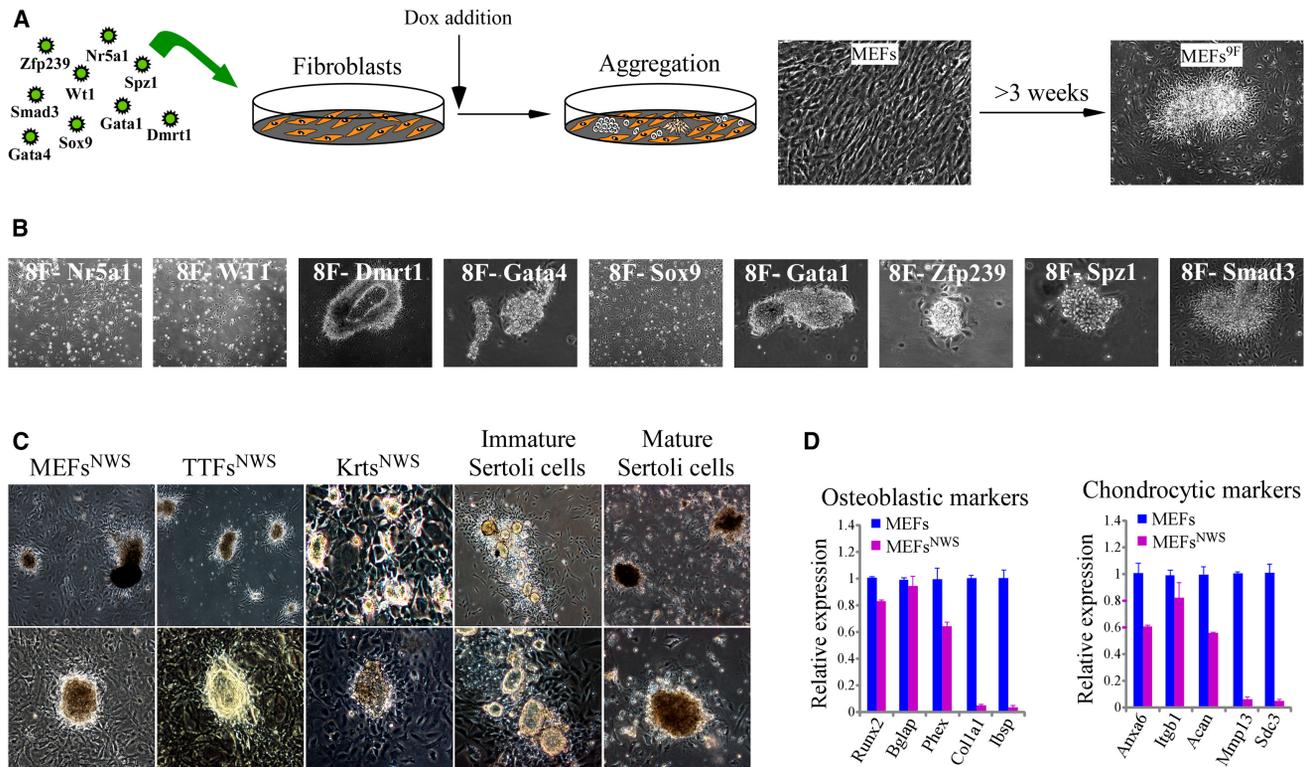


Figure 2. Nr5a1, Wt1, and Sox9 Induce Cell Aggregation

(A) Schematic representation of the strategy to test candidates that induce cell aggregation.
 (B) Systematic approach to discover the candidates that are responsible for cell aggregation. In each infection a different factor was removed from the pool of nine factors. After 3 weeks, cells were seeded as single cells and tested for aggregates 24 hr later.
 (C) Formation of cell aggregates in the indicated cells following the introduction of Nr5a1, Wt1, and Sox9 for 3 weeks.
 (D) qRT-PCR of the indicated genes normalized to the *Hprt* housekeeping gene in MEFs and MEFs^{NWS}. Error bars represent standard deviation of technical duplicates of the same experiment.

expression levels of several osteoblastic and chondrocytic markers in MEFs^{NWS} and found no elevation in their expression (Figure 2D). Our results suggest that Nr5a1, Wt1, and Sox9 promote cell aggregation.

Nr5a1, Wt1, Dmrt1, Gata4, and Sox9 Convert Fibroblasts into ieSCs

Since Gata4 has been shown to drive eSC differentiation (Manuylov et al., 2007) and promote transdifferentiation of cardiomyocytes and hepatocytes (Huang et al., 2011; Ieda et al., 2010), we included it in the cocktail of the selected four factors (Nr5a1, Wt1, Dmrt1, and Sox9). Transduction of the five factors into MEFs (MEFs^{NWDG4S}) (Figure 3A) or TTFs (TTFs^{NWDG4S}) from both sexes resulted in epithelial foci after 2 weeks (Figures 3B). Since eSC polarization requires basement membrane attachment (Mackay et al., 1999), long-term culture was done on Matrigel (Figure 3C).

We observed that very high levels of transgenes in MEFs (MEFs^{NWDG4S-high}) (Figure S3A) upregulated several Leydig-cell-specific markers, such as *Hsd3b1*, *Hsd3b6*, *Dhcr7*, and *Cyp11a1*, along with some germ cell markers like *Dazl* and *Tdrd6* (Figures S3B and S3C). Since Nr5a1 is a steroidogenic factor and a key regulator of Leydig cells and since Dmrt1 is expressed in germ cells as well, it is tempting to speculate that the

extremely high levels of Nr5a1 and Dmrt1 in MEFs^{NWDG4S-high} are responsible for the nonspecific induction of these genes. None of these genes were upregulated in cells with lower transgene expression (MEFs^{NWDG4S-low}). Also, high activation levels of endogenous *Nr5a1*, *Wt1*, and *Dmrt1* were observed in MEFs^{NWDG4S-low} compared to MEFs^{NWDG4S-high} and MEFs (Figure S3D). These results suggest that controlled levels of transgenes are crucial for proper transdifferentiation.

To test whether the morphology of the ieSCs (i.e., MEFs^{NWDG4S-low} after culturing on Matrigel) was similar to endogenous Sertoli cells, we cocultured ieSCs transduced with the H2b-GFP construct (Beronja et al., 2010) with freshly isolated endogenous immature Sertoli cells for 2 days. The morphology of the ieSCs was comparable to that of endogenous immature Sertoli cells (Figure 3D), integrating into the colonies formed by the endogenous cells in contrast to H2b-GFP-MEFs (Figure S3E). As opposed to MEFs^{NWD}, ieSCs expressed the mesenchymal Sertoli markers *Vim*, *Cdh2*, *Sparc*, and *Fn1* at a level comparable to that of immature and mature Sertoli cells (Figure 3E). Epithelial morphology of the cells was consistent with high expression of eSC epithelial markers *Krt18*, *Dsp*, *Ocln*, and *Cgn* (Figures S4A and S4B). These results suggest that ieSCs underwent MET and retained proper epithelial Sertoli cell characteristics.

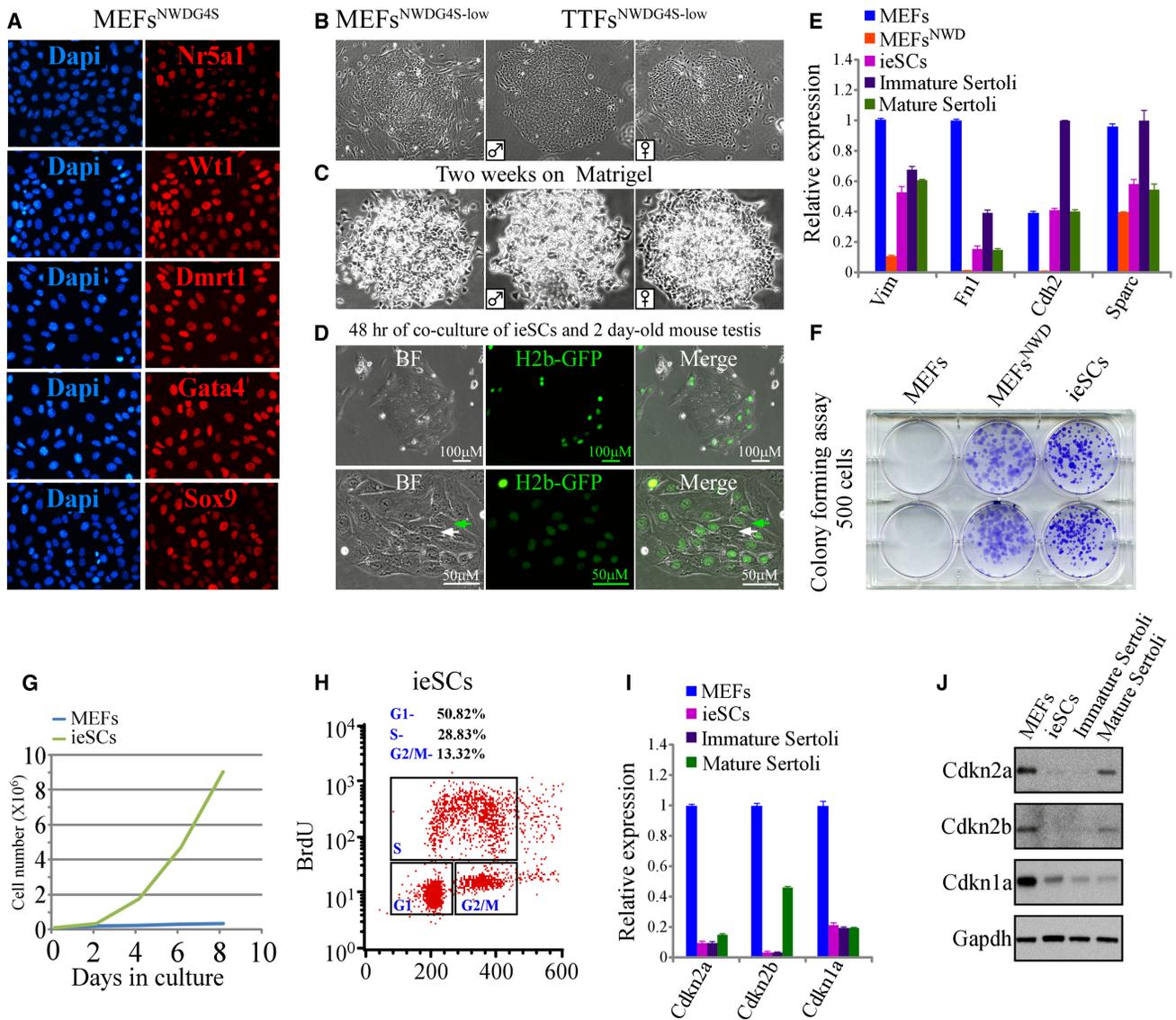


Figure 3. ieSCs are Epithelial Cells with High Proliferative Capability

(A) Immunostaining of Nr5a1, Wt1, Dmrt1, Gata4, and Sox9 (red) in MEFs (MEFs^{NWDG4S}) 3 weeks postinfection. (B) Bright field of epithelial focus generated from transduced MEFs and TTFs from both sexes. (C) Bright field of cells from the upper panel after 2 weeks of cultivation on Matrigel. (D) H2b-GFP-ieSCs were cocultured with 2-day-old testis suspension for 48 hr. Cells were imaged using the bright field and GFP channel. White arrow marks endogenous Sertoli cell and green arrow marks H2b-GFP ieSCs. (E) qRT-PCR of the indicated genes normalized to the *Hprt* housekeeping gene in MEFs, MEFs^{NWD}, ieSCs, and immature and mature Sertoli cells. (F) Colony forming assay of the indicated cells. Five hundred cells were seeded and cultured for 1 week. The colonies were stained with crystal violet and imaged. (G) Proliferation curve of MEFs and ieSCs during 8 days in culture as indicated by cell number (cells were trypsinized and counted on the indicated days). (H) FACS analyses demonstrating the percentage of cells that are actively synthesizing DNA after 30 min of BrdU pulse. (I) qRT-PCR of the indicated genes normalized to the *Hprt* housekeeping gene in MEFs, ieSCs, and immature and mature Sertoli cells. (J) Western blot analysis of the indicated proteins in MEFs, ieSCs, and immature and mature Sertoli cells. Error bars represent standard deviation of technical duplicates of the same experiment. See also [Figures S3 and S4](#) and [Movie S1](#), [Movie S2](#), [Movie S3](#), and [Movie S4](#).

Proliferation of eSCs is essential for testis morphogenesis with rapid proliferation beginning as early as 11.5 dpc (Schmahl et al., 2000). Consistent with that, ieSCs exhibited colony forming capability (Figure 3F), high proliferation rate (Figure 3G), and high percentage of cells (28%) in S phase (Figures 3H).

Also, in contrast to MEFs, we found decreased expression of the Cdk inhibitors Cdkn2b, Cdkn2a, and Cdkn1a in ieSCs, immature Sertoli cells, and, to a lesser extent, the mature Sertoli cells, (Figures 3I and 3J) consistent with their high proliferative capacity.

Whole genome expression
profile

Embryonic Sertoli markers

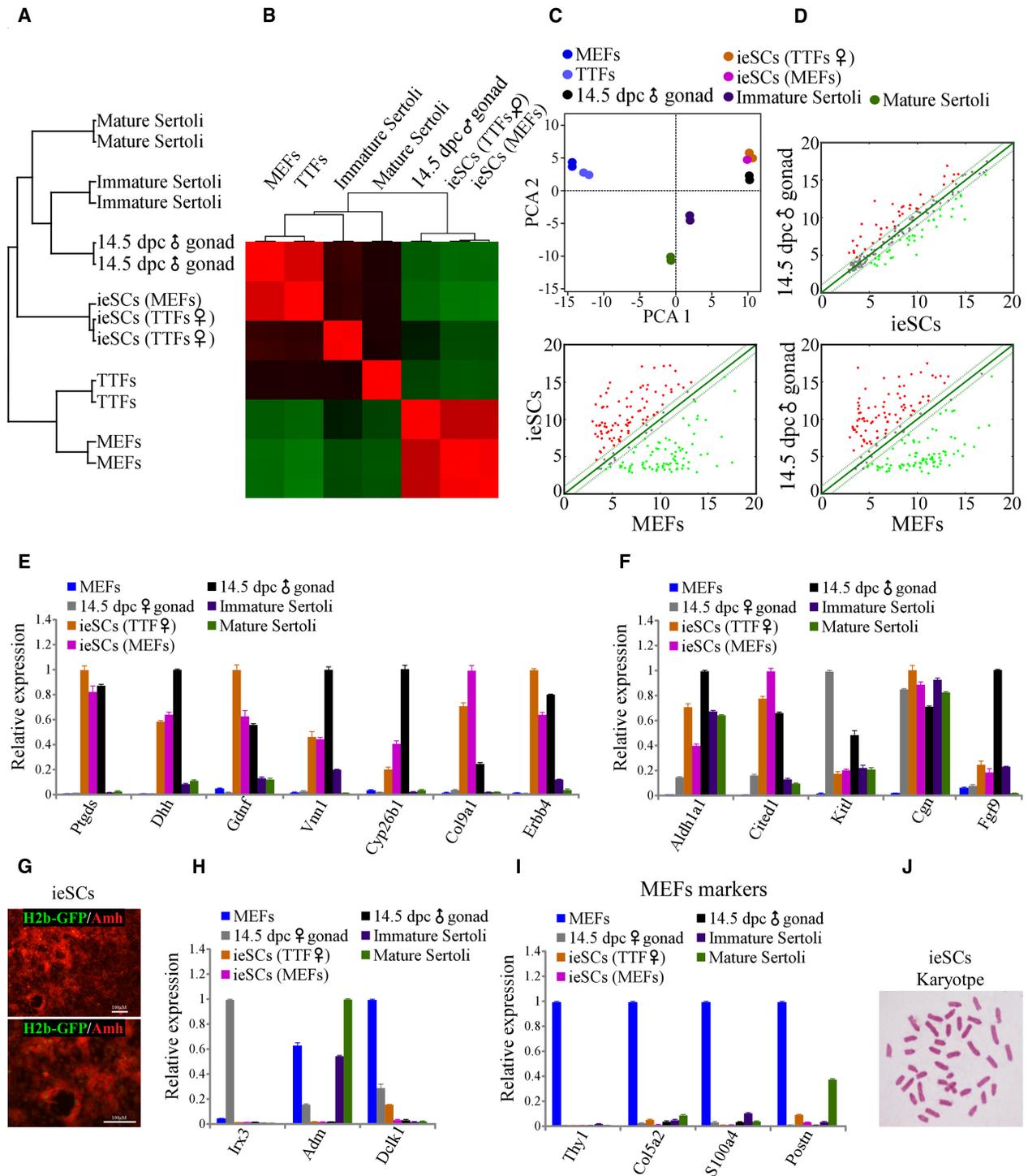


Figure 4. ieSCs Exhibit a Normal Karyotype and Express Embryonic Sertoli-Specific Transcription Profile

(A) Hierarchical clustering of gene expression array profiles as measured by mouse SurePrint G3 Gene Expression Microarrays-8x60K (Agilent). Complete linkage hierarchical clustering analysis was performed using Pearson's correlation metric. The dendrogram includes individual samples from MEFs, TTFs, ieSCs (MEFs), ieSCs (TTFs female), 14.5 dpc male gonad, and immature and mature Sertoli cells.

(B) Hierarchical clustering dendrogram using embryonic Sertoli-specific profile ($n = 200$) generated mostly from two independent studies (Bouma et al., 2010; Boyer et al., 2004). Samples and genes were clustered using Cluster 3.0. Shades of red show the pairwise Pearson correlations of gene expression. Clustering tree is shown on top of the heatmap.

ieSCs Exhibit a Normal Karyotype and Express Embryonic Sertoli Profile

We next compared gene expression profiles of MEFs, TTFs, ieSCs (derived from MEFs), ieSCs (derived from female TTFs), 14.5 dpc male gonad, and immature and mature Sertoli cells using cDNA microarray. Hierarchical clustering analysis demonstrated that the transcriptome profiles of ieSCs were more similar to each other and to the endogenous Sertoli cells than to the donor fibroblasts (Figure 4A), but not identical. Because the isolated 14.5 dpc male gonad contained, in addition to the eSCs, high numbers of germ, Leydig, and peritubular myoid cells, we focused our analysis on the expression of 200 eSC markers identified mainly in two studies that sorted eSCs from 10.5–13.5 embryos (Bouma et al., 2010; Boyer et al., 2004). Notably, hierarchical clustering analysis (Figure 4B), principle component analysis (PCA) (Figure 4C), and scatter plots (Figure 4D) showed that ieSCs were clustered together with the 14.5 dpc male gonad, and far from MEFs, TTFs, and immature and mature Sertoli cells. To confirm the microarray results, we used 14.5 dpc female gonads as a negative control and found that *Ptgds*, *Dhh*, *Gdnf*, *Vnn1*, *Cyp26b1*, *Col9a1*, and *ErbB4* were upregulated mostly in ieSCs and in male 14.5 dpc gonad but not in immature or mature Sertoli cells, female 14.5 gonads, or MEF controls (Figure 4E). Expression of *Aldh1a1* and *Cited1* was high in ieSCs, lower in female 14.5 gonads, and absent in MEFs (Figure 4F). *Kitl*, which is important for germ cell survival (Yan et al., 2000), and the epithelial marker *Cgn* were upregulated in all of the groups, but not in MEFs (Figure 4F). Furthermore, the secretion of the Amh hormone was observed in the ieSC culture (Figure 4G). In contrast, expression of *Fgf9*, which is upregulated in eSCs and forms a positive feedback loop with *Sox9*, was low in ieSCs (Figure 4F). The embryonic female-specific genes *Irx3*, *Adm*, and *Dclk1* were downregulated in 14.5 dpc male gonad and in ieSCs (Figure 4H). Consistent with acquiring an embryonic Sertoli cell-like transcriptional profile, ieSCs showed reduced MEF-specific marker expression (Figure 4I). A clear difference in the transcriptional profile between ieSC lines and MEFs was observed also after 2 weeks of dox withdrawal, suggesting that the ieSCs reached at least partially a stable state that is transgene independent (Figure S4C). Importantly, ieSCs in the absence of dox retained relatively high levels of *Aldh1a1*, *Dhh*, *Kitl*, and *Wt1* (Figure S4D) similarly to endogenous immature Sertoli cells during prolong culturing (Figure S1G).

Cytogenetic analysis of ieSCs cultured for 17 passages revealed that they exhibited normal karyotype with 40 chromosomes in 15 of the 17 analyzed metaphases (Figure 4J). Combined, these results show that the ieSCs expressed a specific eSC profile and maintained normal chromosome numbers.

ieSCs Migrate, Aggregate, Attract Endothelial Cells, and Form Tubular-like Structures

Since some endogenous eSCs migrate from the coelomic epithelium to the gonad and continue their migration inside the gonad to generate testicular cords, we tested whether ieSCs have a potential to migrate. H2b-GFP-MEFs, H2b-GFP-ieSCs, and H2b-GFP-immature Sertoli cells were seeded on a microporous membrane (8 μ M) Boyden chamber that contained serum-free medium in the upper part and 10% FBS medium in the lower part. The number of GFP-positive cells that had migrated through the membrane to the lower side within 24 hr was significantly higher for ieSCs and immature Sertoli cells than for MEFs (Figure 5A). Cell motility assay for ieSCs and MEFs following serum stimulation for 24 hr revealed high motility capability for ieSCs (Movie S1, Movie S2, Movie S3, and Movie S4). These results suggest that the ieSCs have migratory potential resembling that of eSCs.

eSCs and PGCs aggregate in the fetal gonad. Similarly, when cells were cultured at a high density single cell suspension, ieSCs formed aggregates within 3 hr in contrast to MEFs (Figure 5B) with the shape and density closely resembling those of freshly isolated immature Sertoli cells (Figure 5C). eSC polarization and aggregation is thought to be mediated by ECM proteins such as Col4a and Lama1 (Davis et al., 1990; Skinner and Griswold, 2005), both of which were highly enriched in the ECM produced by ieSCs, in contrast to MEFs (Figure 5D).

Endothelial cell migration and induction of peritubular myoid cells are two fundamental processes in the generation of the testicular cords. Migration of endothelial cells has been suggested to be mediated by eSCs and undifferentiated mesenchyme (Brennan et al., 2002; Cool et al., 2011). To test whether the ieSCs can attract endothelial cells, we employed the Boyden chamber-based cell migration assay (8 μ M pore) and found after 4 hr a 2-fold higher cell number of human umbilical vein endothelial cells (HUVECs) in conditioned medium from ieSCs than in conditioned medium from MEFs (Figure S5).

To test whether ieSCs can form tubule-like structures, we cultured immature Sertoli cells on Matrigel, which can induce cell aggregation and 3D hollowed cord-like structures (Gassei et al., 2006). Figures 5E, S6A, and S6B show that ieSCs, when cultured on Matrigel with or without dox for 3 days in 10% FBS, formed spherical cellular aggregates. These 3D cord-like structures were hollowed like a tubule and generated a highly regular hexagonal array in the Matrigel. When the cells were cultured on Matrigel in 2% FBS, web-like structures were formed, consistent with the initiation of tubulogenesis (Figure 5F). Sparsely seeded cultures generated highly organized ring-like structures (Figure 5G) and, in the absence of Matrigel, dense

(C) Scatter plots of the indicated samples (legend on the top) on the principle component 1 (PCA1) and PCA2 axes derived from principle component analysis (PCA) of the samples.

(D) Scatter plots of gene expression values (means of the indicated samples) in log₂. 2-fold (1 log₂) lines were drawn. y axis high genes were drawn in red, while x axis high genes were drawn in green.

(E and F) qRT-PCR of the indicated genes normalized to the *Hprt* housekeeping gene in MEFs, 14.5 dpc female gonad, ieSCs (derived from female TTFs), ieSCs (derived from male MEFs), 14.5 dpc male gonad, and immature and mature Sertoli cells.

(G) Immunostaining of Amh (green) in ieSC culture.

(H and I) qRT-PCR of the indicated genes normalized to the *Hprt* housekeeping gene in MEFs, 14.5 dpc female gonad, ieSCs (derived from female TTFs), ieSCs (derived from male MEFs), 14.5 dpc male gonad, and immature and mature Sertoli cells.

(J) Cytogenetic analysis of ieSCs shows normal karyotype after 17 passages.

Error bars represent standard deviation of technical duplicates of the same experiment. See also Figure S4.

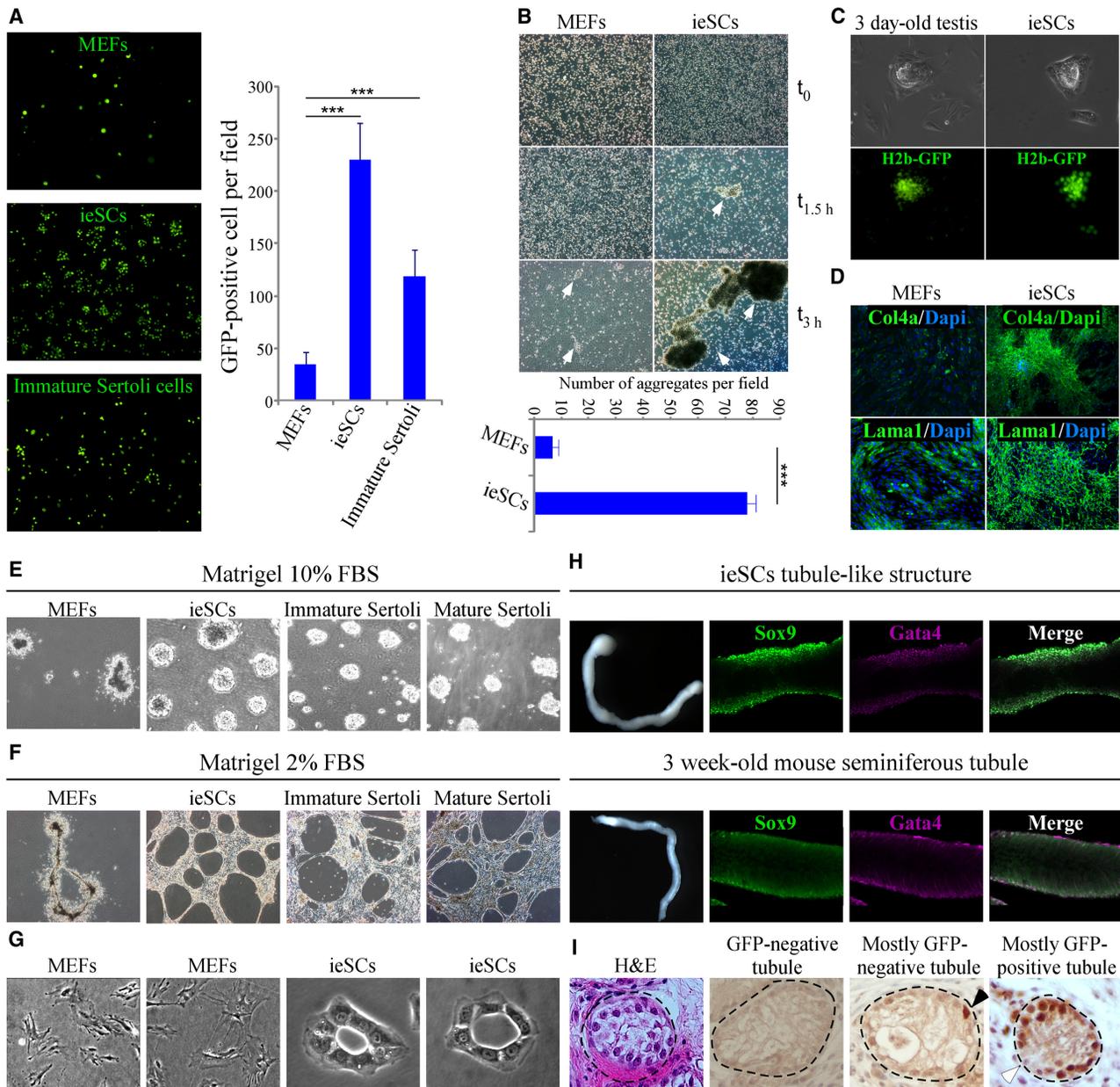


Figure 5. ieSCs Migrate, Aggregate, and Form Tubular-like Structures

(A) Migration assay of H2b-GFP-MEFs, ieSCs, and immature Sertoli cells during 24 hr of incubation using Boyden chamber (8 μ m pore). 3×10^5 cells were seeded in the top of the insert in serum-free medium, while serum was placed in the well below. After 24 hr the cells that migrated to the other side of the membrane were trypsinized and counted. Left panel: representative fields of MEFs, ieSCs, and immature Sertoli cells that had migrated through the membrane. Right panel: graph depicts the average number of cells that were migrated through the other side of the membrane, taken from ten independent fields.

(B) Comparison between MEFs and ieSCs in their aggregation potential. High dense single cell suspension (5×10^6 cells) of MEFs and ieSCs were cultured for 3 hr and imaged in the indicated time points. Right: a graph summarizing the average number of aggregates from ten fields from five independent experiments.

(C) Representative images of the generated aggregates 24 hr postplating of freshly isolated (24 hr) H2b-GFP endogenous immature Sertoli cells (isolated from 3-day-old mouse testis) and H2b-GFP-ieSCs.

(D) Immunostaining of Col4a and Lama1 (green) in MEFs and ieSCs.

(E and F) Comparison between MEFs, ieSCs, and immature and mature Sertoli cells in their capability to form cord-like structures and tubulogenesis when cultured with 10% FBS or 2% FBS medium containing dox on Matrigel for 72–96 hr.

(G) Comparison between MEFs and ieSCs in their capability to form ring-like structures when seeded sparsely.

(H) Spontaneously formed 3D tubule-like structure by ieSCs that was generated in 1–2 weeks culture without passaging in dox containing medium (upper panel) and endogenous seminiferous tubule isolated from 3-week-old mouse testis. The tubules were fixed and stained for Gata4 and Sox9 and subjected to confocal microscopy.

(I) Generation of tubule-like structure in vivo by ieSCs. H2b-GFP-ieSCs or H2b-GFP-MEFs were mixed with 5-day-old mouse testis suspension and centrifuged for 5 min to obtain a solid pellet (each pellet contains 2×10^6 MEFs or ieSCs and one testis). The pellets were then transplanted under the skin of the back of

cultures formed occasionally complete 3D tubules with a structure similar to that of testicular tubules (Figure 5H).

Finally, to examine whether ieSCs can form tubule-like structures in vivo in a dox-independent manner, we transplanted subcutaneously a pellet of H2b-GFP-ieSCs together with an extract from immature testis into NOD/SCID mice. Three weeks later the transplant was fixed and stained for GFP, demonstrating the formation of tubular-like structures arising from GFP-positive cells (Figure 5I). In contrast, no contribution of H2b-GFP-MEFs to tubules was seen (data not shown). In summary, our data suggest that ieSCs can migrate, aggregate, recruit endothelial cells, and incorporate into tubule-like structures.

ieSCs Support Germ Cell Survival and Facilitate Neuronal Differentiation and Self-Renewal of Embryonic Cortical Progenitors

To test whether ieSCs can support germ cell survival, testicular cells from 1-day-old postnatal pups were cultured either alone or with MEFs or ieSCs. One week later, the plates were fixed and stained for the germ cell marker Ddx4 (Vasa). A significantly higher number of Ddx4-positive cells were observed in the ieSC coculture as compared to coculture with MEFs or testicular suspension alone, indicating that ieSCs can support germ cell survival in vitro, which is consistent with their role in development (Figures 6A and 6B). Because Gdnf has been shown to facilitate germ cell survival (Sariola and Immonen, 2008) and is highly expressed in eSCs, we measured its expression after 7 days of culture. While ieSCs exhibited high levels of Gdnf with and without dox, immature Sertoli cells expressed low levels comparable to that in MEFs (Figure 6C). It has been shown previously that the trophic effect of Sertoli cells, transplanted into animal models of neurodegenerative diseases, can rescue some of the neural phenotypes (Huang et al., 2010). Trophic factors including Gdnf, Ntn, Tgfa, Tgfb, Igf1, Pdgfa, Pdgfb, Il1a, Il6, Cntf, and Dhh have been reported to exert this trophic effect and to stimulate self-renewal of neural progenitors and neuronal differentiation (Roussa et al., 2006; Wang et al., 1995; Willing et al., 1998; Yue et al., 2006). To test whether conditioned medium of ieSCs can stimulate neuronal differentiation and survival of embryonic cortical progenitors, we cultured embryonic cortical progenitors for 8 days in basic differentiating medium supplemented by conditioned medium from MEFs or ieSCs. Figure 6D shows that ieSC conditioned medium enhanced cell proliferation and self-renewal of the cortical progenitor cells as assessed by the number of Pax6-positive cells. Moreover, ieSC conditioned medium facilitated neuronal differentiation of the cortical progenitors as evidenced by an elevation of Tubb3 (Tuj1)-positive cells with elongated neuronal branches (Figures 6D and 6E), in contrast to MEF conditioned medium, which supported neural differentiation to a lesser extent. Accordingly, higher levels of Gdnf, Tgfa, Pdgfb, and Ntn was found in ieSCs compared to MEFs (Figure 6E). In contrast, other factors, such Pdgfa and Cntf, were expressed at similar levels in MEFs and

ieSCs (data not shown), possibly explaining the partial effect of MEF conditional medium. In contrast, astrocyte differentiation was comparable in the three groups as estimated by Gfap staining (Figure 6D). Our results suggest that ieSCs secrete trophic factors that contribute to survival, differentiation, and self-renewal of cultured neural progenitors and germ cells.

ieSCs Cooperate with Endogenous Embryonic Sertoli and PGCs in the Formation of the Testicular Cords in the Fetal Gonad

The first sign of testicular differentiation begins at 12.5 dpc with the formation of cylindrical cords, the precursors of adult seminiferous tubules. To test whether ieSCs can contribute to the generation of the testicular cords in the gonad, we injected H2b-GFP-ieSCs, which formed large spheres when cultured on Matrigel, into XY gonads of 12.5/13.5 dpc embryos that had been explanted into organ culture in the presence of dox (Figure 7A). After 4 days the gonads were fixed, stained, and subjected to confocal microscopy. PGCs were identified by Ddx4 (blue) and/or by the detection of a Pou5f1-eGFP transgene carried in transgenic embryos used as recipients. Sertoli cells were identified by staining for Sox9 (red) and the ieSCs were detected using H2b-GFP and Sox9. Figure 7A shows that the injected cells kept their spherical shape without evidence of migration during the first day (Figure 7A, lower panel). After injection, tubules fused and often generated one big tubule, possibly due to damage to the testicular cords. Many ieSCs migrated from the spheres, contributed to the generation of tubules (Figure 7B), and migrated long distances, contributing to the generation of new cylindrical cords (Figures 7C and S7A and Movie S5). Many ieSCs incorporated close to the PGCs, which maintained Ddx4 and Pou5f1 expression. Although the injected ieSCs were derived from single cells, Sox9 expression levels varied (i.e., the color of the various ieSC nuclei ranged between greenish, yellowish, and reddish). Variation in Sox9 expression levels was also observed in endogenous eSCs, albeit to a lesser extent. Integration of three independent ieSC lines (two from MEFs and one from female TTFs) into tubules was observed in 20 of 30 tested XY gonads, but no contribution of H2b-GFP-MEFs was observed (Figure S7B). These results suggest that ieSCs can cooperate with eSCs in the formation of the testicular cords and interact with PGCs while maintaining their characteristics as estimated by Pou5f1-eGFP and Ddx4 expression.

Endothelial cells are attracted by both XX and XY gonads; however, the vasculature of XY gonads undergoes major structural alterations by 12.5/13.5 dpc (Figure S7C), suggesting that construction of a male-specific vasculature plays a role in the functioning of the testis (Brennan et al., 2002). To test whether ieSCs can attract endothelial cells and induce large blood vessel formation, we injected H2b-GFP-ieSC spheres into XY and XX gonads at 12.5 dpc. Four days postinjection the gonads were fixed and stained for the germ cell and endothelial cell marker Pecam1 (Cd31-blue). Consistent with the in vitro results (Figure S5A), ieSCs were capable of attracting endothelial cells

NOD/SCID mice. Three weeks postinjection, the transplants were removed, fixed, and stained for GFP. Black broken line marks tubule's borders. Black arrow marks GFP-positive ieSCs. White arrow marks endogenous Sertoli cell. Asterisks indicate statistical significance of differences in the mean of the number of positive cells between the indicated groups (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$, Mann-Whitney U test). Error bars represent standard deviation of technical duplicates of the same experiment. See also Figures S5 and S6.

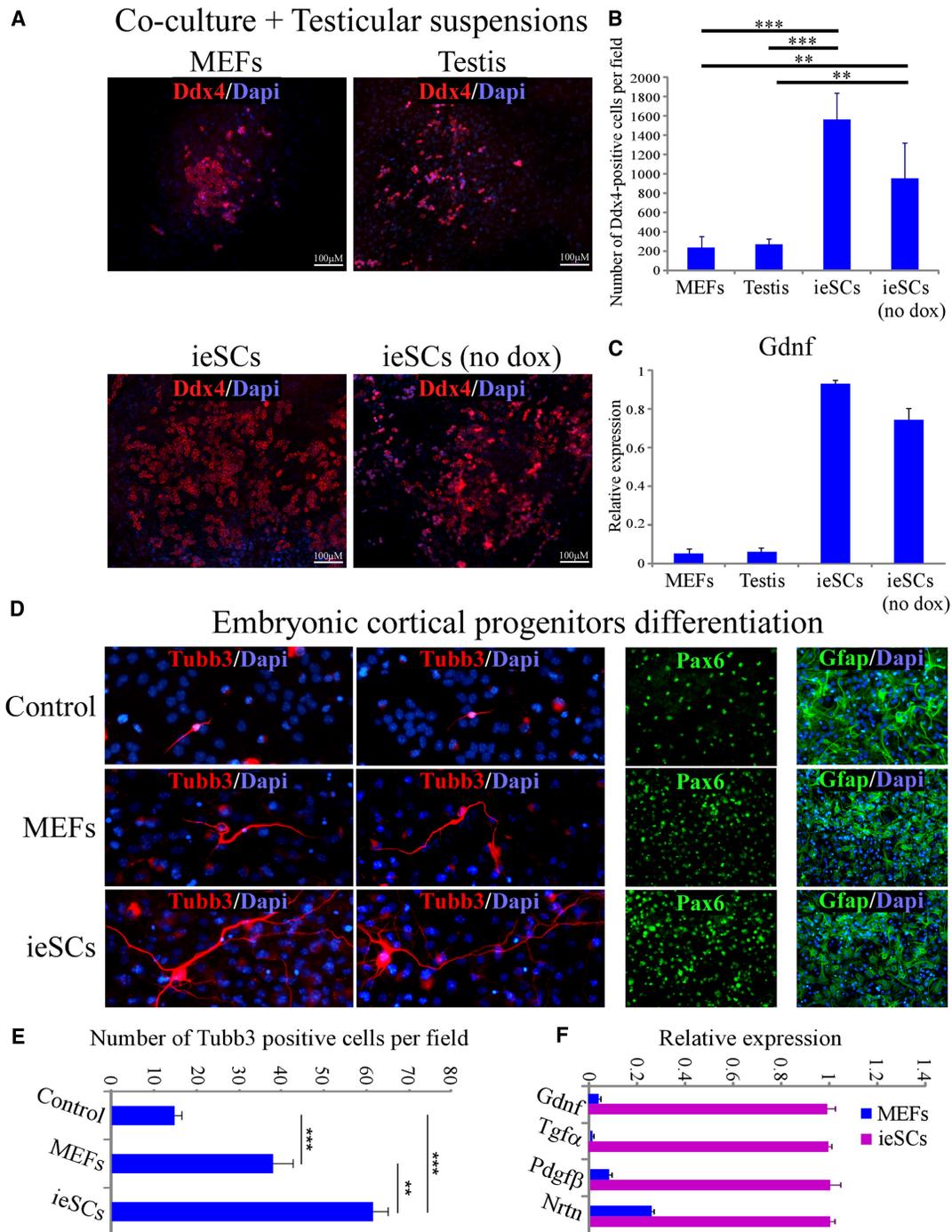


Figure 6. ieSCs Facilitate Germ Cell Survival In Vitro

(A) Immunostaining of Ddx4 (red) after 7 days of coculture of testicular suspensions from 1-day-old postnatal pups with MEFs or ieSCs. During the entire experiment the cells were grown in mouse ES medium. The ieSC-testicular suspension coculture was grown with or without dox.

(B) A graph depicting the average number of Ddx4-positive cells per field in five independent fields in the indicated groups.

(C) qRT-PCR of *Gdnf* normalized to the *Hprt* housekeeping gene in MEFs, testis alone, ieSCs, and ieSCs that were cocultured in a dox-free medium.

(D) Immunostaining of Tubb3 (red), Pax6 (green), and Gfap (green) after 8 days of embryonic cortical progenitor differentiation. The cortical progenitor cells received conditioned medium from MEFs, ieSCs, or fresh basic differentiating medium on a daily basis. Two representative fields from Tubb3 are depicted showing long and branched axons.

(E) A graph depicting the average number of Tubb3-positive cells per field in five independent fields.

(F) qRT-PCR of the indicated genes normalized to the *Hprt* housekeeping gene in MEFs and ieSCs. Asterisks indicate statistical significance of differences in the mean of the number of positive cells between the indicated groups (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$, Mann-Whitney U test).

Error bars represent standard deviation of technical duplicates of the same experiment.

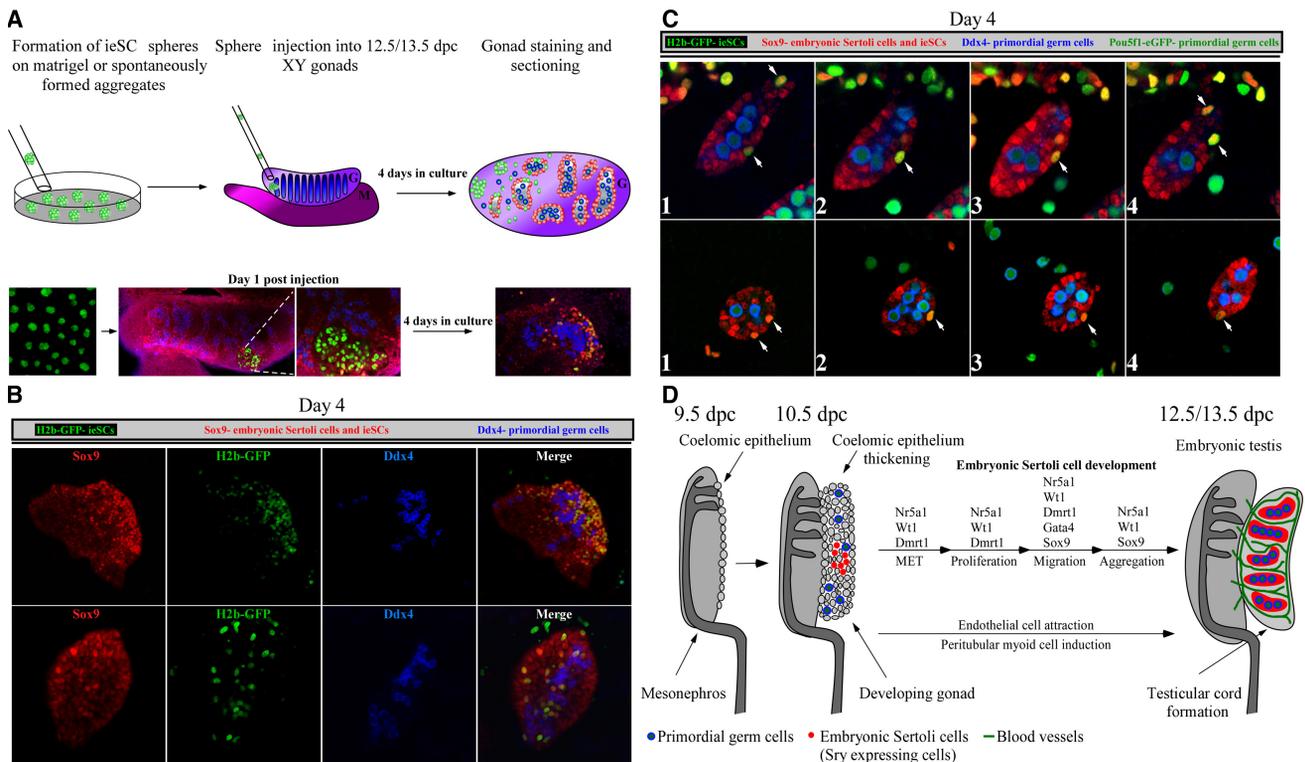


Figure 7. ieSCs Cooperate with Endogenous Embryonic Sertoli Cells and PGCs in the Formation of the Testicular Cords

(A) Schematic representation of the technique to inject ieSCs into the gonad. Left: H2b-GFP sphere formation after 1 week on Matrigel. Middle: injection of the spheres into 12.5/13.5 dpc XY gonads (magnification of ieSC H2b-GFP sphere 24 hr after injection to the gonad). Right: culturing for 4 days with dox, then gonad staining (Sox9, red; Ddx4, blue; ieSCs, green), sectioning, and confocal microscopy analysis.

(B) Representative z axis confocal scans for two testicular cords 4 days postinjection to 13.5 XY gonads.

(C) Representative z axis confocal scans from two testicular cords 4 days postinjection into 12.5 XY gonads. Sections with number indicate sequential z axis confocal scan through one testicular cord. White arrow marks ieSC.

(D) A scheme of the developing testis and the various processes eSCs undergo during differentiation (modified from Skinner and Griswold, 2005). Key factors that were demonstrated to facilitate these processes in vitro are shown.

See also Figure S7 and Movie S5.

and inducing large blood vessel formation in the developing ovary environment, which lacks large blood vessels (Figure S7D). Our results suggest that ieSCs can contribute to the formation of testicular cords and induce large blood vessel formation when injected into cultured fetal gonads.

DISCUSSION

In this study we demonstrated that concomitant expression of five transcription factors (Nr5a1, Wt1, Dmrt1, Gata4, and Sox9) efficiently reprogrammed MEFs into ieSCs. Overall, we isolated seven ieSC clones from MEFs, three clones from male TTFs, and three clones from female TTFs. These clones exhibited very similar morphology and Sertoli-specific gene expression profiles. We showed that different combinations of transcription factors regulate different processes in ieSC generation, with Nr5a1, Wt1, and Dmrt1 initiating cell proliferation and MET, and Nr5a1, Wt1, and Sox9 inducing cell aggregation (Figure 7D).

Our results show that ieSCs are migratory epithelial cells that have the capability to aggregate, form tubule-like structures, and recruit endothelial cells. Additionally, the cells expressed an eSC-specific transcriptional profile, secreted trophic factors

that supported germ cell survival, and interacted with endogenous eSCs and PGCs in the generation of testicular cords. In addition, after dox withdrawal, the cells retained high expression levels of some Sertoli-specific markers and kept their capability to aggregate, form tubule-like structures, and support germ cell survival in vitro. However, like endogenous Sertoli cells, ieSCs did not grow for long periods in the absence of dox without losing some Sertoli cell characteristics, most probably due to lack of optimal growth conditions for Sertoli cells. These results support the notion that ieSCs resemble endogenous eSCs. However, because the expression profile was only comparable to endogenous eSCs (for example: ieSCs do not express high *Fgf9* levels and exhibited high variation of Sox9 expression), our results are consistent with the cells not being identical to endogenous eSCs. Induced expression of *Fgf9* by eSCs is an important stage in eSC differentiation because it stabilizes Sox9 expression and induces differentiation in adjacent cells (Kim et al., 2006). One explanation for the absence of *Fgf9* expression is that a viral promoter drove Sox9 expression and that high levels of Sox9 negatively regulated the endogenous *Fgf9* promoter. This is supported by the observation that *Fgf9* levels were dramatically downregulated in MEFs^{NWDG4S-high}, which expressed very high

levels of *Sox9* transgene (data not shown). In the absence of *Fgf9*, the levels of the endogenous *Sox9* are not stable, which possibly explains the high variation in *Sox9* expression observed in ieSCs in gonad cultures. In contrast, *Ptgds*, which has been shown to activate *Sox9* expression as well in an *Fgf9*-independent manner (Moniot et al., 2009), was highly expressed in ieSCs (Figure 4E).

ieSCs as a Model for the Study of Sertoli Biology and Therapeutic Applications

The ieSCs described here may facilitate research on Sertoli cell function by providing an accessible supply of cells with reliable and predictable characteristics. To date, no cell line has been established with all the properties of Sertoli cells. Primary immature and mature Sertoli cells as well as established cell lines lose their characteristics during prolonged culture (Figure S1 and Skinner and Griswold, 2005). Here we demonstrate that the expression of five transcription factors (*Nr5a1*, *Wt1*, *Dmrt1*, *Gata4*, and *Sox9*) is sufficient to maintain proper function of ieSCs even after 1 year of culturing in the presence of dox. Although these factors are highly induced in the embryonic stage, they remain expressed until and after maturation. Therefore, lack of expression of these five factors in the mouse Sertoli cell line TM4 or in isolated immature Sertoli cells that were cultured for 30 days (Figure S1) might explain their epithelial shape loss, their inability to aggregate, and the silencing of several pivotal Sertoli markers. In a previous study Sertoli cells were derived in vitro from ESCs (Bucay et al., 2009). The cells were cultured, however, for only a limited time and their Sertoli characteristics were not tested. Finally, in contrast to direct transdifferentiation, the differentiating ESCs resulted in a heterogeneous population of cells that contained at least three cell types, PGCs, Sertoli-like cells, and a small fraction of Leydig cells.

Infertility affects approximately 20% of couples, with half of the cases attributable to deficient spermatogenesis in men (McLachlan et al., 1998). Proper spermatogenesis is dependent on Sertoli cells, which are in constant and intimate interaction with all stages of the differentiating germ cells inside the seminiferous tubules. The main role of Sertoli cells is to provide support and nutrition to the developing sperm cells. It has been demonstrated that Sertoli cells can support human early germ cell differentiation of spermatogonia and spermatocytes, isolated from testicular biopsies of azoospermic patients or patients with germ cell maturational defects (Sanberg et al., 1997). However, Sertoli cells isolated from biopsies degenerate within 10–15 days in culture. Thus, a stable Sertoli-like cell line could potentially enable longer Sertoli-germ cell cocultures, allowing a more complete germ cell differentiation for infertility treatments. Also it may be of interest to assess whether, by providing required growth factors and differentiation signals, conditioned medium from ieSCs can contribute to this process.

In addition, Sertoli cells have been demonstrated to possess trophic properties that have been utilized for the protection of nontesticular cellular grafts in transplantations (Mital et al., 2010). This trophic effect by the Sertoli cells has been demonstrated to be mediated by many trophic factors that facilitate the survival of the transplanted graft cells (Sanberg et al., 1997). In agreement with this notion, high levels of the prosurvival gene *Gdnf* were observed in the ieSC culture even in the absence

of dox. In summary, we suggest that concomitant expression of five key embryonic Sertoli cell factors can induce conversion of fibroblasts into ieSCs that possess many characteristics of endogenous eSCs and can be used for basic research and, potentially, for clinical applications.

EXPERIMENTAL PROCEDURES

Cell Culture and Mice

MEFs, TTFs, and keratinocytes were isolated and cultured as previously described (Wernig et al., 2008). Sertoli cells were isolated from testes of 1- to 5-day-old pups (immature Sertoli cells) or 6- to 8-week-old mice (mature Sertoli cells) using an enzymatic digestion procedure as previously described (Ogawa et al., 1997). MEFs and TTFs were grown in DMEM supplemented with 10% FBS, 1% nonessential amino acids, 2mM L-glutamine, and antibiotics. ieSCs were grown in DMEM/F12 supplemented with 10% or 2% FBS as indicated, 1% nonessential amino acids, 2mM L-glutamine, 2 mg/ml dox (unless stated otherwise), 2×10^4 units of *Lif*, and antibiotics. HUVECs were obtained from Invitrogen and cultured following the manufacturer's instructions. For the primary infection, MEFs, TTFs, and keratinocytes were isolated from mice homozygous for the reverse tetracycline-dependent transactivator (*M2rtTA*) inserted into the *Gt(ROSA)26Sor* locus (Beard et al., 2006). The *Pou5f1-eGFP* transgenic mice were obtained from the Jackson Lab and have been backcrossed to the C57BL/6 strain for more than 11 generations. TetO-H2B-GFP mice were generated and described previously (Foudi et al., 2009). Matrigel was absorbed to culture dishes in a ratio of 1:1 with (2% or 10% FBS) medium 1 day before seeding the cells. 5×10^6 to 10×10^6 cells were seeded in 2% FBS and 0.5×10^6 to 1×10^6 cells were seeded in 10% FBS medium and imaged 72–96 hr later.

Quantitative Real-Time PCR

Total RNA was isolated using Rneasy Kit (QIAGEN). Five hundred to two thousand nanograms of total RNA was reversed transcribed using a First Strand Synthesis kit (Invitrogen). Quantitative PCR analysis was performed in duplicates using 1/100 of the reverse transcription reaction in an ABI Prism 7300 (Applied Biosystems) with Platinum SYBR green qPCR SuperMix-UDG with ROX (Invitrogen). Specific primers flanking an intron were designed to the different genes (see Supplemental Experimental Procedures).

Colony Forming Assay

Fifty or five hundred cells from MEFs, MEFs^{NWD}, or ieSCs were seeded in 6-well plates in duplicates. One to two weeks postplating, the colonies were fixed and stained with crystal violet. The colonies were imaged using a light microscope.

Microarray Analyses

Whole genome expression profiling was performed using the mouse SurePrint G3 Gene Expression Microarrays-8x60K (Agilent). Expression values were quantile normalized (Smyth and Speed, 2003; Wettenhall and Smyth, 2004). Probe values were summarized into medians as gene values. Gene expression values were obtained by collapsing probe values targeting the same gene into median. Relative expression was calculated as a log₂ ratio of gene expression in the different cell types to MEFs. Hierarchical clustering was performed on whole genome transcriptional profile and on 200 selected eSC specific markers that were chosen mainly from two previous studies (Bouma et al., 2010; Boyer et al., 2004). Hierarchical clustering was performed using the Cluster 3.0 package (gene-centered by median, Pearson Correlation, Complete-linkage) and visualized using JavaTreeView (de Hoon et al., 2004; Saldanha, 2004). PCA was performed using R function *prcomp* (R: Development Core Team, 2004). Scatter plots were performed on mean expression values of the indicated samples.

ieSC Injection into 12.5/13.5 dpc Gonads

H2b-GFP-ieSCs were grown on 50% Matrigel (BD Biosciences) for 1 week until large spheres were formed. Mouse embryos were collected at 12.5/13.5 dpc. The entire urogenital ridges, including gonads, mesonephroi, dorsal part of mesentery, and dorsal aorta, were isolated and placed in grooves of

1.5% agar blocks. Two or three spheres were then injected into each gonad using mouth pipette with a fine pulled glass needle. Gonads were incubated in agar blocks placed in 35 mm tissue culture dishes with DMEM medium containing 2% FBS, 1% nonessential amino acids, 2mM L-glutamine, 2 mg/ml dox, and antibiotics at 37°C in 5% CO₂ for 4 days. For the whole-mount immunohistochemistry, the gonads were fixed in 4% PFA at 4°C overnight and then blocked with 3% BSA/5% donkey serum/0.1% Triton X-100/PBS overnight. Then they were washed with 0.1% Triton X-100/PBS and incubated with Sox9 (AB5535, Millipore), Ddx4 (AF2030, R&D Systems), or Pecam1 (550274, BD Biosciences) antibodies in 1% donkey serum/0.1% Triton X-100/PBS for 16 hr at 4°C. After intensive washes, they were incubated with secondary antibodies conjugated with Rhodamine Red X or DyLight 649 (Jackson ImmunoResearch) in 1% donkey serum/0.1% Triton X-100/PBS for another 16 hr at 4°C. The images were taken by a LSM710 confocal microscope (Zeiss).

ACCESSION NUMBERS

The Agilent microarray accession number for the data in this paper is GSE35653.

SUPPLEMENTAL INFORMATION

Supplemental Information for this article includes seven figures, Supplemental Experimental Procedures, and five movies and can be found with this article online at <http://dx.doi.org/10.1016/j.stem.2012.07.019>.

ACKNOWLEDGMENTS

This work was supported by National Institutes of Health Grants R37-CA084198 and RO1-HD045022 (R.J.) and the Howard Hughes Medical Institute (D.C.P. and Y.-C.H.). We thank Y. Katz for technical assistance and for commenting on the manuscript. We also thank W.C. Salmon for microscopy assistance.

Received: September 23, 2011

Revised: February 22, 2012

Accepted: July 30, 2012

Published: September 6, 2012

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