Spring 2024 – Systems Biology of Reproduction
Lecture Outline – Gametogenesis/ Stem Cells/ Cloning
Michael K. Skinner – Biol 475/575
CUE 418, 10:35-11:50 am, Tuesday & Thursday
March 19, 2024
Week 11

Gametogenesis/ Stem Cells/ Cloning

- Gametogenesis
- Spermatogenesis
  – Cell Development Stages
  – Meiosis
- Male Germline Stem Cell and Niche
  – Cell Biology
  – Niche
  – Regulatory Factors
- Germ Cell Transplantation
- Oogenesis
  – Cell Development
  – Meiosis
- Female Germline Stem Cells
- Embryonic Stem Cells
- Cloning

Required Reading

McCarrey JR. (2018) Gametogenesis Overview. in: Encyclopedia of Reproduction 2nd Edition, Ed: MK Skinner. Elsevier. Vol 3:2-4.

Dobrinski, et al. (2018) Regulation of Spermatogonial Stem Cell Function. in: Encyclopedia of Reproduction 2nd Edition, Ed: MK Skinner. Elsevier. Vol 3: 100-104.

Westhusin and Long (2018) Reproductive Cloning. in: Encyclopedia of Reproduction 2nd Edition, Ed: MK Skinner. Elsevier. Vol 6:776-781.

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GAMETOGENESIS

Gametogenesis Overview

John R McCarrey, University of Texas at San Antonio, San Antonio, TX, United States

Gametogenesis

As the name implies, gametogenesis refers to the process by which the gametes—oocytes or eggs in females and spermatozoa or sperm in males—are generated. The gametes are the final, differentiated product of the germ line in each sex. However, unlike all other differentiated cells present in the adult, the gametes do not represent terminally differentiated cell types because they participate in fertilization to give rise to an entirely new individual. In this regard, it has been suggested that the germ line is essentially immortal. The gametes are also the only haploid cell type found in the adult. This is because the developing germ cells undergo the process of meiosis which includes one round of DNA replication followed by two rounds of cytokinesis or cellular division. Upon fertilization, the union of the two haploid gametes (sperm + egg) restores diploidy in the zygote.

Development of the Germ Line

The germ line is the first individual cell type allocated during development of the embryo proper. In mammals, the first manifestation of the germ line in each sex is the formation of primordial germ cells (PGCs) at about the stage of gastrulation in the embryo. This process is common to germline development in both the male and female and at this stage the PGCs appear identical in each sex. The PGCs arise in the epiblast and subsequently migrate to the sight of the developing genital ridges that will eventually give rise to the gonads. As with the PGCs, the early stages of gonadal development are not significantly different in males and females, although subsequent to this stage both the gonads and the germ cells undergo sexually dimorphic development. The developing gonads are pre-programmed to form ovaries in (XX) females, but expression of the Y-linked *Sry* gene in developing Sertoli cells in the testes in (XY) males induces the indifferent, bipotential gonad to develop into a testis instead of an ovary. Once this gonadal sex differentiation is initiated in the somatic cells of the developing gonads in each sex, the previously indifferent PGCs are induced to initiate sex-specific gametogenesis. Thus, PGCs in a developing ovary are induced to enter the oogenic pathway, whereas those in a developing testis are induced to enter the spermatogenic pathway. It is the sexual phenotype of the somatic elements of the developing gonad that directs the initial choice of which sex-specific gametogenic differentiation pathway the PGCs will enter, regardless of the genetic sex of the PGCs. Normally, genotypically female XX PGCs will be present in a phenotypically female developing ovary and will be induced to initiate oogenesis, whereas genotypically male XY PGCs will be present in a phenotypically male developing testis so will be induced to initiate spermatogenesis. However, even when XY PGCs are present in a developing ovary, they will be induced to initiate oogenesis, just as XX PGCs present in a developing testis will be induced to initiate spermatogenesis. Nevertheless, although the sex-specific somatic phenotype of the developing gonad directs the sex-specific initiation of either oogenesis or spermatogenesis, the ability of the germ cells to complete gametogenesis by forming either functional oocytes or sperm is limited by their sex-specific genotype, such that XY germ cells do not normally produce functional oocytes (though this has been reported on occasion) and XX germ cells never produce functional spermatozoa.

The first manifestation of sexually dimorphic gametogenesis is the entry by oogenic cells into prophase of meiosis I during fetal development while at this same stage spermatogenic cells remain mitotic and do not enter meiosis until well after birth in association with puberty. In both males and females, entry of germ cells into meiosis is stimulated by retinoic acid (RA) produced by gonadal somatic cells. This takes place during the fetal stages in both the developing ovary and the developing testis. However, in the fetal testis, there is also production of an inhibitor of RA (cytochrome P450 26B1 encoded by the *Cyp26b1* gene) which effectively blocks the meiosis-inducing signaling function of RA, and so prevents the induction of entry into meiosis by germ cells in the fetal testis. Prior to and at the time of puberty in the postnatal testis, RA is again produced by somatic cells in the pubertal testis in the absence of any simultaneous production of cytochrome P450 26B1, resulting in induction of entry of spermatogonia into meiosis at this time.

In response to production of RA in the fetal ovary, PGCs enter first meiotic prophase and progress to the diplotene stage. But they then suspend further progression through subsequent phases of meiosis and remain meiotically quiescent until stimulated to resume progression through meiosis upon ovulation in the adult. This prolonged suspension of meiotic progression at the stage when homologous chromosomes have paired on the first meiotic metaphase plate is the source of the marked increase in aneuploid offspring of older mothers due to non-disjunction of the paired homologues.

At the same time when female germ cells are entering into meiosis followed by entry into meiotic quiescence, fetal male germ cells remain mitotic, but also enter a state of mitotic quiescence. This quiescence is more transient than that observed in developing oocytes, lasting only until resumption of expansion of the spermatogonial pool in the immature, postnatal testis.

Nevertheless, it is very intriguing to note that both female and male germ cells enter a stage of quiescence during the latter portion of fetal development. This period of quiescence corresponds to a period of genome-wide epigenetic reprogramming unique to the developing germ line. Whether or not proliferative quiescence is necessary to facilitate germ line-specific epigenetic reprogramming is not known.

Postnatal Gametogenesis

Another striking dimorphism in the manner in which gametogenesis takes place in females and males, respectively, is that the cohort of primary oocytes that develops during the fetal period represents the entirety of the lifelong oogenic pool under normal circumstances in the female. It appears that all of the developing female germ cells enter first meiotic prophase during the fetal period, after which they retain the ability to complete the meiotic divisions, but are not able to further propagate their numbers, thereby limiting the oogenic pool to those primary oocytes that are present at birth. Indeed, many of the early primary oocytes undergo cell death, thus further limiting the pool of oocytes available during the reproductive lifespan of the female. Typically, exhaustion of the primary oocyte pool correlates with menopause in female mammals.

In contrast to the developmental dynamics of oogenesis, the dynamics of spermatogenesis are quite distinct. In the fetal testis, male PGCs develop into prospermatogonia that continue to divide mitotically, thus increasing their numbers significantly. After birth, these mitotically expanded prospermatogonia can either give rise directly to progenitor and/or differentiating spermatogonia, or they can enter a cell death pathway, or, in the case of a small subpopulation of the prospermatogonial pool, they can form spermatogonial stem cells (SSCs). SSCs are unique in that they can either self-renew or proceed through the spermatogenic differentiation pathway. The SSCs are the only cell type in the entire spermatogenic lineage capable of undergoing self-renewal. To accomplish both maintenance of the SSC pool and contribution of cells that will continue through the spermatogenic pathway, the SSCs must undergo, directly or indirectly, an asymmetric division whereby a portion (likely half) of the mitotic progeny of SSCs retain SSC identity and function, while the other portion become committed to entering the spermatogenic pathway. The latter progenitor spermatogonia undergo further mitotic expansion before initiating the spermatogenic pathway as differentiating spermatogonia.

Meiosis

Meiosis is a critical, unique feature of gametogenic cell lineages. Meiosis facilitates multiple beneficial outcomes. Meiotic recombination generates unique rearrangements of the paternal and maternal genomes prior to fertilization. In addition, meiosis achieves a reduction division that yields haploid eggs or sperm that can then re-establish diploidy upon the union of a sperm and egg at fertilization. However, the manner in which meiosis is achieved is also dimorphic during female and male gametogenesis. As noted above, during fetal development in females, all PGCs give rise to oogonia that then proceed into first meiotic prophase. From this stage on, there are no premeiotic (mitotic) female germ cells, and there is therefore no subsequent opportunity to expand the oogenic cell pool beyond completion of the two meiotic divisions. As a result, females possess their entire lifetime supply of germ cells when they are born and this pool will then be steadily reduced by ovulation or cell death throughout the remainder of the female's reproductive lifespan. In contrast, the mitotic SSCs persist throughout the reproductive lifespan of the male and, in addition to sustaining the SSC pool by self-renewal, give rise to progenitors and then differentiating spermatogonia that then progress into meiosis during each succeeding wave of spermatogenesis in the testis.

Albeit on the basis of very different developmental schedules, germ cells progress through the same stages of meiosis in both males and females. This includes an initial round of DNA replication, followed by the various stages of first meiotic prophase (preleptotene, leptotene, zygotene, pachytene, and diplotene) and completion of the first meiotic division as primary oocytes or spermatocytes followed by completion of the second meiotic division as secondary oocytes or spermatocytes prior to formation of the haploid products of meiosis—the ovum in the female and the spermatids in the male. These steps occur as distinct stages during spermatogenesis as cells progress from premeiotic spermatogonia to meiotic primary spermatocytes and then secondary spermatocytes to postmeiotic round and then elongating spermatids to spermatozoa. During oogenesis, these steps occur in rapid succession following resumption of first meiotic prophase in the female, and are triggered by ovulation and fertilization.

Postmeiotic Gametogenesis

The completion of the first and second meiotic divisions is very disproportionate in oocytes, yielding the very small first and second polar bodies while leaving the large majority of the ooplasm intact in the ovum. Thus, only one of the four products of female meiosis yields a functional gamete, but that one gamete will contain abundant maternal nutrients and factors that will sustain preimplantation embryogenesis following fertilization. In the male, all four products of meiosis emerge as round spermatids that then undergo the differentiative process of spermiogenesis to progress from small, round haploid cells to elongating spermatids and finally to the highly differentiated spermatozoa, all in the absence of any further cellular division. The resulting testicular spermatozoa are then released from the seminiferous epithelium by the process of spermiation and are then transported through the lumen of the seminiferous tubule to the rete testis which serves as a collection area prior to transport of spermatozoa out of the testis and into the epididymis via the efferent ducts. The spermatozoa then traverse the various sections of the epididymis, including the caput, corpus, and cauda regions before moving into the vas deferens from where they are available for release from the male via ejaculation. Transit of the testicular spermatozoa through the epididymis is critical to the acquisition of motility and fertility, yielding mature spermatozoa competent to participate in fertilization.

The Gametogenic Transcriptomes

Unique patterns of gene expression are required to facilitate the unique gametogenic processes of oogenesis and spermatogenesis. Because the gametes are, in many ways, distinct from any of the somatic cell types, there are numerous oogenesis- or spermatogenesis-specific members of gene families, and/or variants of mRNAs (e.g., splicing variants) or proteins (e.g., post-translational modifications). The transcriptomes associated with oogenesis and spermatogenesis are highly complex and largely unique, rivaled only by the brain in these respects. Coordination of expression of these unique transcriptomes is facilitated by many gametogenesis-specific regulatory factors including oogenesis- or spermatogenesis-specific transcription factors, chromatin remodeling factors, and components of other regulatory networks.

Germline-Specific Epigenetic Reprogramming During Gametogenesis

In addition to undergoing meiosis common to both oogenesis and spermatogenesis, as well as the sex-specific differentiation patterns associated with oogenesis or spermatogenesis to yield the egg (ovum) or spermatozoon (sperm), respectively, the germ cells also undergo a unique process of epigenetic reprogramming coincident with the process of gametogenesis in each sex. As noted above, germ cells are unique in that they undergo extensive differentiation to yield the male and female gametes, but these gametes then participate in fertilization to give rise to a complete new individual in which all cell types will subsequently re-emerge. Thus, distinct regulatory mechanisms are required to (1) direct the gametogenic differentiation processes and (2) prepare the gametogenic genomes to contribute to development of the embryo following fertilization. This is achieved by reprogramming of the epigenome in both the preimplantation embryo and the developing germ lines.

Embryonic reprogramming is achieved by erasing a majority of the epigenetic programming that is inherited via the gametes at the time of fertilization. This is followed by resetting of epigenetic programming that is common to the precursors of both somatic and germline cells and is complete by the time of gastrulation in the developing embryo. Subsequently, all somatic cell lineages retain the majority of this reset epigenetic programming. However, a second round of germline-specific epigenetic programming is initiated in the PGCs in which extant epigenetic programming is once again erased—to an even greater extent than the erasure that occurs in the preimplantation embryo, and this is followed by yet another round of resetting of epigenetic programming that follows distinct patterns in oogenic and spermatogenic cells, respectively. This germline-specific epigenetic reprogramming facilitates the processes of gametogenesis in both sexes, including meiosis and either oogenesis or spermatogenesis, as well as the preparation of the gametogenic genomes to direct subsequent early embryonic development following fertilization.

Maintenance of Enhanced Genetic Integrity During Gametogenesis

A critical function of the germ line that must be maintained during gametogenesis in each sex is the integrity of the gametogenic genomes, such that the genetic information transmitted from one generation to the next is preserved in an optimally pristine state. While a relatively small amount of genetic variation among individuals and between generations is beneficial, most spontaneous or induced genetic variation is not advantageous. Therefore it is not surprising that genetic integrity is maintained more stringently and faithfully in the germ line than in any somatic cell lineage. This is achieved by elevated levels of expression and corresponding activity of multiple cell death and DNA repair pathways. The former are typically induced in response to the occurrence of large scale genetic defects or damage such as polyploidy, aneuploidy, major chromosomal rearrangements or extensive DNA damage genome wide, thus dealing with these defects retrospectively. However, more minor genetic defects such as point mutations which are the most common disease-related genetic defects do not typically induce cell death and can only be minimized by prospectively preventing their occurrence. Enhanced DNA repair activities serve to reduce the initial occurrence of point mutations by increasing the likelihood that initial DNA damage will be corrected prior to becoming stabilized as a heritable point mutation. These elevated levels of cell death and DNA repair activities exemplify a situation in which a long-term evolutionary benefit offsets the evolutionary expense of the elevated investment of additional energy required to achieve the beneficial outcome.

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Regulation of Spermatogonial Stem Cell Function

Ina Dobrinski, Whitney Alpaugh, and Taylor Goldsmith, University of Calgary, Calgary, AB, Canada

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Glossary

SSC Spermatogonial stem cell
Rhox10 Reproductive homeobox 10
A$_s$ A single spermatogonia
A$_{pr}$ A paired spermatogonia
A$_a$ A aligned spermatogonia
PI3K Phosphoinositide 3-kinase
AKT Protein kinase B (PKB)
MAP2K1 Mitogen-activated protein kinase kinase 1
Etv5 ETS Variant 5
CXCR4 C-X-C chemokine receptor 4
RARɣ Retinoic acid receptor gamma
mTORC1 Mamalian target of rapamycin complex 1
REDD1 Regulated in development and DNA damage response 1
STAT3 Signal transducer and activator of transcription 3

Spermatogonial Stem Cell Dynamics

The majority of our understanding of SSC biology comes from rodent models; therefore, this chapter will focus on evidence from the mouse model. SSCs arise postnatally from the transient pro-spermatogonia or gonocyte population. Prospermatogonia are the only germ cells present in the neonatal testis cords until expression of the transcription factor reproductive homeobox 10 (*Rhox10*) provides the signal to differentiate and establish the SSC pool (Song et al., 2016). In an adult testes, SSCs are rare and believed to constitute only about 0.03% of all germ cells (Tegelenbosch and De Rooij, 1993). The low proportion of SSCs in the total germ cell population can support spermatogenesis through multiple mitotic amplification divisions. The number of amplification divisions differs between species which greatly influences their spermatogenic output (Hess and Renato de Franca, 2008). In a mouse testes, SSCs are a subset of A$_{single}$ (A$_s$) spermatogonia, which divide to A$_{paired}$ (A$_{pr}$) and then A$_{aligned}$ (A$_{al}$) (4, 6, 8,16 and rarely 32 cells) spermatogonia. A$_{al}$ cells then differentiate to A1 spermatogonia without division, then divide to form A2, A3, A4, intermediate and type B spermatogonia before entering meiosis as a spermatocyte (De Rooij, 2001). These spermatogonia clones are all linked by intercellular bridges resulting from incomplete cytokinesis. The main hypotheses for the role of these intercellular bridges involve the ability for the cellular clone to share cytoplasmic content and facilitate synchronous cell division in the later spermatogonia clones (A1-type B spermatogonia). This becomes especially important for coordinating meiotic entry (Greenbaum et al., 2011).

It has long been thought that once A$_s$ spermatogonia divide to become A$_{pr}$ spermatogonia, the cells are committed to differentiation. However, more recently, a series of studies demonstrated that early progenitor cells are capable of reverting back to a stem cell phenotype (Barroca et al., 2009; Nakagawa et al., 2010; Hara et al., 2014). Through transplantation experiments, Barroca et al. (2009) found that isolated progenitor cells were able to repopulate a germ-cell-depleted testes albeit at a much lower efficiency than isolated stem cells. Through in vivo imaging studies, Nakagawa et al. (2010) established that, during steady state spermatogenesis in mouse, dividing clones up to A$_{al-8}$ have the ability to fragment from their dividing sister cells, become an A$_s$ spermatogonium and regain stem cell function. The rate of fragmentation was increased considerably during regeneration after toxic insult to the testes. Similar findings were previously reported in *Drosophila melanogaster* (Brawley and Matunis, 2004) which demonstrates that this phenomenon of transient amplifying progenitor cells being capable of de-differentiating is highly conserved.

Spermatogonial Stem Cell Markers

One persistent challenge in the field of SSC biology is the ability to specifically identify and isolate this cell population for study. Currently, the only conclusive way to determine if a population of cells contains stem cells is by transplanting them into a germ cell-depleted recipient animal and examine the recipient testes 2 months post transplantation for donor derived colonization and spermatogenesis. However, the dynamic nature of SSCs described above confounds this as well as other approaches to examine SSCs.

The identification of several differentially expressed proteins have helped to characterize subpopulations of spermatogonia that have increased stem cell potential (Table 1). In Shinohara et al., 1999, Shinohara et al. found that cell populations enriched

Table 1   Summary of factors involved in SSC regulation discussed in this article.

Name	Role in SSC Biology	Evidence
β1-integrin	Positive population enriched in stem cell activity	Transplantation
α6-integrin	Positive population enriched in stem cell activity	Transplantation
THY-1	Positive population enriched in stem cell activity	Transplantation
ID4	SSC marker	Immunohistochemistry and transplantation
PAX7	SSC marker	Immunohistochemistry, lineage tracing and transplantation
GDNF	Required for SSC self-renewal signaling through GFRα1/RET	Knock-out studies and in vitro culture system
FGF2	Important for SSC self-renewal	In vitro culture system
CSF1	Important for SSC self-renewal	Microarray studies, immunohistochemistry and in vitro culture system
CXCL12	Required for SSC maintenance signaling through CXCR4	Immunohistochemistry, in vitro culture system and knockout model studies
Retinoic acid	Pulsatile retinoic acid stimulation is required for differentiation and spermatogenesis	Vitamin A deficiency models, immunohistochemistry, knockout model studies and in vitro culture system
PLZF	Undifferentiated spermatogonia marker and required for SSC self-renewal and maintenance	Immunohistochemistry, knock-out studies, transplantation and in vitro culture system
GFRα1/RET	Undifferentiated spermatogonia marker and required for SSC self-renewal	Immunohistochemistry, in vivo imaging and in vitro culture system
BCL6B	Expression required for SSC self-renewal	Microarray studies, in vitro culture system and knockout model studies
RB1	Required for SSC self-renewal and maintenance of the pool	In vitro culture system and conditional knockout studies
NANOS2	Undifferentiated spermatogonia marker, required for SSC self-renewal and maintenance	Immunohistochemistry, lineage tracing, knockout and overexpression models
Reactive oxygen species	Finite amounts required for SSC self-renewal	In vitro culture system, inhibitor/enhancer studies and transplantation studies
SALL4	Early differentiating spermatogonia marker required for SSC differentiation	Immunohistochemistry, co-immunoprecipitation, knockout studies and ChIP-seq
NGN3	Early differentiating spermatogonia marker required for SSC differentiation	Immunohistochemistry, lineage tracing experiments, transplantation and in vitro culture system
SOX3	Expression required for early spermatogonia differentiation	Immunohistochemistry and knockout model studies

for β1-integrin and α6-integrin expressing spermatogonia have higher stem cell capacity following transplantation than non-enriched cells. Transplantation experiments using THY-1$^+$ (CD90.2) spermatogonia have also established this population to be enriched in stem cell activity (Kubota et al., 2003). Glial cell line-derived neurotrophic factor receptor α 1 (GFRα1) is expressed in A$_s$, A$_{pr}$ and some A$_{al}$ spermatogonia and has become a well established protein marker of the undifferentiated spermatogonia population that contains stem cells (Hofmann et al., 2005).

Recently, two other proteins, inhibitor of DNA binding 4 (ID4) and paired box transcription factor 7 (PAX7), have been introduced to the field as more specific stem cell markers (Oatley et al., 2011; Aloisio et al., 2014). ID4 expression is restricted to a subset of A$_s$ spermatogonia and, upon transplantation of ID4 positive spermatogonia, recipient testes are extensively colonized (Oatley et al., 2011; Chan et al., 2014). PAX7 is expressed in a very rare subpopulation of A$_s$ spermatogonia. Lineage tracing experiments found that a single PAX7 positive spermatogonium can differentiate to germ cells of all spermatogenic stages. Additionally, PAX7 positive spermatogonia are resistant to germ cell ablation by chemo- and radiotherapy and expand in number after these toxic insults (Aloisio et al., 2014). This regenerative cell behavior has been establish as a stem cell hallmark in other stem cell systems.

Spermatogonial Stem Cell Niche Regulation

The specific cellular, molecular and structural environment that supports stem cell function is known as its niche. The SSC niche is located at the periphery of the seminiferous tubules along the basement membrane produced by Sertoli and peritubular myoid cells. A schematic representation of the cell associations and factors contributing to the stem cell niche in the testis is presented in Fig. 1. Time-lapse imaging experiments have also revealed that undifferentiated type A spermatogonia are over-represented along sections of the seminiferous tubules adjacent to blood vessels and migrate away from this vascular-associated niche upon differentiation (Yoshida et al., 2007).

Several niche-cell secreted ligands are important for SSC maintenance (Table 1). The best studied ligand, glial cell-line derived neurotrophic factor (GDNF), is secreted by Sertoli as well as peritubular myoid cells under testosterone pulsatile controlled release
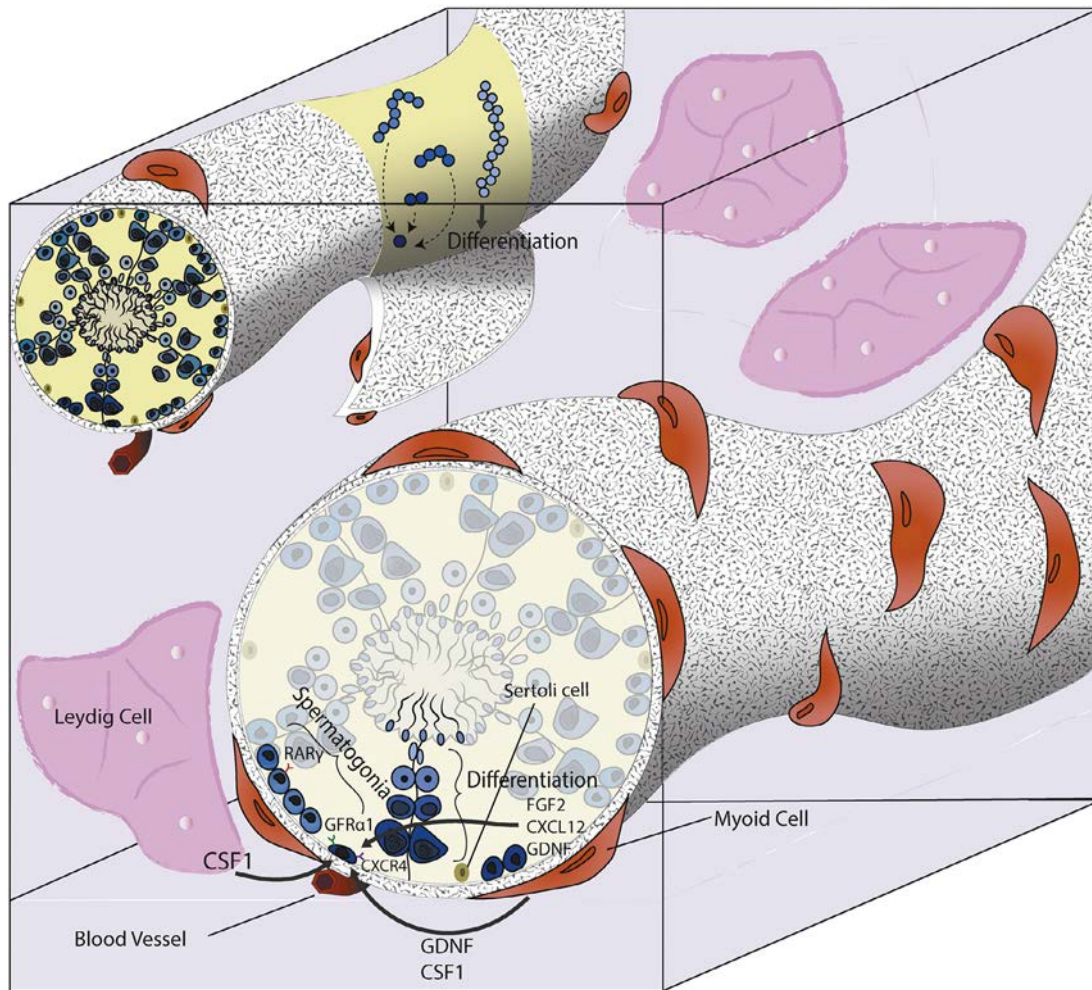
Fig. 1   Schematic illustration of the SSC niche in the testis.

(Meng et al., 2000; Tadokoro et al., 2002; Yomogida et al., 2003; Chen et al., 2016). GDNF signals through the GFRα1/RET tyrosine kinase receptor dimer located on undifferentiated spermatogonia to activate the PI3K-Akt pathway which promotes proliferation and self-renewal (Braydich-Stolle et al., 2007; Lee et al., 2007; Oatley et al., 2007). Another important factor for SSC self-renewal is fibroblast growth factor 2 (FGF2) which activates MAP2K1 and ultimately up-regulates *Etv5* and *Bcl6b* gene expression (Kubota et al., 2004; Ishii et al., 2012). Mouse spermatogonia in vitro culture systems require these two growth factors, GDNF and FGF2, for expansion of undifferentiated spermatogonia.

A microarray screen revealed that colony stimulating factor 1 receptor (*Csf1r*) is up-regulated on THY1$^+$ compared to THY$^-$ testis cells (Oatley et al., 2009). Colony stimulating factor 1 (CSF1) ligand is secreted by both Leydig and peritubular myoid cells and promotes self-renewal of SSCs in vitro. Another ligand-receptor interaction that is important for SSC self-renewal is CXCL12 secreted by Sertoli cells and CXCR4 which is expressed on a subset of undifferentiated spermatogonia (Yang et al., 2013b). Upon inhibition of CXCR4, spermatogonia display increased propensity to differentiate in vitro upon retinoic acid (RA) stimulation (a potent inducer of differentiation) and this leads to disrupted spermatogenesis in vivo.

RA is synthesized from dietary vitamin A and RA signaling from Sertoli and other germ cells is essential for spermatogenesis (Morales and Griswold, 1987; Sugimoto et al., 2012). As GFRα1 positive undifferentiated spermatogonia begin to differentiate, they start expressing the transcription factor neurogenin-3 (NGN3). NGN3$^+$ spermatogonia express retinoic acid receptor gamma (RARγ) and are therefore responsive to retinoic acid induced differentiation (Ikami et al., 2015).

Transcriptional Regulation of Spermatogonial Stem Cells

Our understanding of the transcriptional regulation of SSC maintenance is constantly evolving and a hot area of research. Several factors that play a key role in SSC self-renewal and differentiation have been identified (Table 1).

Self-Renewal

In 2004, the *luxoid* mutant mouse, which has a mutation in the gene encoding *Plzf*, was observed to have severe spermatogenic defects (Buaas et al., 2004; Costoya et al., 2004). PLZF is expressed in undifferentiated spermatogonia and its absence leads to progressive germ cell loss. More recently, Hobbs et al. (2010) demonstrated that PLZF decreases mammalian target of rapamycin complex 1 (mTORC1) activity, which promotes proliferation and differentiation in multiple stem cell systems, via inducing mTORC1 inhibitor regulated in development and DNA damage response 1 (REDD1). There is evidence that this regulation of mTORC1 by PLZF affects the responsiveness of undifferentiated spermatogonia to GDNF self-renewal signaling. Microarray analysis of established spermatogonia cultures grown in the presence or absence of GDNF identified expression of the transcription repressor B-cell lymphoma member 6 (*Bcl6b*) as being regulated by GDNF signaling and important for SSC self-renewal (Oatley et al., 2006). Further analysis of *Bcl6b* knock-out testes revealed degenerating tubules, devoid of spermatogenesis confirming the role of BCL6B in SSC self-renewal in vivo.

Expression of a tumor suppressor gene that encodes for retinoblastoma protein (RB) has also been shown to be important for SSC maintenance and self-renewal (Hu et al., 2013; Yang et al., 2013a). Conditional knockout studies of *Rb1* in the germline show that first wave spermatogenesis occurs followed by progressive loss of all germ cells due to failure of the GRF$\alpha 1^+$ A$_s$ spermatogonia population to self-renew. Lineage tracing experiments demonstrated undifferentiated spermatogonia expressing RNA-binding protein NANOS2 give rise to the entire spermatogenic lineage (Sada et al., 2009). Conditional knockout and over-expression studies in vivo show that NANOS2 is required for SSC self-renewal and long-term maintenance of the pool. In addition to expression of these specific genes, the generation of finite amounts of reactive oxygen species (ROS) within SSCs was shown to be required for self-renewal in vitro (Morimoto et al., 2013). When ROS generation was inhibited in vitro, numbers of spermatogonia in cultures decreased but when ROS production is slightly elevated, SSC doubling time decreases which, in turn, increases SSC cell numbers.

Differentiation

Several transcriptional mechanisms of SSC differentiation have also been identified. Expression of the transpiration factor spalt-like 4 (Sall4) in undifferentiated spermatogonia promotes differentiation by suppressing PLZF activity (Hobbs et al., 2012; Gassei and Orwig, 2013; Lovelace et al., 2016). PLZF promotes SSC self-renewal, in part, by suppressing gene networks required for differentiation. SALL4 removes this repression by interacting with PLZF to facilitate differentiation. Similar to the antagonist relationship of SALL4 with PLZF, increasing expression of neurogenin 3 (NGN3) in the GFR$\alpha 1^+$ undifferentiated spermatogonia population coincides with differentiation (Yoshida et al., 2004; Nakagawa et al., 2010). NGN3 expression was found to be regulated by STAT3 activation and to ultimately induce differentiation in vitro and in vivo following transplantation of *Ngn3* deficient spermatogonia (Kaucher et al., 2012). Sry-related homeobox 3 (SOX3) has also been associated with NGN3 expression and early spermatogonia differentiation (Raverot et al., 2005). Undifferentiated spermatogonia fail to differentiate in *Sox3* knockout testes resulting in tubules containing undifferentiated spermatogonia and Sertoli cells only.

Summary

In summary, regulation of SSC function to achieve a balance between self-renewal and differentiation is essential for male fertility. While several key factors and pathways active in mammalian SSCs have been elucidated, predominantly in the mouse model (summarized in Table 1 and Fig. 1), a more complete picture and translation to other non-rodent species are still under intense investigation. Translating these mouse model findings as well as elucidating novel mechanisms in human SSC regulation are important for increasing our understanding of clinical male infertility. Knowing which pathways are critical for normal SSC maintenance provides potential targets for diagnosis and ultimately treatment of male infertility.

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Reproductive Cloning

Mark Westhusin and Charles Long, College of Veterinary Medicine and Biomedical Sciences, College Station, TX, United States

Reproductive Cloning

The goal of reproductive cloning is to produce one or more genetically identical animals that carry the same genetic makeup (genotype). Many examples of genetically identical offspring can be found in nature and range from monozygotic (identical) twins in humans to the seven-banded armadillo that can produce up to 15 genetically identical pups in a single litter. These identical animals are formed from the division of a single embryo into two or more parts, each of which can develop into a fully formed individual. Although these animals are genetically identical, they are technically not considered clones as they result from an embryo produced sexually (the combination of the genetic material of a sperm and egg) that somehow splits into several embryos during development. Reproductive cloning involves the production of genetically identical animals by employing a specific technology developed by scientists, nuclear transplantation.

In its simplest form, nuclear transplantation refers to the transfer of the nucleus (chromosomes/DNA) of one cell into another. The utilization of this technique to produce cloned animals involves transferring the nucleus of a single cell derived from the animal being cloned (cell donor), into an unfertilized oocyte collected from a female (preferably from the same animal species), and that has had its own DNA removed (enucleated oocyte). When this happens, components contained within the cytoplasm of the enucleated oocyte have the ability to reprogram the donated nucleus such that it resets the developmental clock back to the one-cell stage of development, similar to an egg just after fertilization by a sperm cell, prior to the first cell division. If successful, the reconstructed embryo derived from this process will begin to develop and can be transferred into the reproductive tract of a surrogate mother where it will develop to term and result in an animal with the same genetic traits as the cell donor (Figs. 1 and 2.)

Historical Overview

The idea of developing methods to produce, and reproduce genetically identical animals, has been around since the early 1900s. Hans Spemann, often referred to as the "father of cloning", performed extraordinary studies involving nuclear transplantation using cells derived from developing salamander embryos, resulting in the production of genetically identical offspring. He later referred to a "fantastical experiment" in his book, *Embryonic Development and Induction* (1938), where he outlined the process of nuclear transplantation, and described the basic methodology still used today to clone animals (Speman, 1938).

At the time Spemann described this procedure, the methodology and equipment needed to perform such a difficult task were not available. Therefore, it was not until the early 1950s that Robert Briggs and Thomas King were able to perform such experiments. Using a small glass pipet they removed the DNA from a *Rana pipiens* egg, replaced it with the nucleus from a blastula stage embryo, and demonstrated that cloned frogs could be produced by transferring nuclei obtained from early stage embryos into enucleated oocytes (Briggs and King, 1952).
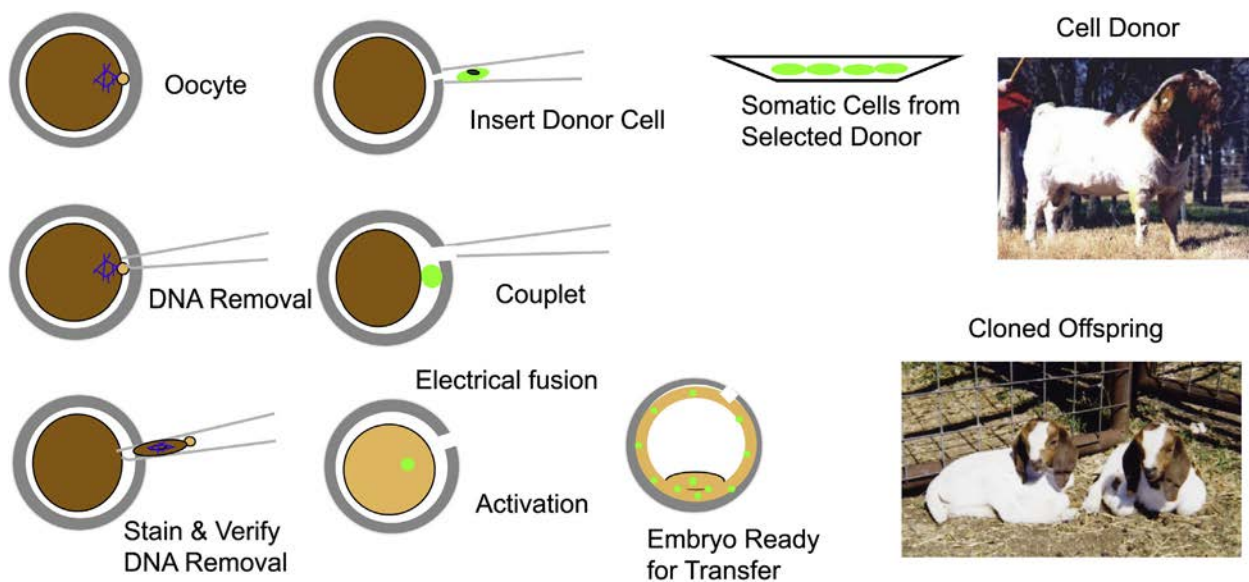

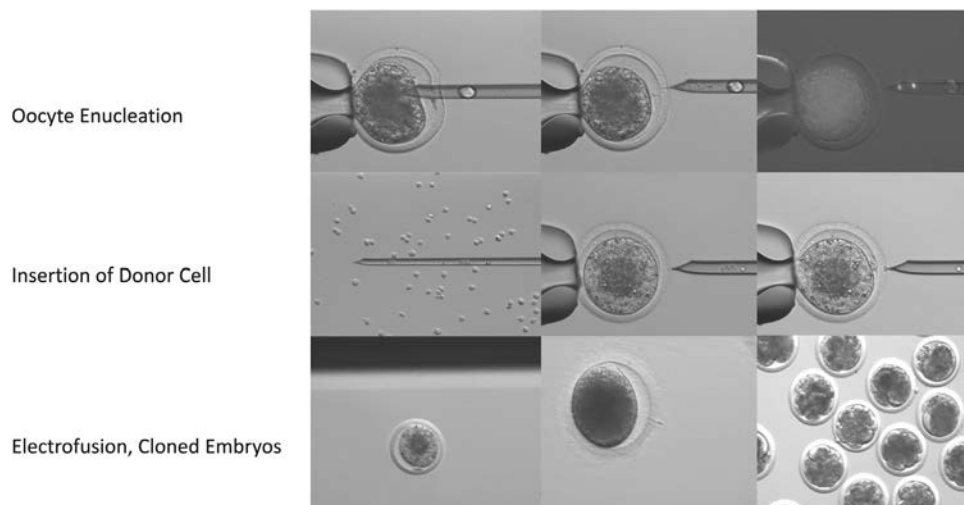**Fig. 1** Reproductive cloning by nuclear transplantation.

Fig. 2 Reproductive cloning by nuclear transplantation.

It is important to note that cells utilized for cloning in these early experiments were derived from growing embryos rather than adult animals. The dogma at that time was embryonic cells could be used for cloning because the cells had not yet differentiated into a specific cell type and thus remained totipotent and capable of developing into any tissue in the body. However, a major break-through, supporting the idea that nuclear transplantation could potentially be utilized to clone adult animals using cells that were more differentiated came in 1962 when John Gurdon reported the utilization of nuclear transfer to produce cloned frogs using cells obtained from the gut of feeding tadpoles. The transferred nucleus in these experiments had originated from cells that had clearly differentiated into a somatic cell type and thus dispelled the prevailing theory that nuclei were incapable of directing development after differentiation. The importance of this work was recognized by Gurdon being awarded a Nobel Prize in 2012 (Gurdon, 1962).

While work with amphibians was being carried out in the early and mid-1900s, it was not until the late 1970s that any significant work involving the utilization of nuclear transfer to try and clone mammals was performed. This was likely due to the much smaller size of mammalian eggs compared to amphibians, limitations in the equipment and difficulties with adapting the technology to other species. Procedures had to be developed that would allow micromanipulation of individual cells, ova and embryos in addi-tion to the expertise needed to transfer cloned embryos into the reproductive tract of the appropriate surrogate mother. Moreover, simply obtaining large numbers of oocytes from mammals that could be utilized for experiments involving nuclear transfer was in itself a challenge.

The great majority of early work with nuclear transfer in mammals involved the manipulation of ova and embryos derived from mice. Peter Hoppe and Karl Illmensee reported the successful production of cloned mice by transferring cells derived from the inner cell mass of a developing embryo into enucleated zygotes, then transferring the cloned embryos into recipient females (Illmensee and Hoppe, 1981). However, many attempts by other laboratory groups failed to repeat this work; and in 1984 Jim McGrath and Davor Solter published the results of their research stating that "cloning mammals by simple nuclear transfer was biologically impossible" (McGrath and Solter, 1984). Solter, McGrath and other scientists working with mice at that time, again thought the problem was related to embryonic differentiation. In spite of the experiments performed previously by Gurdon that demonstrated differentiated cells could be used for cloning frogs. Researchers working with mice hypothesized that in mammals, as embryonic cells became more differentiated, they could not be used for cloning. McGrath and Solter were successful at producing cloned mice by nuclear transplantation when 2-cell embryos were used as nucleus donors, but not when nuclei were derived from the 4-cell stage or beyond. In mice, the 2-cell stage of development represents the time at which the embryonic genome is activated; in other words, the first time in mouse development when cells actually begin to use their own genes to transcribe mRNA, produce new protein and differentiate into unique cell types.

Early work in other mammalian species continued to support the idea that differentiated cells sequentially lose the ability to regain totipotency following nuclear transfer. Steen Willadsen reported the successful cloning of sheep, and then cattle, using 8–16 cell embryos as nuclei donors (Willadsen, 1986). In terms of development, this is similar to mice, as maternal/zygotic transition, the time point in development when an embryo starts producing its own mRNA and protein (embryonic genome activation), begins at the 2-cell stage in mice and the 8–16 cell stage in sheep and cattle.

Willadsen's work with cattle, a species with substantial economic value, no doubt played the major role in efforts to move cloning from the research laboratory to commercial application. The initial business model was based on cloning to propagate (replicate) cattle representing the top 5%–10% in terms of phenotypic performance. If cloning could be used to increase the number of animals representing genetics of the top producers, large increases could be made in the efficiency of milk and meat production.

In the late 1980s, Willadsen moved from England to the United States to work for Granada Genetics Inc., and commercialize cloning in cattle. His efforts proved extremely successful, however, he left Granada after only about a year and took his talents

to Canada where he started a similar program focused on cloning cattle for Alta Genetics. Granada, Alta, and shortly thereafter American Breeders Services (ABS) represented the first 3 commercial entities in the world to offer commercial cloning to the cattle industry.

Early on, this approach proved to be viable, and a large number of cloned cattle were produced for various cattle producers. However, a number of unanticipated problems also occurred, and the dream of producing large numbers of cloned livestock representing the top individuals of beef or dairy cattle and utilized for the production of food and fibre never materialized. A major problem that interfered with the large scale production of clones was founded in the inefficiency of nuclear transfer when measured by the actual number of live offspring produced. Regardless of the countless experiments that were conducted to try and improve the process, the great majority of embryos produced by nuclear transfer failed to result in a viable pregnancy and/or a normal calf. This problem could be overcome by simply producing and transferring more cloned embryos. However, it soon became clear that in most cases, the costs far outweighed the benefits. Only 10%–20% of embryos produced by nuclear transplantation resulted in a viable pregnancy. In addition, another serious and unexpected problem was that a significant number (approximately 30%) of the offspring born as a result of cloning exhibited abnormal development, most notably, large birth weights associated with placental anomalies. It was not uncommon to see calves born weighing almost 2 X normal, which caused significant health problems at birth for both the calf and surrogate mothers (Wilson *et al.*, 1993). As a result, all recipient cows carrying cloned pregnancies had to be carefully monitored and many needed assistance at birth. This was simply unacceptable to the cattle industry. A final issue that also clearly played a major role in the failure to adopt cloning to produce large numbers of high-producing cattle was the unexpected observation that even though the animals were genetically identical, clones could exhibit remarkable differences in their phenotypes. As mentioned above, this first became obvious when looking at birth weights and the enormous variation that resulted. However, as more cloned animals were born, including not only cattle but sheep, goats, pigs, horses, etc., it was clear that while the genotypes were identical, the phenotypes were not. The cause of this was not clear and remains uncertain even today. However, obvious culprits include the environment in which fetal development occurs and unexplained differences in nuclear reprogramming within the oocyte needed to support normal development. In addition, since cloned animals were derived using enucleated oocytes collected from other animals, the possibility existed that the mitochondria which are exclusively inherited through the cytoplasm of the ova were also having an effect on the final phenotype.

Although various problems with producing cloned livestock using cells derived from early stage embryos prevented the large scale utilization of this technology for production agriculture, other potential applications continued to drive the research and development of cloning. The primary interest changed from using the technology to produce large numbers of genetically identical animals to producing a few, or even "one" animal representing for example, a genotype that was extremely rare or had been lost (i.e., unique animals of extremely high value that had died or were close to death, endangered and extinct animal species). Although clones did not always exhibit similar phenotypes, their genotypes were identical, therefore clones were extremely useful for breeding, thus a powerful tool that could be used for the conservation and/or propagation of a particular genotype. The other driving force was founded in the utilization of cloning as a potential tool for producing genetically modified animals. However, both of these applications required methods that would allow the utilization of genetically identical cells that had differentiated beyond embryonic, and which could be expanded in culture to obtain large numbers of cells.

One of the first key experiments which provided strong support for addressing this challenge and test the hypothesis that differentiated cells could be reprogrammed by nuclear transplantation and used to produce cloned animals was reported by Sims and First in 1994 and again by Campbell *et al.* (1996). Sims and First derived cell lines from the inner cell mass of a blastocyst stage bovine embryo and cultured these cells using methods and conditions designed to expand the cell population while at the same time maintain pluripotency (Sims and First, 1994). Campbell *et al.* utilized the embryonic disc of a sheep embryo that during culture differentiated to an epithelial type cell (Campbell *et al.*, 1996). In both cases, nuclear transfer to an enucleated oocyte produced live offspring, proving that differentiation in vitro during cell culture did not prevent reprogramming to a totipotent state when the nucleus of these cells was transferred into an enucleated oocyte. In short, differentiated cells growing in culture could be successfully utilized for reproductive cloning of mammals, a situation similar to that demonstrated by Jon Gurdon when using cells derived from frogs, over 30 years earlier.

From a historical point of view these initial experiments involving cells growing in culture predicted that reproductive cloning with cells derived from a living adult animal would soon follow; and in 1997, the scientific community, indeed the entire world was shocked by the announcement that a living mammal had been cloned (Wilmut *et al.*, 1997). The birth of a cloned sheep derived by employing nuclear transplantation and a single mammary epithelial cell obtained from an adult animal was reported. Being a female (ewe) and given the cell type utilized for cloning, she was named "Dolly" (Fig. 3). The birth of Dolly, as with many great scientific breakthroughs represented a serendipitous surprise. Keith Campbell and Ian Wilmut were using fetal cells (fibroblasts) growing in culture for nuclear transfer, with the primary objective being to genetically modify the cells prior to nuclear transfer so to produce transgenic sheep. In one series of experiments, adult cells derived from mammary epithelia were utilized as a control treatment and not expected to develop to term, but resulted in the birth of Dolly.

It is clear that the birth of Dolly was a complete surprise to everyone involved. The overwhelming response from the general public and the world as a whole was also surprising given the long history of cloning animals and having previously produced cloned sheep and cattle from cells growing in culture. The major difference of course was that Dolly was derived from a cell obtained from an adult animal and as such it revived all sorts of scary scenarios about the possibility of cloning humans. The idea of being able to clone human beings was now too close to reality, sending a shock-wave throughout society. This resulted in an avalanche of controversy and ethical debates all over the world, not to mention legal actions quickly being taken at all levels of government to
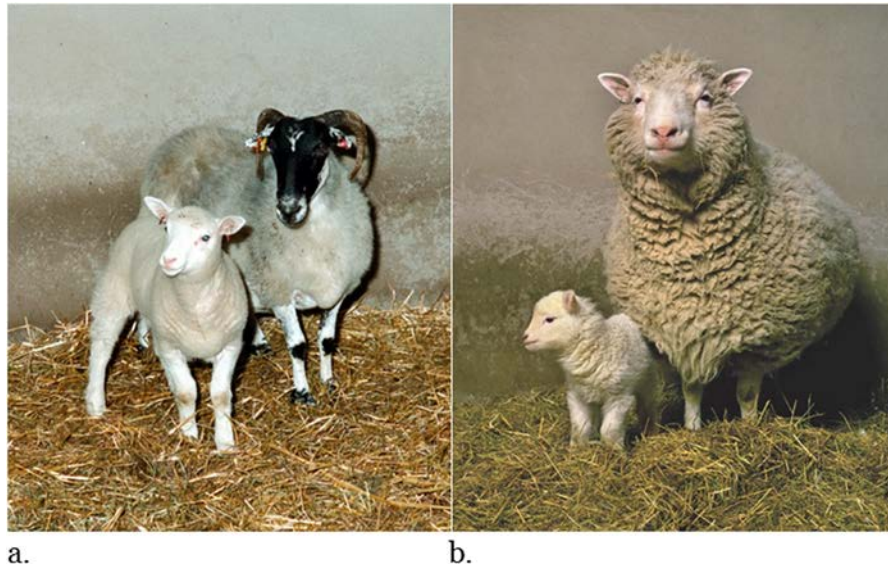
Fig. 3 (a) World's first cloned mammal, a sheep named Dolly derived from a mammary epithelial cell collected from an adult animal. The black-faced sheep is her surrogate mother (Wilmut *et al.,* 1997). (b) Dolly as an adult and her first lamb. Photo courtesy of The Roslin Institute, The University of Edinburgh and in the Acknowledgments "Photo courtesy of The Roslin Institute, The University of Edinburgh, Roslin, Scotland, United Kingdom."

ban human reproductive cloning. Even now, legislation to regulate the application of reproductive cloning is in place and enforced throughout the world, with some countries even banning reproductive cloning of animals. The birth of Dolly resulted in an immediate and enormous increase in research conducted all over the world, focused on the utilization of reproductive cloning to produce genetically identical animals. Dozens of laboratory groups launched efforts to adapt methods involving nuclear transplantation in attempts to clone a wide variety of different animal species. Cloned animals representing more than 20 different species have now been produced by reproductive cloning, providing strong evidence for the robustness of nuclear transplantation as a tool for producing genetically identical animals.

Current State of the Art

Most reproductive cloning conducted today is focused on applications to livestock species and companion animals. Advancements in the technology have made it possible to produce cloned animals using almost any cell type that can be collected from an adult animal, however, the most common is fibroblasts derived by employing standard methods for tissue culture to a skin biopsy. Also, as indicated above, the goal in the vast majority of cases is not to produce large numbers of genetically identical animals, rather to obtain just a few, even one, of a very unique animal with a highly valued genotype. Examples of this include cloning prize winning steer to obtain a bull(s) that can then be used for breeding, cloning deceased pets, and even cloning animals that are deceased but which had cells cryopreserved for storage years prior to the time cloning was even thought possible (Westhusin *et al.,* 2007). In at least one case, cloned mice have been reported that were derived from cells obtained from whole carcasses that were stored in a normal freezer (Wakyama *et al.,* 2008).

The efficiency of cloning animals by nuclear transplantation remains low, with on average only 5%–10% of the cloned embryos transferred into surrogate mothers resulting in live offspring. The outcome also remains variable and uncertain. However, over time small changes have been introduced into the protocols to control cell cycle or changes in chromatin structure of the donor cells that have, at minimum, reduced the variability of outcomes and decreased the incidence of abnormal development including large birth weights. Early on there were concerns that cloned animals would age more quickly due to shortening of telomeres as a result of using cells derived from adult animals (and older animals) for nuclear transplantation. Experiments in our own laboratory and others demonstrated this not to be the case. CC, the world's first cloned cat is now almost 16 years old (**Fig. 4**), and the world's first cloned white-tailed deer (also derived from work in our laboratory) is 15 years old, well beyond the average age these animal species normally live.

Although 5%–10% of cloned embryos will develop into normal offspring, it is important to point out that the efficiency of nuclear transplantation is oftentimes misunderstood and seemingly much more robust than it truly is. The process involves several steps and losses occur at each of these steps. For example, one may need to begin with 1000 oocytes to obtain 500 that are usable. Enucleation may leave only 200 which survive the procedure to serve as recipients for donor cells, 100 may survive transfer of the donor cell into the enucleated oocyte, and only 10 develop into a normal cloned embryo. Following the transfer of these 10 cloned

Fig. 4 The world's first cloned cat, CC. (a) Cat from which donor cells were collected. (b) CC and her surrogate mother. Note that the cell donor is a calico but CC does not exhibit this coat color pattern, indicative that cloned animals do not always exhibit the same phenotype as the cell donor. It is also noteworthy that failure of the orange allele to express may indicate the inactive X chromosome was not completely reprogrammed by nuclear transplantation. Taken from Shin, T., Kraemer, D., Pryor, J., *et al.*, 2002. A cat cloned by nuclear transplantation. Nature 415, 859.

embryos one could expect to get on average only 1 cloned offspring. Again, this is extremely variable, but the example is not unusual and explains the considerable amount of time, effort and expense that is still required to produce animals by reproductive cloning.

This example also exposes the enormous challenges that can arise when attempting to apply reproductive cloning to certain animals. In dogs for example, surgical procedures must be employed to obtain viable oocytes from the oviducts following natural ovulation. Dogs do not respond to hormone treatments that are used in other species for superovulation and estrus synchronization, and they only exhibit estrus every 6 months – 1 year. As a result, considerable time and effort is required simply to produce a few cloned embryos which have to be surgically implanted into the surrogate female. Other species present even greater challenges, most of which have nothing to do with the process of nuclear transplantation itself, but the logistics of all the other steps required for "reproductive cloning". In recent years, a few scientists have suggested the possibility of cloning long extinct species. Simple logistics and common sense make this highly unlikely. Where would one obtain oocytes to produce cloned embryos? What would serve as the surrogate mother? How would embryos ever be transferred, where and when? It is not unreasonable to expect reproductive cloning could potentially be used to re-derive some animal species that have become extinct, but the list would likely be pretty short.

At present, the overall inefficiency of reproductive cloning keeps the costs high, so the technology remains primarily a tool for the reproduction of elite commercial genotypes, i.e., animals with a specific/special purpose. Market demand has been estimated at approximately 100 horses per year, <500 cattle, and a few hundred pets (dogs and cats, Fig. 4). There is also a smaller but real demand for cloning in pigs, sheep and goats. The cost of cloning varies but ranges from 10 s of thousands of dollars to over $100,000 depending on the animal and specific circumstances. Besides the application of cloning to reproduce specific genotypes, it is also important to point out that reproductive cloning continues to play a significant role in the production of genetically modified (GM) animals, in particular livestock (Wheeler, 2007). In fact, a common method currently employed to produce GM livestock is to first produce cells growing in culture that have the desired genetic modification, then utilize these as donor cells for nuclear transplantation (reproductive cloning).

Today there are literally hundreds if not thousands of scientific publications reporting various success rates with cloning animals by nuclear transplantation. Regardless of how the data are analysed and interpreted, reproductive cloning in animals remains a very inefficient process. Although numerous experiments have been carried out to identify a variety of different culprits potentially responsible for the inefficiency of cloning, such as abnormal epigenetic regulation of gene expression or problems with cell cycle synchrony; the true mystery that remains to be explained is how it works at all. It is unremarkable that embryos produced by nuclear transplantation fail to develop. The true "miracle" is that it actually sometimes works, ultimately resulting in a normal animal.

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Campbell, K. H. S., McWhir, J., Ritchie, W. A., & Wilmut, I. (1996). Sheep cloned by nuclear transfer from a cultured cell line. *Nature, 380*, 64–66.
Gurdon, J. B. (1962). The developmental capacity of nuclei taken from intestinal epithelium cells of feeding tadpoles. *J Embryol Exp Morphol, 10*, 622–640.
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Wakyama, S., Ohta, H., Hikichi, T., et al. (2008). Production of healthy cloned mice from bodies frozen at −20 degrees C for 16 years. *Proc Natl Acad Sci USA, 105*, 17318–17322.

Westhusin, M. E., Shin, T., Templeton, J. W., Burghardt, R. C., & Adams, L. G. (2007). Rescuing valuable genomes by animal cloning: A case for natural disease resistance in cattle. *J Anim Sci, 85*, 138–142.

Wheeler, M. B. (2007). Agricultural applications for transgenic livestock. *Trends Biotechnol, 25*, 204–210.

Willadsen, S. M. (1986). Nuclear transplantation in sheep embryos. *Nature, 320*, 63–65.

Wilmut, I., Schnieke, A. E., McWhir, J., Kind, A. J., & Campbell, K. H. S. (1997). Viable offspring derived from fetal and adult mammalian cells. *Nature, 385*, 810–813.

Wilson, J. M., Williams, J. D., Bondioli, K. R., et al. (1993). Comparison of birth weight and growth characteristics of bovine calves produced by nuclear transfer (cloning), embryo transfer and natural mating. *Anim Reprod Sci, 38*, 73–83.

Slide 1

"Systems Biology of Reproduction"

Spring 2024 (Even Years) – Course Syllabus
Biol 475/575 Undergraduate/Graduate (3 Credit)
SLN: (475) – 06763, (575) – 06764
Time - Tuesday and Thursday 10:35 am-11:50 am
Course Lectures in person and recorded on Canvas/Panopto and Discussion Sessions live in person and on WSU Zoom for all campuses (Hybrid Course)
Room – CUE 418
Course Director – Michael Skinner, Abelson Hall 507, 335-1524, skinner@wsu.edu
Co-Instructor – Eric Nilsson, Abelson Hall 507, 225-1835, nilsson@wsu.edu
Learning Objective -
Current literature based course on the Systems Biology of Reproduction. Learning Systems approaches to the biology of reproduction from a molecular to physiological level of understanding.
Schedule/Lecture Outline –

January 9 & 11	Week 1	Systems Biology Introduction
16 & 18	Week 2	Molecular/ Cellular/ Reproduction Systems
23 & 25	Week 3	Sex Determination Systems
Jan /Feb 30 & 1	Week 4	Male Reproductive Tract Development & Function
February 6 & 8	Week 5	Female Reproductive Tract Development & Function
13 & 15	Week 6	Gonadal Developmental Systems Biology
20 & 22	Week 7	Testis Systems Biology
27 & 29	Week 8	Ovary Systems Biology
March 5 & 7	Week 9	Epigenetics and Transgenerational Gonadal Disease
11 – 15	**Week 10**	**Spring Break**
19 & 21	Week 11	Gametogenesis/ Stem Cells/ Cloning
26 & 28	Week 12	Hypothalamus-Pituitary Development & Function
April 2 & 4	Week 13	Reproductive Endocrinology Systems
9 & 11	Week 14	Fertilization & Implantation Systems
16 & 18	Week 15	Fetal Development & Birth Systems
23 & 25	Week 16	Assisted Reproduction/Contraception
Apr/May 30 & 2	Week 17	Exam or Grant Review

Slide 2

Spring 2024 – Systems Biology of Reproduction
Lecture Outline – Gametogenesis/ Stem Cells/ Cloning
Michael K. Skinner – Biol 475/575
CUE 418, 10:35-11:50 am, Tuesday & Thursday
March 19, 2024
Week 11

Gametogenesis/ Stem Cells/ Cloning

– Gametogenesis
– Spermatogenesis
  – Cell Development Stages
  – Meiosis
– Male Germline Stem Cell and Niche
  – Cell Biology
  – Niche
  – Regulatory Factors
– Germ Cell Transplantation
– Oogenesis
  – Cell Development
  – Meiosis
– Female Germline Stem Cells
– Embryonic Stem Cells
– Cloning

Required Reading

McCarrey JR. (2018) Gametogenesis Overview. in: Encyclopedia of Reproduction 2nd Edition, Ed: MK Skinner. Elsevier. Vol 3:2-4.

Dobrinski, et al. (2018) Regulation of Spermatogonial Stem Cell Function. in: Encyclopedia of Reproduction 2nd Edition, Ed: MK Skinner. Elsevier. Vol 3: 100-104.

Westhusin and Long (2018) Reproductive Cloning. in: Encyclopedia of Reproduction 2nd Edition, Ed: MK Skinner. Elsevier. Vol 6:776-781.

Slide 3

Spring 2024 – Systems Biology of Reproduction
Discussion Outline – Gametogenesis / Stem Cells / Cloning
Michael K. Skinner – Biol 475/575
CUE 418, 10:35-11:50 am, Tuesday & Thursday
March 21, 2024
Week 11

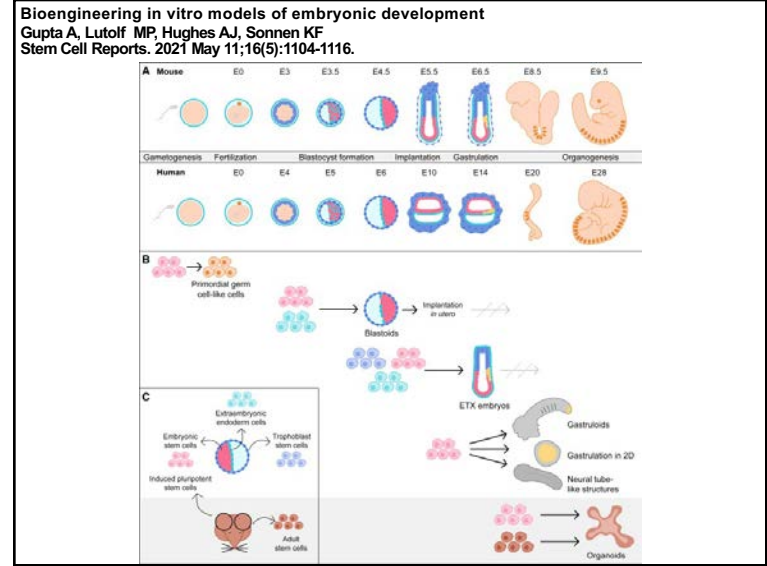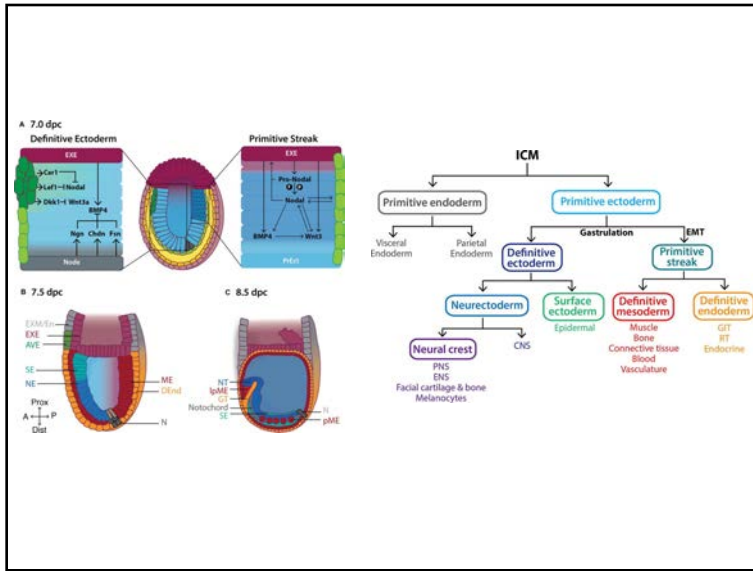### Gametogenesis / Stem Cells / Cloning

Primary Papers:

1. Ball, et al. (2016) BMC Genomics 17:628
2. Yoshino, et al. (2021) Science 373:298
3. Guo, et al. (2020) Cell Stem Cell 26:262-276

Discussion

Student 3: Reference 1 above
- What regulatory factor was identified?
- How was spermatogonial stem cell renewal identified?
- What insights into spermatogonial stem cells was provided?

Student 4: Reference 2 above
- What are the technologies used?
- What are the limitations of organ cultures?
- What clinical applications could be considered?

Student 5: Reference 3 above
- What developmental stage and cell types were investigated?
- What technology was used, and advantages compared to other technologies?
- What conclusions were made about the onset of testis function and gametogenesis?

Slide 4

Specification of trophoblast from embryonic stem cells exposed to BMP4.
Roberts RM, Ezashi T, Sheridan MA, Yang Y.
Biol Reprod. 2018 Jul 1;99(1):212-224.
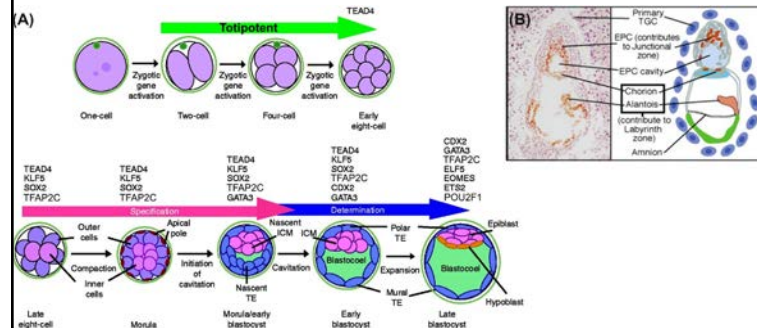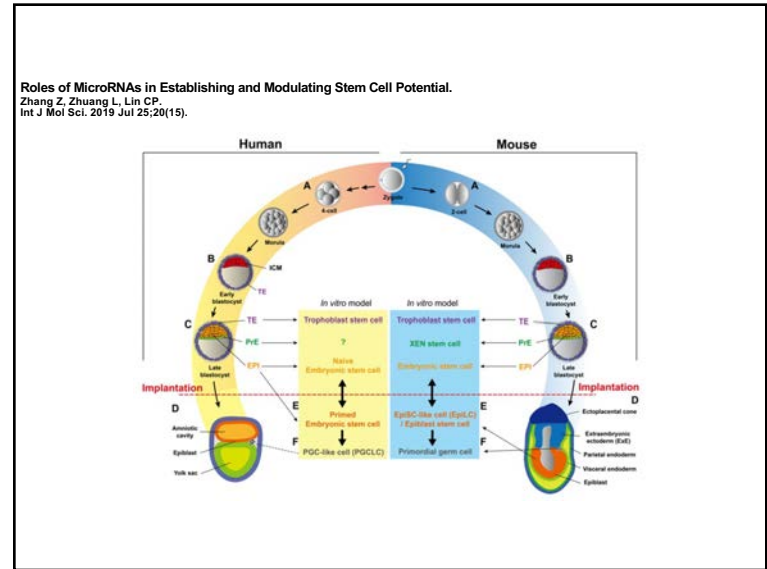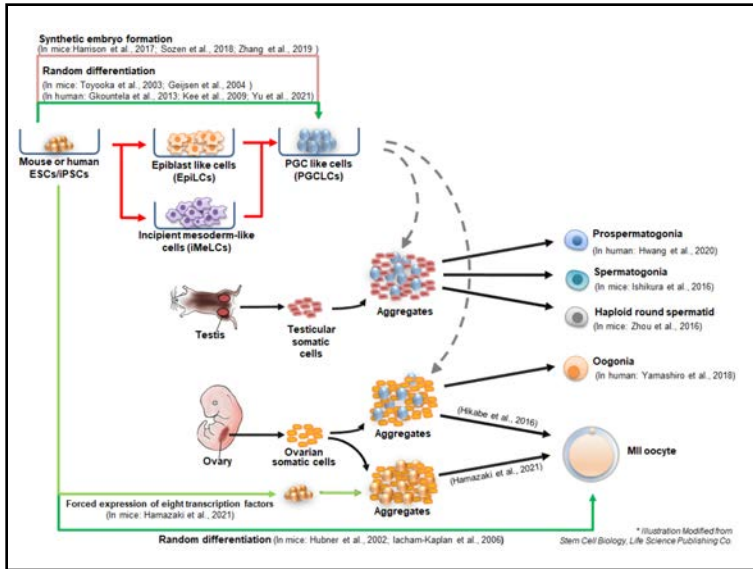
Bioengineering in vitro models of embryonic development
Gupta A, Lutolf MP, Hughes AJ, Sonnen KF
Stem Cell Reports. 2021 May 11;16(5):1104-1116.

Roles of MicroRNAs in Establishing and Modulating Stem Cell Potential.
Zhang Z, Zhuang L, Lin CP.
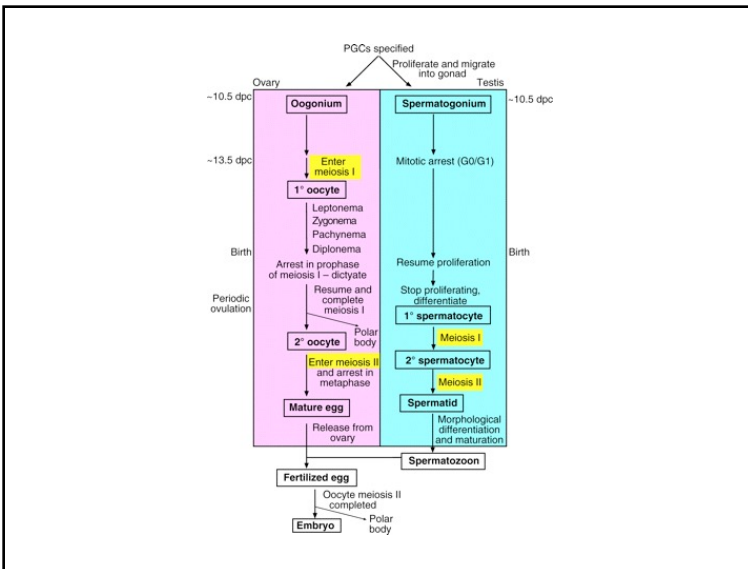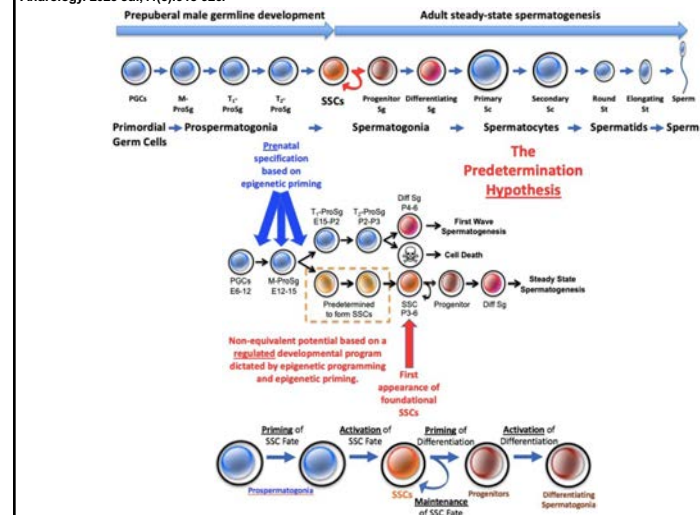Int J Mol Sci. 2019 Jul 25;20(15).

Germer Cell Derivation from Pluripotent Stem Cells for Understanding In Vitro Gametogenesis
Hong T-K, Song J-H, Lee S-B, Do J-T.
Cells. 2021 Jul 26;10(8):1889.
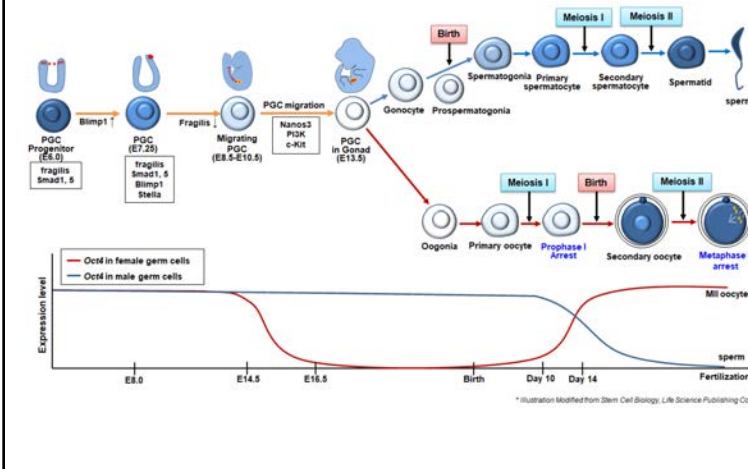
Primordial Germ Cells

Epigenetic priming as a mechanism of predetermination of spermatogonial stem cell fate.
McCarrey JR.
Andrology. 2023 Jul;11(5):918-926.

3

DMRT1 regulates human germline commitment.
Irie N, Lee SM, Lorenzi V, Xu H, et al.
Nat Cell Biol. 2023 Oct;25(10):1439-1452.

FIG. 1. Flowchart of differences in the cell-cycle progression during mammalian spermatogenesis and oogenesis (Wolgemuth *et al.*, 1995). [Modified from Alberts B, Bray D, Lewis J, Raff M, Roberts K, Watson J 1983 Molecular Biology of the Cell. New York: Garland Publishing, Inc.]

FIG. 4. Possible restriction points during mitosis and meiosis.

Meiosis initiation: a story of two sexes in all creatures great and small
Sou IF, Pryce RM, Tee W-W, McClurg UL.
Biochem J. 2021 Oct 29;478(20):3791-3805.

4

A Oogenesis

B Spermatogenesis

The molecular regulatory mechanisms of meiotic arrest and resumption in Oocyte development and maturation.
Pei Z, Deng K, Xu C, Zhang S.
Reprod Biol Endocrinol. 2023 Oct 2;21(1):90

Oogenesis in Women: From Molecular Regulatory Pathways and Maternal Age to Stem Cells.
Krajnik K, Mietkiewska K, Skowronska A, Kordowitzki P, Skowronski MT.
Int J Mol Sci. 2023 Apr 6;24(7):6837.

Epigenetic transgenerational inheritance, gametogenesis and germline development.
Ben Maamar M, Nilsson EE, Skinner MK.
Biol Reprod. 2021 Sep 14;105(3):570-592

5

Spermatogenesis

FIGURE 1
The human infant preformed in the sperm as depicted by Nicolas Hartsoeker (1694).
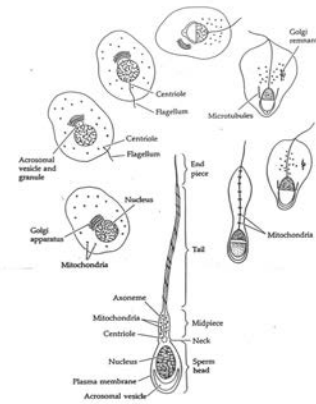
FIGURE 2
The modification of a germ cell to form a mammalian sperm. The centriole produces a long flagellum at what will be the posterior end of the sperm, and the Golgi apparatus forms the acrosomal vesicle at the future anterior end. The mitochondria (hollow dots) collect about the flagellum near the base of the haploid nucleus and become incorporated into the midpiece of the sperm. The remaining cytoplasm is jettisoned, and the nucleus condenses. The size of the mature sperm has been enlarged relative to the other figures. (After Clermont and Leblond, 1955.)
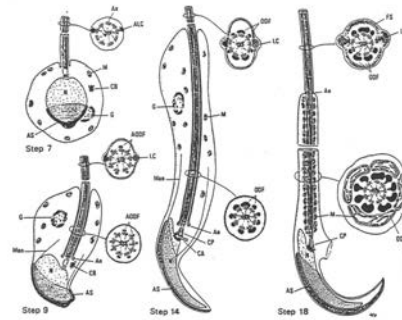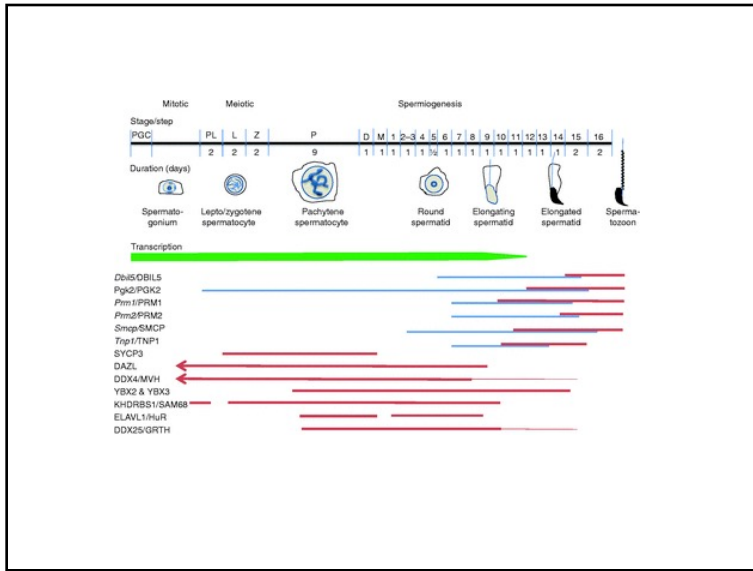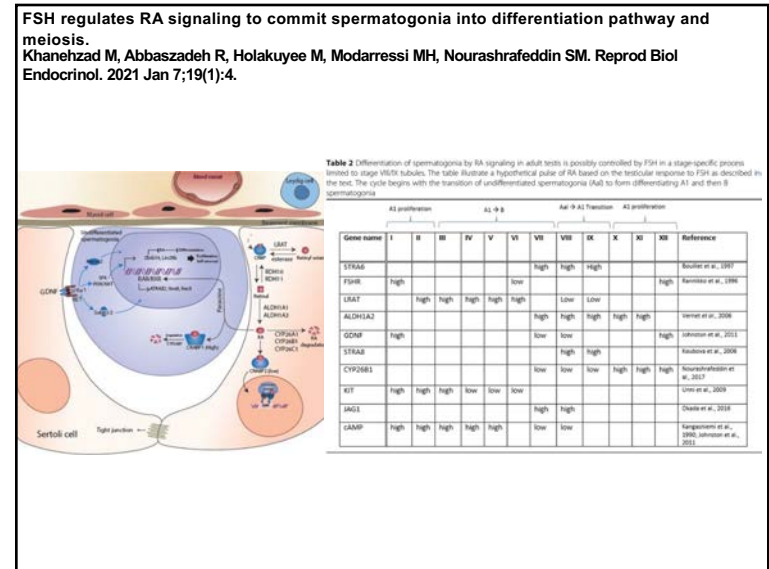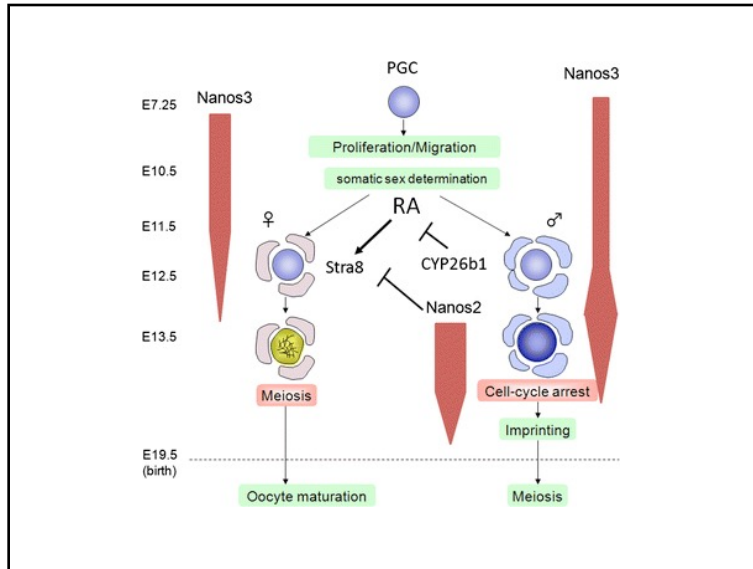

FIGURE 7. Diagram showing the main ultrastructural changes taking place in the developing tail of the rat spermatid. Four steps of spermiogenesis are illustrated, i.e., steps 7, 9, 14, and 18. Cross-sectional outlines of the proximal and distal regions of the tail are shown with the various components, i.e., outer dense fibers (ODF) and their anlagen (AODF), and fibrous sheath with its longitudinal column (LC) and its anlagen (ALC) and the ribs. The development of the principal piece with its fibrous sheath is illustrated in the four electron microscope cross-sectional profiles above, while the development of the middle piece with the outer dense fibers (ODF) and their anlagen (AODF) are illustrated in the three electron microscope cross-sectional profiles below. Note that until the end of step 14, the tail structures are enclosed within a perinuclear compartment delimited in its proximal portion by a double plasma membrane which extends down to the annulus (An), located near the connecting piece. Early in step 15, the annulus slides along the axoneme and concomitantly, the ODF increase in diameter and mitochondria migrate toward the tail. Other lettering: Man, manchette; AS, acrosomic system; N, nucleus; M, mitochondria; CB, chromatoid body; G, Golgi apparatus; CA, centriolar adjunct; CP, connecting piece.

6

FIG. 5. Summary of the expression of selected cell-cycle-regulating genes in the mouse testis and ovary. This figure represents a composite of the results of studies from our laboratory on the levels of expression of the genes denoted on the left. The references containing the data supporting this compilation may be found in a review by Wolgemuth et al. (1995) and in references cited in this review. The relative levels of expression are depicted by shading of the bars: solid black, most robust expression of a particular gene; white bar, no detection of expression.

Regulatory complexity revealed by integrated cytological and RNA-seq analyses of meiotic substages in mouse spermatocytes.
BMC Genomics. 2016 Aug 12;17(1):628.
Ball RL, Fujiwara Y, Sun F, Hu J, Hibbs MA, Handel MA, Carter GW.

Stra8 and its inducer, retinoic acid, regulate meiotic initiation in both spermatogenesis and oogenesis in mice

Ericka L. Anderson,* Andrew E. Baltus,* Hermien L. Roepers-Gajadien,† Terry J. Hassold,‡ Dirk G. de Rooij,† Ans M. M. van Pelt,† and David C. Page* §

*Howard Hughes Medical Institute, Whitehead Institute, and Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02142; †Center for Reproductive Medicine, Departments of Obstetrics and Gynecology, Academic Medical Center, University of Amsterdam, 1012 ZA, Amsterdam, The Netherlands; and ‡School of Molecular Biosciences and Center for Reproductive Biology, Washington State University, Pullman, WA 99164 § To whom correspondence should be addressed. E-mail: dcpage@wi.mit.edu

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FSH regulates RA signaling to commit spermatogonia into differentiation pathway and meiosis.
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CRISPR/Cas9 mediated chicken Stra8 gene knockout and inhibition of male germ cell differentiation.
PLoS One. 2017 Feb 24;12(2):e0172207.
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Abstract

An efficient genome editing approach had been established to construct the stable transgenic cell lines in the domestic chicken (Gallus gallus domesticus) at present. Our objectives were to investigate gene function in the differentiation process of chicken embryonic stem cells (ESCs) into spermatogonial stem cells(SSCs). Three guides RNA (gRNAs) were designed to knockout the Stra8 gene, and knockout efficiency was evaluated in domestic chicken cells using cleavage activity of in vitro transcription of gRNA, Luciferase-SSA assay, T7 endonuclease I assay(T7E1) and TA clone sequence. In addition, the Cas9/gRNA plasmid was transfected into ESCs to confirm the function of Stra8. SSA assay results showed that luciferase activity of the vector expressing gRNA-1 and gRNA- 2 was higher than that of gRNA-3. TA clone sequencing showed that the knockdown efficiency was 25% (10/40) in DF-1 cells, the knockdown efficiency was 23% (9/40) in chicken ESCs. T7E1 assay indicated that there were cleavage activity for three individuals, and the knockdown efficiency was 12% (3/25). Cell morphology, qRT-PCR, immunostaining and FCS indicated that Cas9/gRNA not only resulted in the knockout of Stra8 gene, but also suggested that the generation of SSCs was blocked by the Stra8 gene knockdown in vitro. Taken together, our results indicate that the CRISPR/Cas9 system could mediate stable Stra8 gene knockdown in domestic chicken's cells and inhibit ECSs differentiation into SSCs.
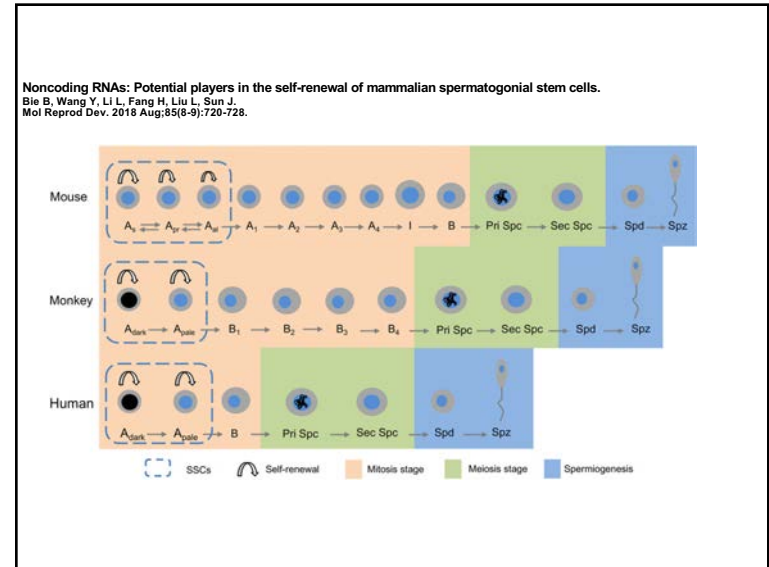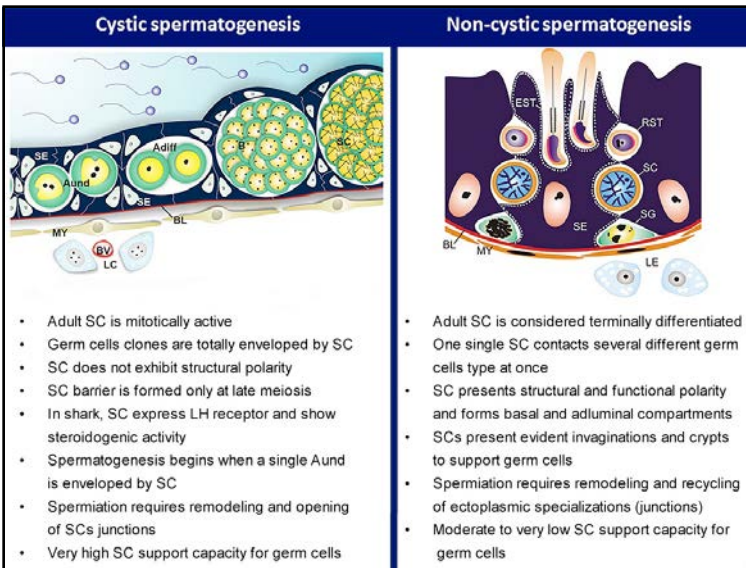

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Sci Rep. 2021 Oct 14;11(1):20472.

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Sertoli cells are the source of stem cell factor for spermatogenesis.
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The Dynamic Transcriptional Cell Atlas of Testis Development during Human Puberty.
Guo J, Nie X, Giebler M, Mlcochova H, et al.
Cell Stem Cell. 2020 Feb 6;26(2):262-276.e4.

Genes Regulating Spermatogenesis and Sperm Function Associated With Rare Disorders.
Linn E, Ghanem L, Bhakta H, Greer C, Avella M.
Front Cell Dev Biol. 2021 Feb 16;9:634536.

Novel Gene Regulation in Normal and Abnormal Spermatogenesis
Du L, Chen W, Cheng Z, et al.
Cells. 2021 Mar 17;10(3):666.

Sperm competition and the evolution of spermatogenesis.
Mol Hum Reprod. 2014 Dec;20(12):1169-79.
Ramm SA, Schärer L, Ehmcke J, Wistuba J.

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Table I Summary of different aspects of spermatogenesis discussed in this review and pertinent evolutionary considerations.

Aspect of spermatogenesis	Evolutionary considerations
Number of sperm produced	Affects likelihood of competitive fertilization success, with greater sperm production being strongly favoured under greater levels of sperm competition; this selects for more sperm-producing tissue and for greater spermatogenic efficiency
Morphology of sperm produced	Affects sperm performance in the external medium or female reproductive tract and thus fertilization success, and is thus strongly affected by sperm competition and cryptic female choice; different sperm morphologies have different spermatogenic requirements
Testicular organization and spermatogonial stem cell system	Several interrelated factors including sperm competition, reproductive lifespan, seasonality and mode of fertilization could all influence the way in which spermatogenesis is organized within the testis and the stem cell system used to support it
Genetics of spermatogenesis	The large number and rapid evolution of testis-specific genes is likely at least partially attributable to sperm competition
Repression of transcription and haploid selection	Between-ejaculate sperm competition selects for males that repress within-ejaculate sperm competition; sperm competition sometimes favours the evolution of sperm cooperation
Germline (selfish spermatogonial) selection	De novo mutations in spermatogonial cells can sometimes selfishly expand their representation within the spermatogonial stem cell population; even small advantages can accumulate, given the very large number of cell divisions (which is ultimately due to the evolution of anisogamy and sperm competition)

See 'Evolutionary forces shaping aspects of spermatogenesis' section of the main text for full details and references.

Male Germline Stem Cell And Niche

Cystic spermatogenesis
- Adult SC is mitotically active
- Germ cells clones are totally enveloped by SC
- SC does not exhibit structural polarity
- SC barrier is formed only at late meiosis
- In shark, SC express LH receptor and show steroidogenic activity
- Spermatogenesis begins when a single Aund is enveloped by SC
- Spermiation requires remodeling and opening of SCs junctions
- Very high SC support capacity for germ cells

Non-cystic spermatogenesis
- Adult SC is considered terminally differentiated
- One single SC contacts several different germ cells type at once
- SC presents structural and functional polarity and forms basal and adluminal compartments
- SCs present evident invaginations and crypts to support germ cells
- Spermiation requires remodeling and recycling of ectoplasmic specializations (junctions)
- Moderate to very low SC support capacity for germ cells

Notch signaling mediates the age-associated decrease in adhesion of germline stem cells to the niche.
PLoS Genet. 2014 Dec 18;10(12):e1004888.
Tseng CY, Kao SH, Wan CL, Cho Y, Tung SY, Hsu HJ.

Greenspan LJ, et al. 2015.
Annu. Rev. Cell Dev. Biol. 31:291–315
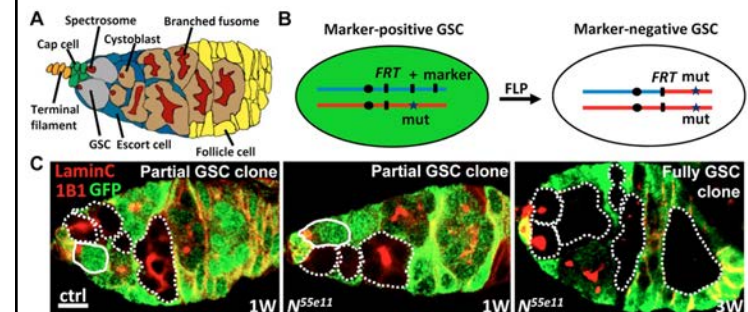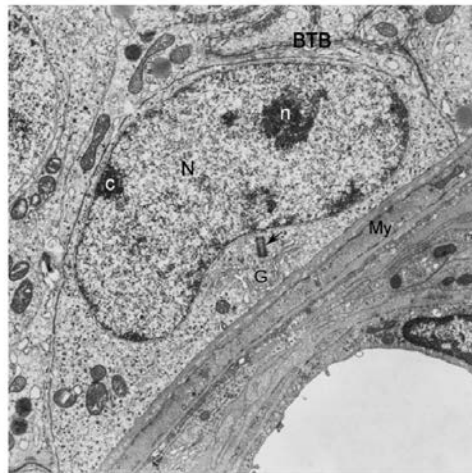
Fig. 4. A more differentiated rat type A (type A₄). The nucleus (N) is oblong, partly indented presenting clumps of heterochromatin (c) at its periphery and a nucleolus (n). Note the Golgi apparatus (G) and an adjacent centriole (arrow). The blood-testis barrier (BTB) is well developed in this field. My, myoid cell. Magnification, ×49,000.
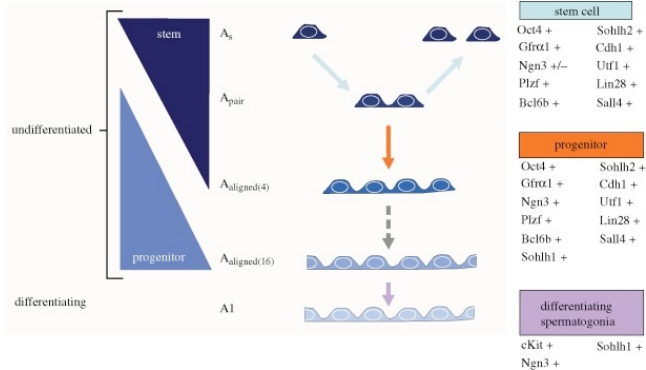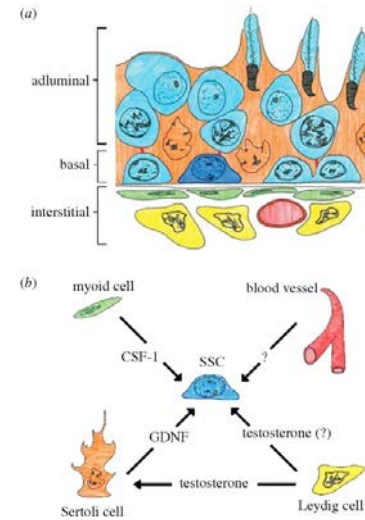
Genes expressed by stem, progenitor and differentiating spermatogonia. The As, seen at the top of the diagram, is responsible for self renewal and differentiation. Self-renewal is represented here by the Apair dividing to form two As. Differentiation is indicated by colour change (from dark to light) and the lengthening chain of germ cells. Genes are listed with their expression at the given stages of spermatogonial development. While stem cell activity is considered to reside in the pool of As spermatogonia, the tapered triangle on the left indicates that stem cell activity may extend to Apr and some Aal spermatogonia.
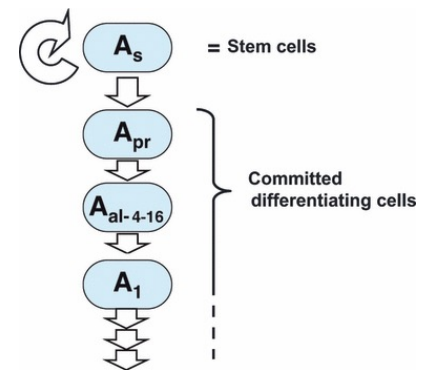
A schematic representation of the "As model". This model suggests that every single As spermatogonium acts as the stem cell. This model also proposes that Apr and subsequent longer cysts are committed to differentiation and do not act as the stem cells.

a Dedifferentiation model

A_s A_{pr} A_{al-4} A_{al-8} A_{al-16}

GFRα1+ Nanos2+

Ngn3+

A_1 spermatogonia (c-kit)

1. Reversible under steady condition
2. Gradual loss of self-renewal activity during differentiation
3. Actual (GFRα1+Nanos2+) versus potential (Ngn3+ or c-kit) stem cells
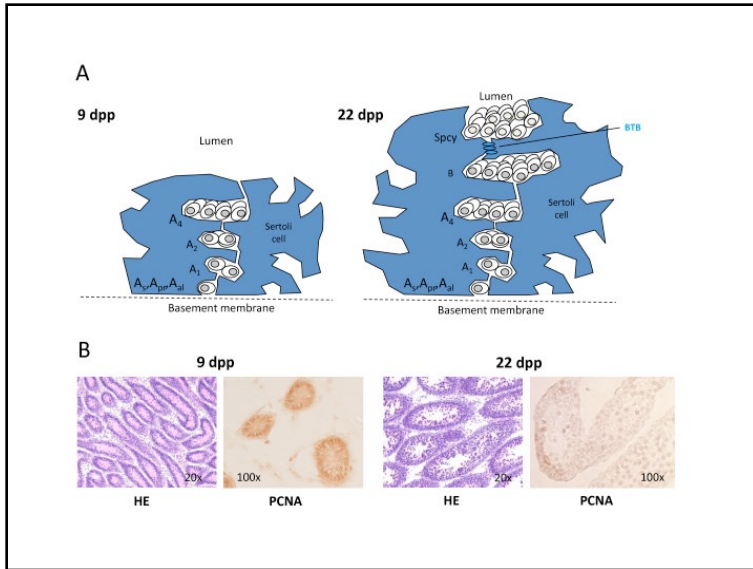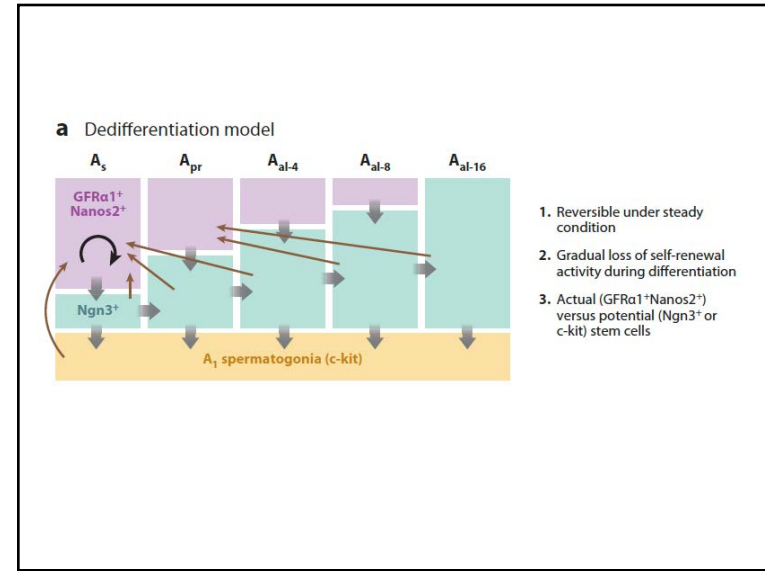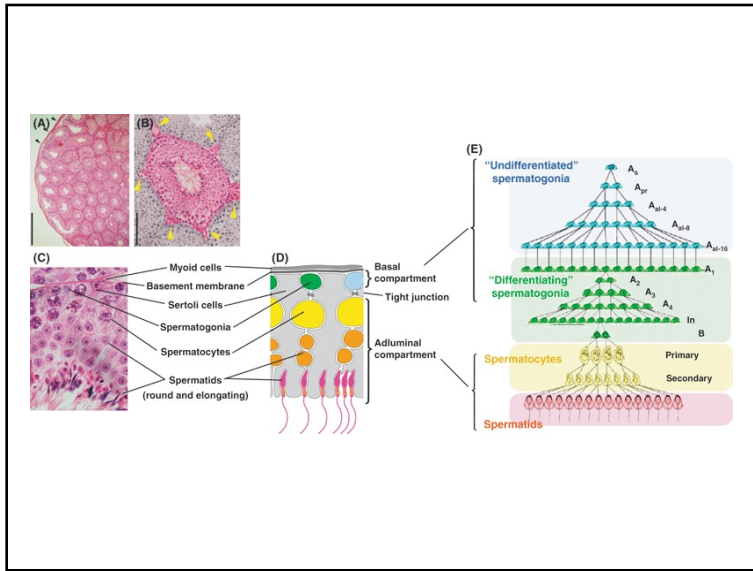
Table. *Summary[a] of important genes expressed in spermatogonia and their putative functions*

Growth Factors	Putative Function	References
cRET, GFRα1	Receptors for GDNF	de Rooij and Russell, 2000
Csf1r	Receptor for CSF-1	Oatley et al, 2009
Transcription Factors		
DMRT1	Maintenance of undifferentiated state	Matson et al, 2010
Plzf (Zfp145)	Transcriptional repressor	Buaas et al, 2004
SOLH1 and 2	Essential for differentiation	Ballow et al, 2006
ID4	Putative SSC marker	Oatley et al, 2011a
Other		
STAT3	Signaling required for differentiation	Kaucher et al, 2012
STRA8	Required for differentiation	Baltus et al, 2006
LIN 28	Regulation of miRNAs	Tong et al, 2011
DAZL	Essential for differentiation, RNA binding	Lin et al, 2008

Abbreviations: CSF-1, colony-stimulating factor 1; DMRT, doublesex-related transcription; GDNF, glial cell–derived neurotrophic factor; miRNA, microRNA; SSC, spermatogonial stem cell.

[a] Factors listed are confined to those discussed in the text, and references are limited to a single introductory reference.

NANOS2 acts downstream of glial cell line-derived neurotrophic factor signaling to suppress differentiation of spermatogonial stem cells.
Sada A, Hasegawa K, Pin PH, Saga Y. Stem Cells. 2012 Feb;30(2):280-91.
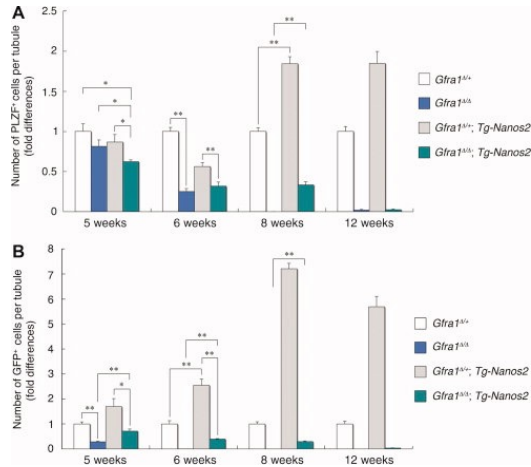
The niche-derived glial cell line-derived neurotrophic factor (GDNF) induces migration of mouse spermatogonial stem/progenitor cells.
Dovere L, et al. PLoS One. 2013 Apr 22;8(4):e59431.
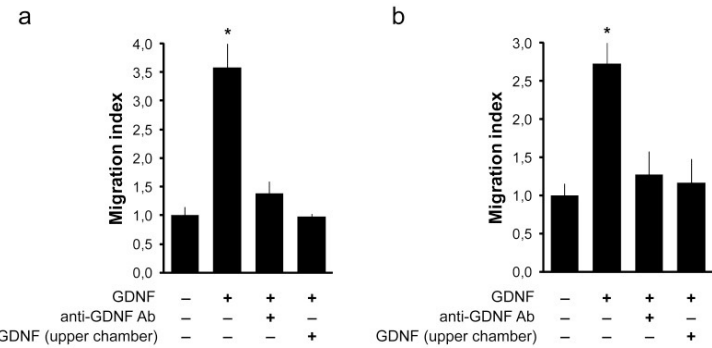
Glial cell line-derived neurotrophic factor is constitutively produced by human testicular peritubular cells and may contribute to the spermatogonial stem cell niche in man.
Spinnler K, et al. Hum Reprod. 2010 Sep;25(9):2181-7.
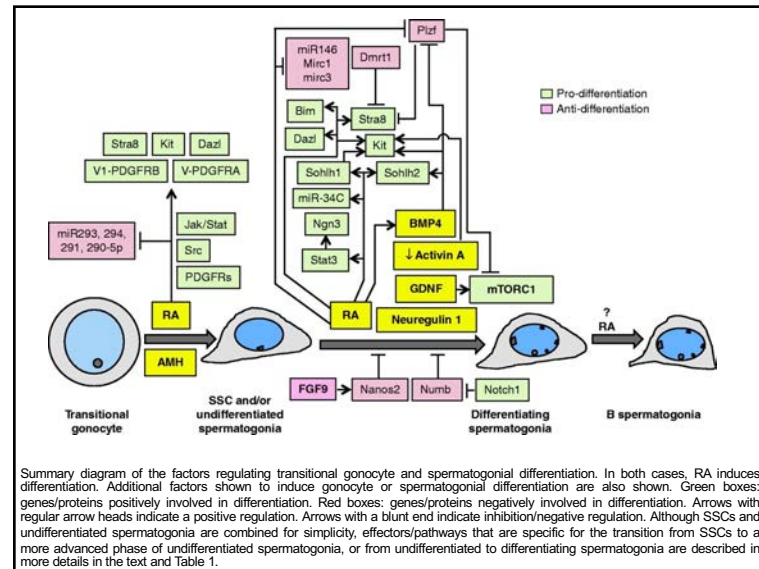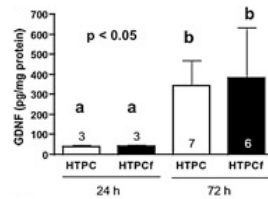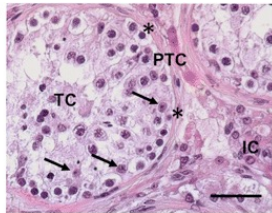
Summary diagram of the factors regulating transitional gonocyte and spermatogonial differentiation. In both cases, RA induces differentiation. Additional factors shown to induce gonocyte or spermatogonial differentiation are also shown. Green boxes: genes/proteins positively involved in differentiation. Red boxes: genes/proteins negatively involved in differentiation. Arrows with regular arrow heads indicate a positive regulation. Arrows with a blunt end indicate inhibition/negative regulation. Although SSCs and undifferentiated spermatogonia are combined for simplicity, effectors/pathways that are specific for the transition from SSCs to a more advanced phase of undifferentiated spermatogonia, or from undifferentiated to differentiating spermatogonia are described in more details in the text and Table 1.

Secretome analysis of testicular peritubular cells: a window into the human testicular microenvironment and the spermatogonial stem cell niche in man.
Flenkenthaler F, et al. J Proteome Res. 2014 Mar 7;13(3):1259-69.
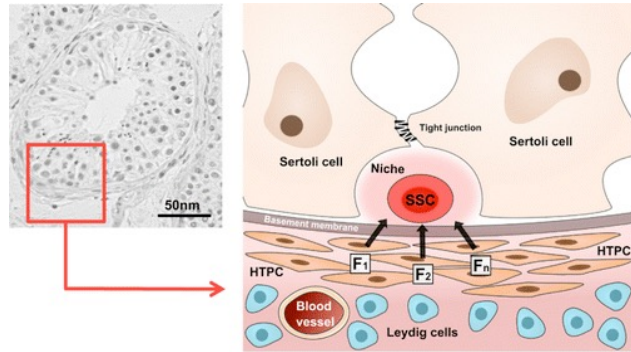
Molecular regulation of spermatogonial stem cell renewal and differentiation.
Mäkelä JA, Hobbs RM.
Reproduction. 2019 Nov;158(5):R169-R187.
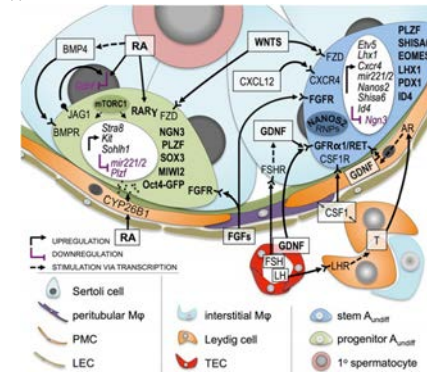
The regulation of spermatogenesis by androgens.
Smith LB, Walker WH.
Semin Cell Dev Biol. 2014 Jun;30:2-13.

Table 1
Phenotypes of mice having cell-specific alterations in AR expression.

Cell specific AR alteration	Phenotype
Germ cell KO	None
Peritubular cell KO	Progressive germ cell loss in adults, incomplete Leydig cell development
Leydig cell KO	Decreased steroidogenic enzyme activity, effect on germ cells not conclusive
Vascular endothelial KO	None
Vascular smooth muscle KO	Normal spermatogenesis, impaired testicular vasomotion, increased interstitial fluid
Sertoli cell KO	Spermatogenesis halted during meiosis, disruption of the BTB, fewer Leydig cells
AR overexpression in Sertoli cells	Accelerated Sertoli cell maturation, fewer Sertoli cells and post meiotic germ cells, decreased Leydig cell proliferation
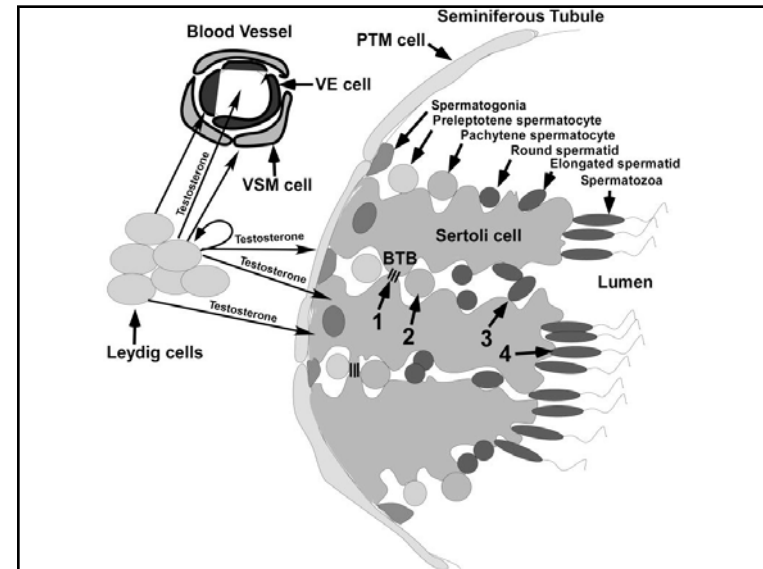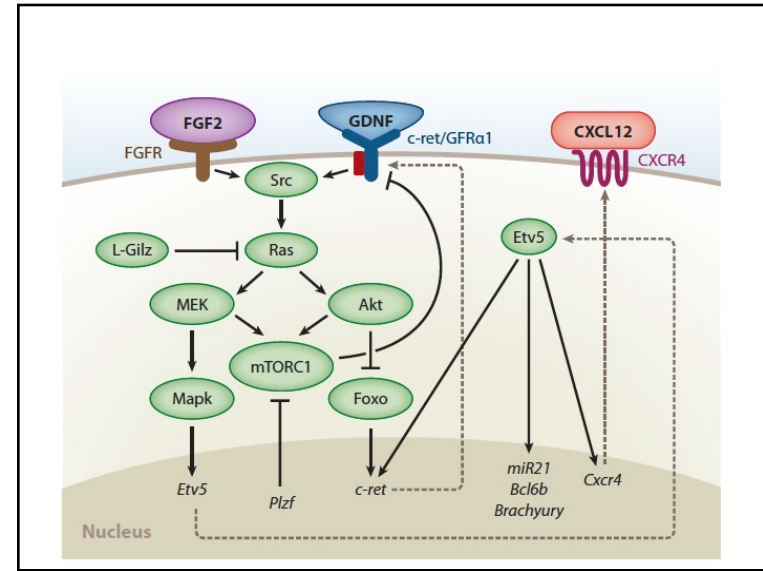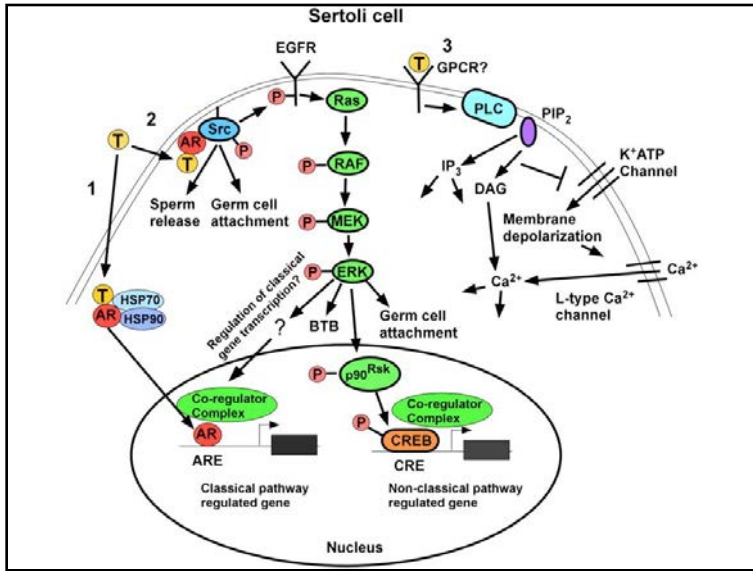
Cycling to and from a stem cell niche: the temporal and spatial odyssey of mitotic male germ cells.
Payne CJ. Int J Dev Biol. 2013;57(2-4):169-77.
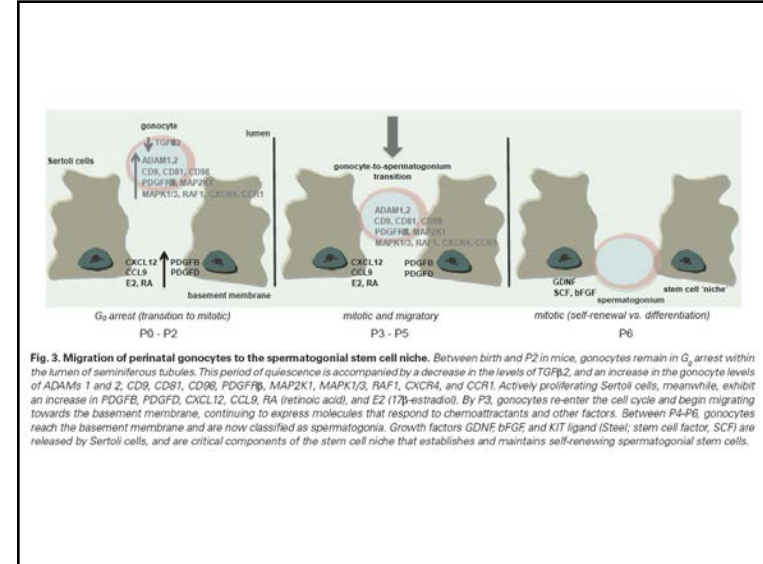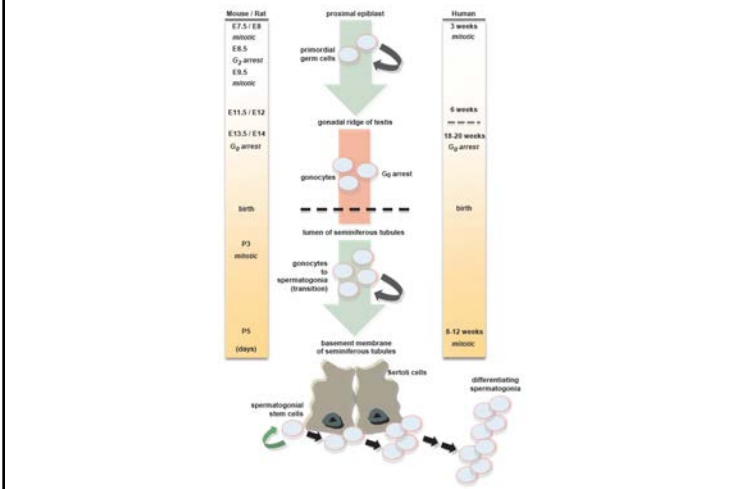
Fig. 3. Migration of perinatal gonocytes to the spermatogonial stem cell niche. *Between birth and P2 in mice, gonocytes remain in G₀ arrest within the lumen of seminiferous tubules. This period of quiescence is accompanied by a decrease in the levels of TGFβ2, and an increase in the gonocyte levels of ADAMs 1 and 2, CD9, CD81, CD98, PDGFRβ, MAP2K1, MAPK1/3, RAF1, CXCR4, and CCR1. Actively proliferating Sertoli cells, meanwhile, exhibit an increase in PDGFB, PDGFD, CXCL12, CCL9, RA (retinoic acid), and E2 (17β-estradiol). By P3, gonocytes re-enter the cell cycle and begin migrating towards the basement membrane, continuing to express molecules that respond to chemoattractants and other factors. Between P4-P6, gonocytes reach the basement membrane and are now classified as spermatogonia. Growth factors GDNF, bFGF and KIT ligand (Steel; stem cell factor, SCF) are released by Sertoli cells, and are critical components of the stem cell niche that establishes and maintains self-renewing spermatogonial stem cells.*
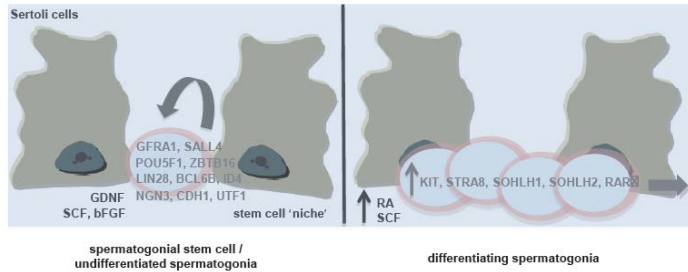
Fig. 4. Maintenance of self-renewing spermatogonial stem cells and differentiating spermatogonia.
Spermatogonial stem cells (SSCs) reside within the SSC niche, expressing intrinsic factors such as GFRA1, POU5F1, ZBTB16, LIN28, SALL4, BCL6B, ID4, NGN3, CDH1, and UTF1. Sertoli cells release growth factors like GDNF, bFGF, and KIT ligand (Steel; stem cell factor, SCF) to maintain SSCs within the niche. In response to RA (retinoic acid) and SCF, spermatogonia commit to differentiation, downregulating SSC molecules and upregulating KIT, STRA8, SOHLH1, SOHLH2, and RARγ (retinoic acid receptor). SSCs are first established shortly after birth, whereas the differentiation signals are expressed beginning at puberty. This balance between SSC self-renewal and differentiation continues throughout adulthood.

Transplantation

Spermatogonial stem cells.
Kubota H, Brinster RL.
Biol Reprod. 2018 Jul 1;99(1):52-74.
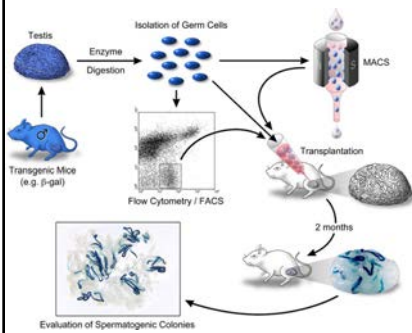
Outline of spermatogonial transplantation method and quantitative assay for SSCs. Single-cell suspension prepared from testes of transgenic mice expressing a reporter gene (e.g., β-galactosidase) by enzymatic digestion is injected into the seminiferous tubules of an infertile recipient mouse. Cells from in vitro culture or cells fractionated by FACS or MACS can be used for a donor cell population. Two months after transplantation, donor-derived spermatogenesis can be detected in the recipient testis as blue colonies. Because each colony of spermatogenesis is developed from a single SSC, the number of colonies represents the number of SSCs in the donor cell suspension. The length of each colony demonstrates the degree of SSC expansion.

## Spermatogonial stem cell transplantation
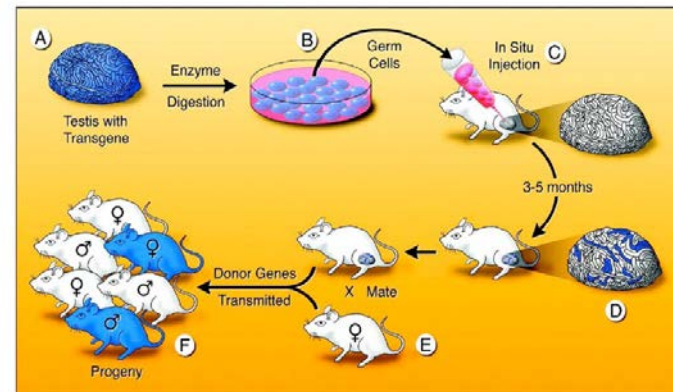
Brinster, 2002
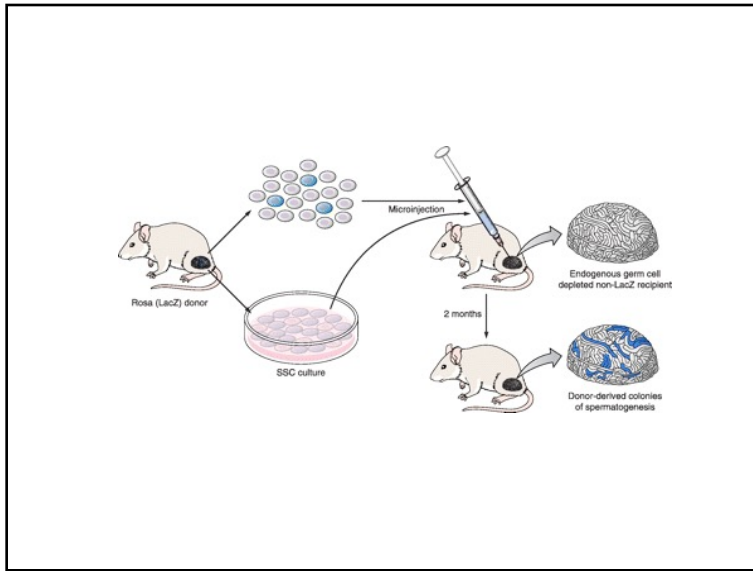
Transplantation of male germ line stem cells restores fertility in infertile mice
Takehiko Ogawa et al.
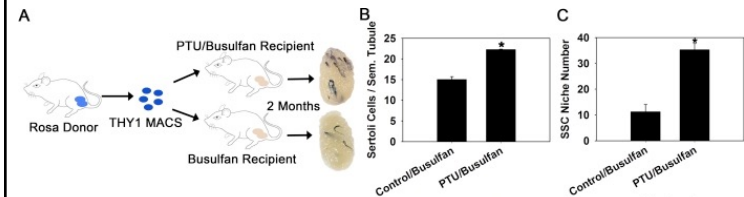Nature Medicine 6, 29 - 34 (2000)

Figure 1: Steel factor (Kit ligand)–Kit receptor interaction in the mouse testis, with phenotypes and testis sizes of mice.

a, Black wild-type C57BL/6 male; testis weight, 107 mg. In wild-type mice, Sertoli cells (S) express both the soluble and the membrane-bound form of Steel factor. Functional Kit receptor dimers on the germ cells (G) interact with the soluble and membrane-bound form of Steel factor. Signal transduction then occurs by autophosphorylation-induced interaction with target proteins. *b*, White *Sl* mutant (*Sl/Sld*) male; testis weight, 14 mg. In *Sl/Sld* mice, Sertoli cells do not express the membrane-bound form of Steel factor because the *Sl* mutation deletes the entire gene, and the *Sld* mutation deletes the transmembrane and intracellular domains. Therefore, only the soluble form of Steel factor is produced to interact with Kit receptors on germ cells. Binding of the soluble form is not sufficient for normal germ cell differentiation. *c*, White *Kit* mutant (*W/W v*) male; testis weight, 15 mg. In *W/Wv* mice, Sertoli cells express both the soluble and the membrane bound form of Steel factor. However, the *W* mutation is a 78-amino-acid deletion that includes the transmembrane domain, and the *Wv* mutation results in a defective receptor on the germ cell because of a point mutation in the tyrosine kinase domain. Therefore, neither the bound nor the soluble form of Steel factor can effectively influence germ cells. Scale bars (insets) represent 1 mm.
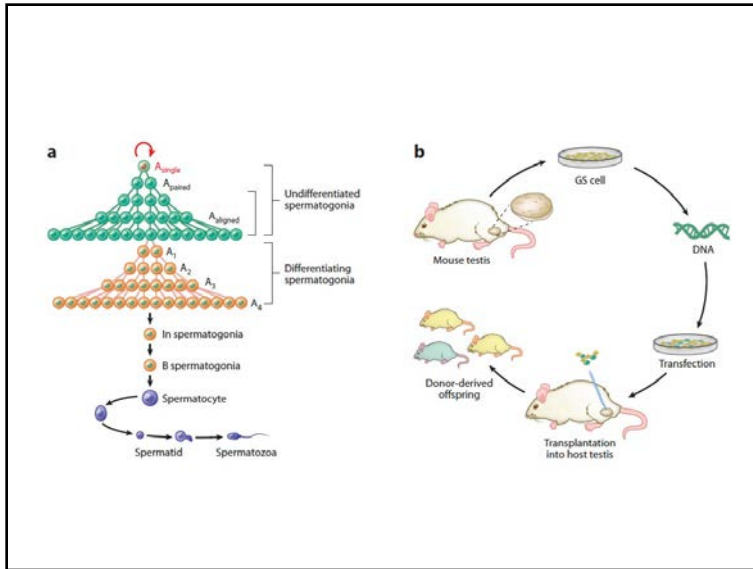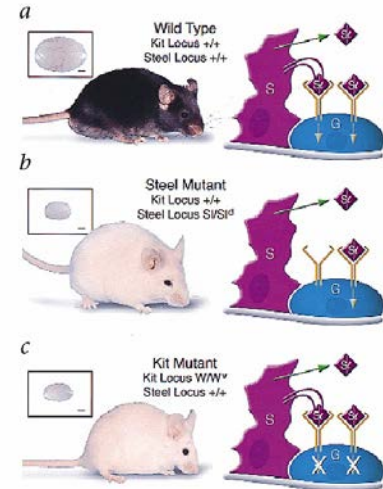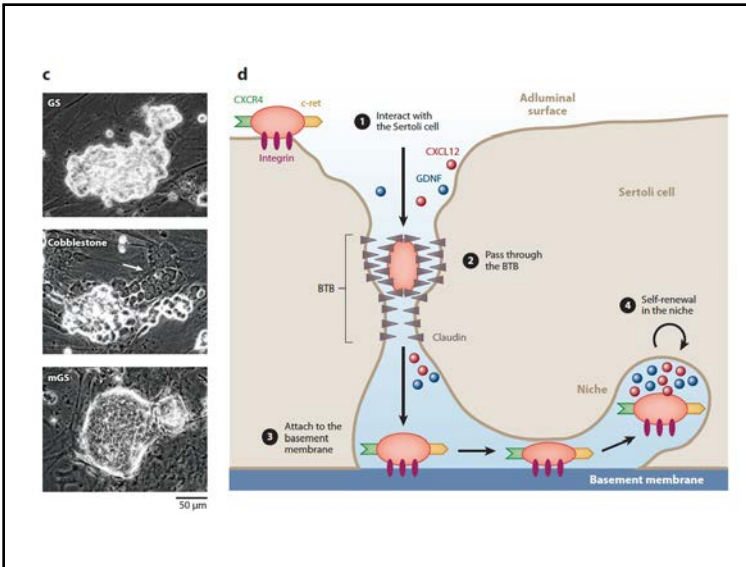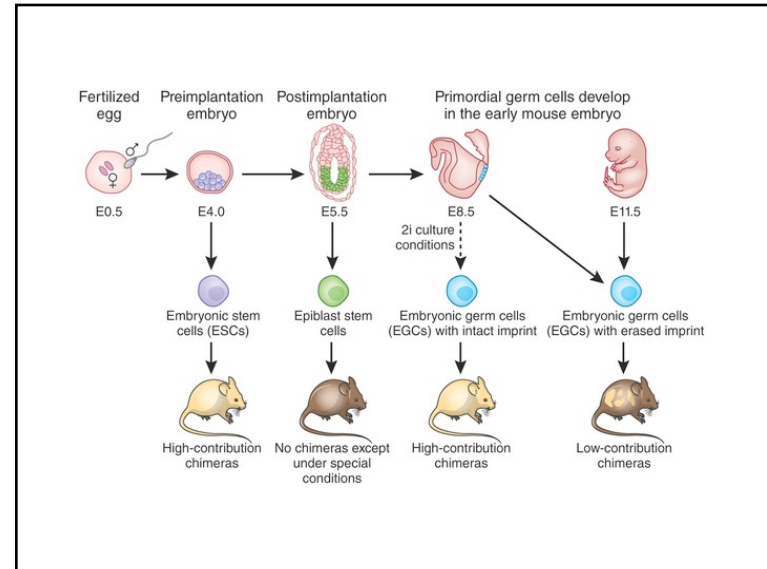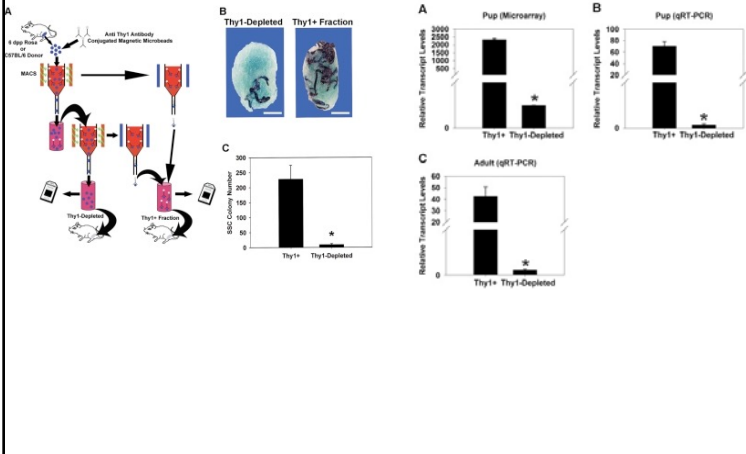

Sertoli cells dictate spermatogonial stem cell niches in the mouse testis.
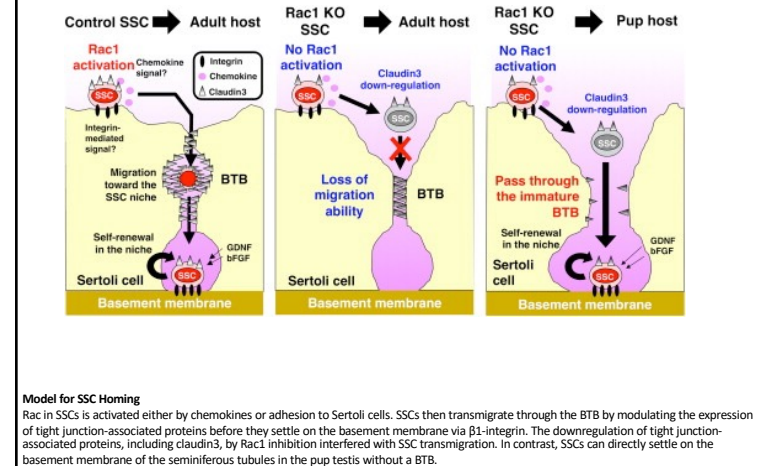Oatley MJ, Racicot KE, Oatley JM. Biol Reprod. 2011 Apr;84(4):639-45.

Colony stimulating factor 1 is an extrinsic stimulator of mouse spermatogonial stem cell self-renewal.
Oatley JM, et al. Development. 2009 Apr;136(7):1191-9.

Rac mediates mouse spermatogonial stem cell homing to germline niches by regulating transmigration through the blood-testis barrier.
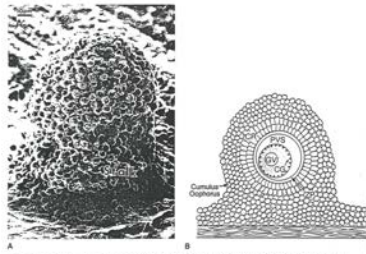Takashima S, et al. Cell Stem Cell. 2011 Nov 4;9(5):463-75.

Model for SSC Homing
Rac in SSCs is activated either by chemokines or adhesion to Sertoli cells. SSCs then transmigrate through the BTB by modulating the expression of tight junction-associated proteins before they settle on the basement membrane via β1-integrin. The downregulation of tight junction-associated proteins, including claudin3, by Rac1 inhibition interfered with SSC transmigration. In contrast, SSCs can directly settle on the basement membrane of the seminiferous tubules in the pup testis without a BTB.

Spermatogonial stem cells – adult stem cells present in the testis

Spradling, 2001

Niche - subset of tissue cells and extracellular substrates that can indefinitely house one or more stem cells and control their self-renewal and progeny production *in vivo*.

Tissue Engineering to Improve Immature Testicular Tissue and Cell Transplantation Outcomes: One Step Closer to Fertility Restoration for Prepubertal Boys Exposed to Gonadotoxic Treatments.
Int J Mol Sci. 2018 Jan 18;19(1).
Del Vento F, Vermeulen M, de Michele F, Giudice MG, Poels J, des Rieux A, Wyns C.

Fertility preservation for peri- and pre-pubertal male patients: experimental clinical practice and future perspectives. SZ: Spermatozoa; ICSI: Intra-Cytoplasmic Sperm Injection; ART: Assisted Reproductive Technology; IVM: In Vitro Maturation; TESE: TEsticular Sperm Extraction; IUI: Intra-Uterine Insemination.

Differentiation of primate primordial germ cell-like cells following transplantation into the adult gonadal niche.
Sosa E, Chen D, Rojas EJ, et al.
Nat Commun. 2018 Dec 17;9(1):5339.

Current scenario and challenges ahead in application of spermatogonial stem cell technology in livestock.
Binsila B, Selvaraju S, Ranjithkumaran R, et al.
J Assist Reprod Genet. 2021 Dec;38(12):3155-3173.

Table 1 Spermatogonial stem cells regulators and their functions in rodents, humans, and livestock

Sl.no	Regulators	Species	Function	References
1.	Chromodomain helicase/ATPase DNA binding protein 1-like (Chd1l)	Mouse	• Chd1l-miR-486-MMP2 regulatory axis increases β-catenin nuclear translocation and SSCs stemness gene expression	[192]
2.	Chd1l	Mouse	• Supports SSC survival and self-renewal partially through a GDNF signaling pathway. • Regulates expression levels of Oct4, Plzf, Gfra1, and Pena genes	[203]
3.	miRNA-21	Mouse	• Regulates the transcription factor ETV5, critical for SSC self-renewal	[204]
4.	microRNA-224	Mouse	• Regulates WNT/β-catenin signaling pathway for SSC self-renewal	[190]
5.	miRNA-10b	Mouse	• Mediates SSC proliferation through Kruppel-like factor 4	[191]
6.	Foxos	Mouse	• Effectors of PI3K-Akt signaling in SSCs • SSC self-renewal and differentiation	[205]
7.	RNA-binding protein DND1	Mouse	• Mediates target mRNA destabilization in adult murine spermatogonia and SSCs	[206]
8.	Nodal	Mouse	• Is a member of the TGFβ superfamily which activates Smad2/3 phosphorylation, Oct-4 transcription, cyclin D1, and cyclin E expression	[119]
9.	Dead-end (RNA-binding protein)	Mouse	• Interacts with some of NANOS2-target mRNAs in undifferentiated spermatogonia. • NANOS2 independent role in differentiated spermatogonia	[207]
10.	Dead-end1 (DND1)	Mouse	• Directly interacts with NANOS2 to load unique RNAs into the CNOT complex and mediates male germ cell development	[208]
11.	Ets related molecule (ERM)	Mouse	• Located in Sertoli cells in the testis and is required for SSC's self-renewal	[209]
12.	TET1	Mouse	• Interact with PCNA and HDAC1 and promote self-renewal and proliferation	[210]
13.	Pten	Mouse	• SSC's maintenance by regulating the expression of PLZF and UTF1	[211]
14.	Pelota	Mouse	• Necessary for the transition of gonocytes to SSCs	[212]
15.	lncRNA AK015322	Mouse	• SSC self-renewal by attenuating the repression of ETV5	[213]
16.	miRNA-663a	Human	• SSCs proliferation, DNA synthesis and suppresses early apoptosis by targeting NFIX via cell cycle regulators cyclin A2, cyclin B1, and cyclin E1	[214]
17.	P21-activated kinase 1(PAK1)	Human	• Promotes the proliferation and inhibits apoptosis of human SSCs via PDK1/KDR/ZNF367 and ERK1/2 and AKT pathways	[215]
18.	miRNA-20 and miRNA-106a	Human	• Promote renewal at the post-transcriptional level via targeting STAT3 and Ccnd1	[216]
19.	Lin28a	Goat	• Self-renewal and proliferation of SSCs through regulation of mTOR and PI3K/AKT	[217]
20.	Lin28a	Goat	• NANOG transcriptional regulation via epigenetic DNA modifications to maintain the stemness of germline stem cells	[218]
21.	Tet1	Goat	• DNA methylation/demethylation and regulation of epigenetic modifications in SSCs	[219]
22.	PLZF	Goat	• Downregulated mir146a and upregulated the expression of CXCR4 protein for germ stem cell proliferation	[220]
23.	miRNA-544	Goat	• Regulates the self-renewal and differentiation of mGSCs via targeting PLZF	[221]
24.	miRNA-302	Goat	• Enhanced the cell proliferation ability and the attachment ability, also, promoted the expression level of CD49f and OCT4	[222]
25.	DDX4 and c-KIT	Porcine	• DDX4 and c-KIT are putative markers of undifferentiated spermatogonia in the prepubertal porcine testis	[223]
26.	EOMES (T-box transcription factor)	Mouse	• Contribute to long-term maintenance of A-single spermatogonia and steady-state spermatogenesis	[224]

Regulation of Cell Types Within Testicular Organoids.
Lara NLEM, Sakib S, Dobrinski I.
Endocrinology. 2021 Apr 1;162(4):bqab033.

Table 1. Summary of testicular organoid reports

Authors, y	Species	Age	Cell types	Organotypic morphology, yes/no	Testosterone production, yes/no	Germ cell differentiation, yes/no	Reference
Baert et al, 2017	Human	Adult and 15 y	Primary Sertoli, Leydig, peritubular myoid, and germ cells	No	Yes	No	(20)
Pendergraft et al, 2017; Skardal et al, 2020	Human	Adult	Immortalized Sertoli and Leydig cells, primary germ cells	No	Yes	Yes	(21, 50)
Alves-Lopes et al, 2017	Rat	5-8, 20, and 60 d	Primary Sertoli, peritubular myoid, and germ cells	Yes	NA	NA	(19)
Sakib et al, 2019	Pig, mouse, monkey, human	7 d (pig), 8-10 d (mouse), 2 y (monkey), 6 mo and 5 y (human)	Primary Sertoli, Leydig, and germ cells	Yes	NA	NA	(23)
Edmonds and Woodruff, 2020	Mouse	5, 12, 21 d, and adult (8-16 wk)	Primary Sertoli, Leydig, peritubular myoid, and germ cells	Yes	Yes	No	(25)
Topraggaleh et al, 2019	Mouse	3-5 d	Primary Sertoli, Leydig, and germ cells	Yes	Yes	Yes	(22)
Vermeulen et al, 2019	Pig	4-7 d	Primary Sertoli, Leydig, peritubular myoid, and germ cells	Yes	Yes	No	(37)
Mall et al, 2020	Rat, human	5-9 d	Primary rat Sertoli, peritubular myoid, and germ cells, human iPSC-derived primordial germ cell–like cells	No	NA	NA	(26)

Abbreviations: iPSC, induced pluripotent stem cell; NA, not available.

Oogenesis

Figure 6-4 • The life cycle of the human ovary. (Modified from Ham A.W., Leeson T.S. Histology, 4th ed. Philadelphia, JB Lippincott, 1968.)

FIGURE 2b.

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Figure 1-1. A. Scanning electron micrograph of an intact mouse granulosa complex grown for 7 days in vitro. The oocyte is contained within the ball of granulosa cells as indicated by the arrowheads. Photomicrograph courtesy of Dr John Eppig. (Reproduced, with permission, from Eppig J. et al. Dev Biol. 140:307, 1990.) B. Representation of this cumulus-granulosa-oocyte complex. Abbreviations: CR, corona radiata; CB, cortical granules; ZP, zona pellucida; GV, germinal vesicle; PVS, perivitelline.

Prior to Gonadotropin Surge   Post Gonadotropin Surge
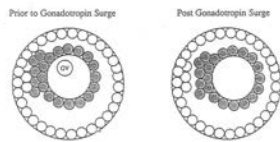
FIGURE 4. Schematic representation of gonadotropin-induced reduction in gap junctions between cumulus and granulosa cells. Prior to the gonadotropin surge, a syncitium of gap junctions is present between the three different cell types present in the follicle. Following the gonadotropin surge, a reduction in the number of gap junctions between the granulosa cells and cumulus cells occurs. Although this is depicted as a physical separation of the two cell types in the figure, they are still physically associated. The significance of this reduction is intercellular communication, however, is that the cumulus cell-oocyte complex is functionally uncoupled from the granulosa cell mass, which appears necessary to maintain meiotic arrest. A reduction in intercellular communication between the oocyte and cumulus cells occurs subsequently. Open circles, granulosa cells; shaded circles, cumulus cells; large circle, oocyte.
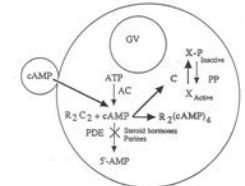
FIGURE 1. Model for maintenance of meiotic arrest by cAMP. Oocyte cAMP is generated by either an oocyte adenylate cyclase or is derived from follicle cells following transfer through the heterologous gap junctions. The cAMP activates a PK-A and the active catalytic subunit C, phosphorylates X, which is active and prevents GVBD, to X-P, which is the inactive form. Steroid hormones and purines may also participate in maintaining inhibitory levels of cAMP by inhibiting the oocyte PDE. R₂C₂, holoenzyme form of PK-A, which is inactive; R₂(cAMP)₄, dissociated regulatory subunits of PK-A; C, active catalytic subunit of PK-A; PDE, cAMP phosphodiesterase; PP, protein phosphatase responsible for converting X-P to X. Species depicted in the outline letters represent the dominant forms present.
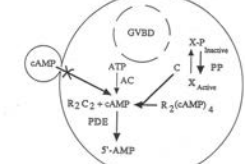
FIGURE 2. Model for resumption of meiosis induced by a decrease in oocyte cAMP. A decrease in oocyte cAMP, which is due in part to the PDE activity, promotes reassociation of regulatory subunits of PK-A with the catalytic subunits. This results in a decrease in PK-A activity. The protein phosphatase then promotes the conversion of X-P to X, which is somehow involved in GVBD. The symbols are described in the legend to Figure 1.

72   Voronina and Wessel

young oocyte   oocyte   MI   MII   egg
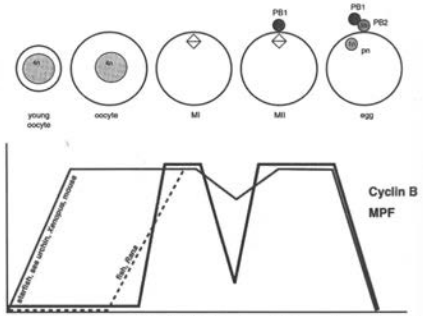
Cyclin B
MPF

Figure 4   Schematic representation of MPF activity changes during growth and maturation of oocytes compared with levels of the regulatory subunit of MPF, cyclin B (see color plate).

74   Voronina and Wessel

Table I   Structure of the Proposed Maturation Inducing Substances (MIS) of Various Species

Species	Name of MIS	Structure of MIS
Mammals	4,4,-Dimethyl-5α-cholesta-8, 14,24-trien-3β-ol (FF-MAS)	
Frog	Progesterone	
Fish	17α,20β-Dihydro-4-pregnen-3-one (DHP) is shown in black; the extra hydroxyl of 17α,20β, 21α-trihydro-4-pregnen-3-one (THP) is shown in parentheses	
Starfish	1-Methyladenine	

See also Fig. 5 for the structure of C. elegans MIS.

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Voronina and Wessel

A. Mouse, *Xenopus*

B. Starfish, fish (?)

Arrest in G2

Induction of meiosis

Identification of Maturation-Specific Proteins by Single-Cell Proteomics of Human Oocytes.
Mol Cell Proteomics. 2016 Aug;15(8):2616-27.
Virant-Klun I, Leicht S, Hughes C, Krijgsveld J.

Female Germline Stem Cells

25
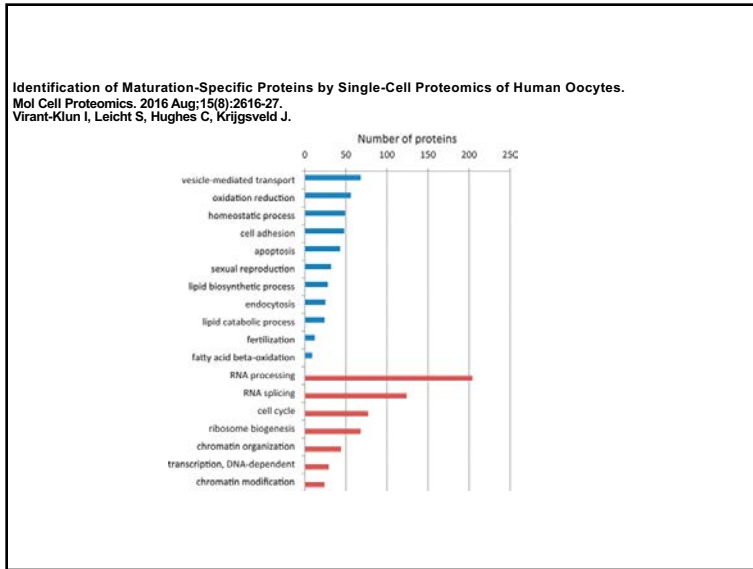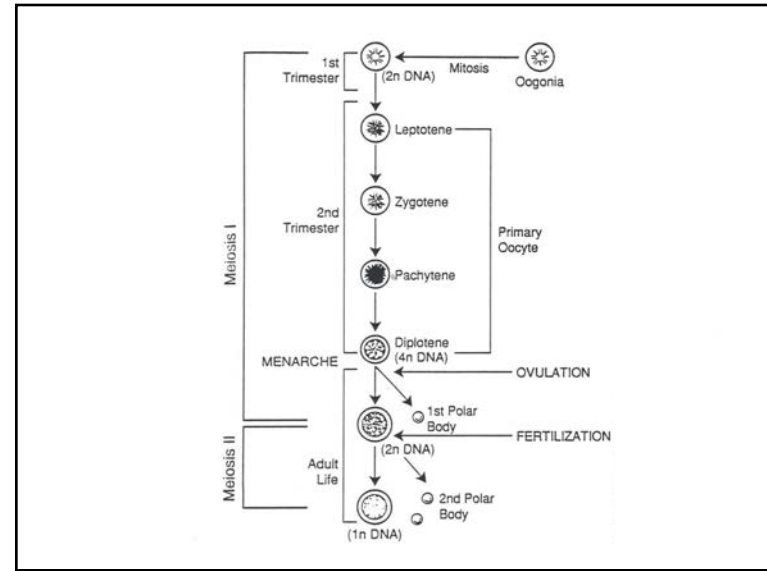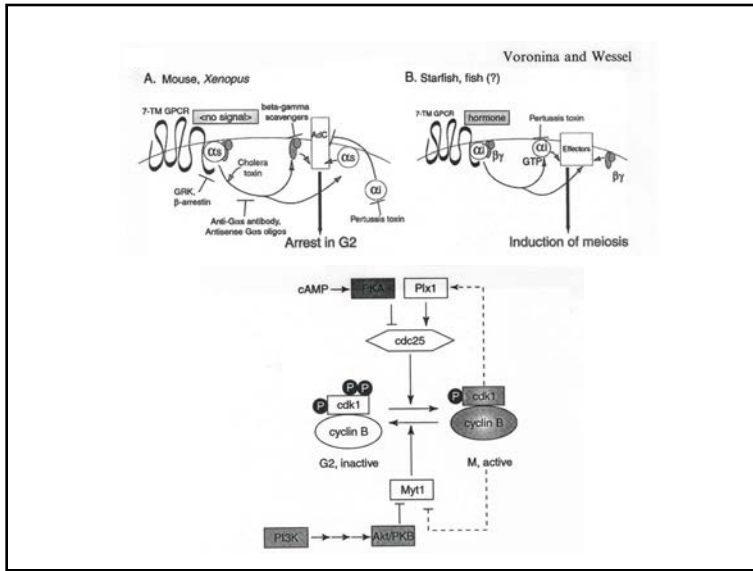
Ultrastructural characterization of mouse embryonic stem cell-derived oocytes and granulosa cells.
Psathaki OE, et al. (2011)
Stem Cells Dev. 20(12):2205-15.
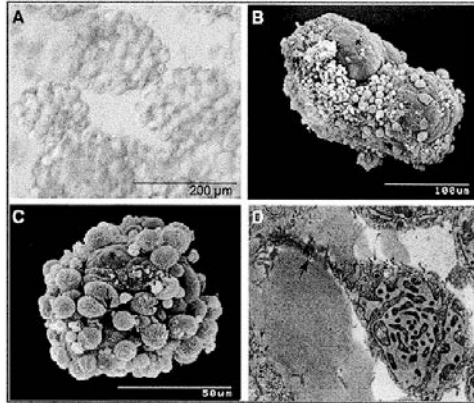
FIG. 1. ESC-derived follicle-like structures. (A) Light microscopy image of ESC-derived follicle-like structures with clusters of granulosa cells loosely surrounding the oocyte. (B) SEM image of a defined follicle structure. Note the layer of densely attached cuboidal cells (asterisk). (C) SEM image of polygonal-shaped granulosa cells around a smooth zona pellucida-like surface (asterisk). The pole of a granulosa cell is facing toward the oocyte (arrow). (D) TEM image of a granulosa cell with an extension stretching towards the oocyte (arrow) (×4,890). ESC, embryonic stem cell; SEM, scanning electron microscopy; TEM, transmission electron microscopy.

Table 2 Some experiments that observe the role of an estimated extra-gonadal source of female GSCs in post-natal oogenesis
(a) Studies that support the role of an estimated extra-gonadal source of female GSCs in post-natal oogenesis

References	Study highlights
Johnson et al. (2005a)	Putative GSCs in BM and peripheral blood may support oogenesis in adult female mice
Lee et al. (2007b)	Putative GSCs reside in the BM, and BMT can stimulate host neo-oogenesis by introducing oocyte precursors in adult female ovary
Bukovsky (2011b); Bukovsky & Caudle (2012); Bukovsky et al. (2007, 2009)	BM-derived cells contribute to the origination of putative germ cells from the OSE stem cells in normal adult rat females and from the medullary somatic stem cells in the neonatally estrogenized mature female rats without OSE
Selesniemi et al. (2009)	Once-monthly infusion of BM-derived cells into young adult female mice ovary maintained the fertility of ageing females long past the time of normal reproductive failure
Ghadami et al. (2012)	Intravenously injection of BM-derived GSCs into the FORKO mice led to expression of the FSH receptor gene, synthesis of FSH receptors, oestrogen hormone production, and folliculogenesis in the ovaries

(b) Studies that refuse the role of an estimated extra-gonadal source of female GSCs in post-natal oogenesis

References	Study highlights
Eggan et al. (2006)	There is no evidence that circulating or BM cells contribute to the generation of oocytes or enhance ovulation of endogenous oocytes in transplanted mice
Begum et al. (2008)	There is no evidence to support the hypothesis that progenitor cells from extra-ovarian sources can replenish the oocytes in adult ovary
Santiquet et al. (2012)	Chemotherapy-sterilized SCID mice cannot produce new oocytes after BMT but fertility of mice improved

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Oogonial stem cells: do they exist and may they have an impact on future fertility treatment?
Ghazal S. Curr Opin Obstet Gynecol. 2013 Jun;25(3):223-8.

KEY POINTS

- Oogonial stem cells have recently been identified in several different species, including humans.

- Given that this finding questions a fundamental tenet of reproductive biology, that women are born with a finite number of oocytes without the ability to self-renew, this research has been met with scientific skepticism.

- The functional capacity and developmental competence of these oogonial stem cells in humans has not been proven.

- The potential impact of oogonial stem cells on female infertility is still unknown, as this area of research is still in its infancy.

Stem Cells
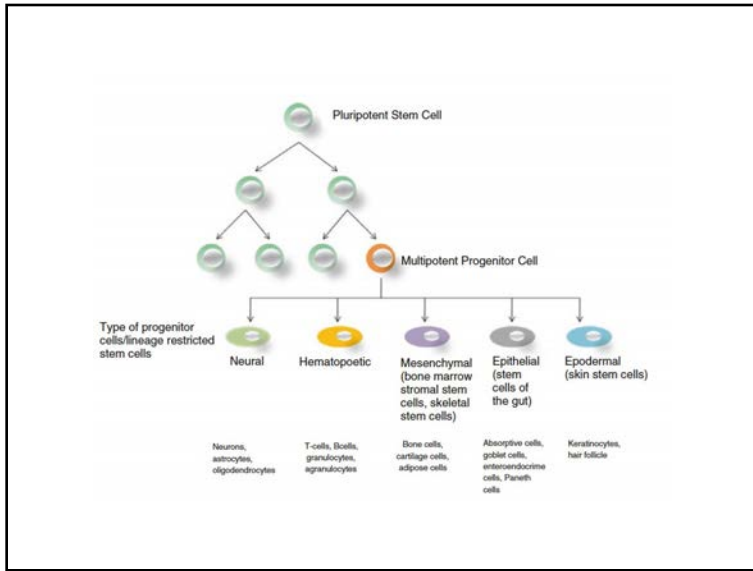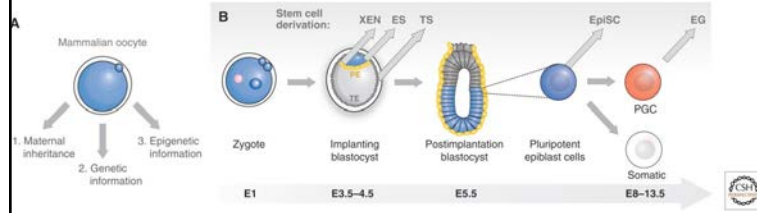
Potential of the mammalian oocyte, zygote, and blastocyst. (A) The mammalian oocyte contains maternal RNAs and proteins (maternal inheritance), which can determine early developmental events, genetic information (maternal chromosomes), and epigenetic information (DNA methylation and chromatin marks). (B) The zygote gives rise to the blastocyst with its inner cell mass (ICM) cells (blue) giving rise to ES cells in culture. The epiblast derivative of the ICM in the postimplantation blastocyst gives rise to all somatic cells and PGCs. A range of pluripotent stem cells (top line) can be derived from the various cell types isolated from early- and late-stage blastocysts and later primitive streak embryos. Types of stem cell include XEN, extraembryonic endoderm; ES, embryonic stem; TS, trophoblast stem; EpiSC, epiblast stem cell; EG, embryonic
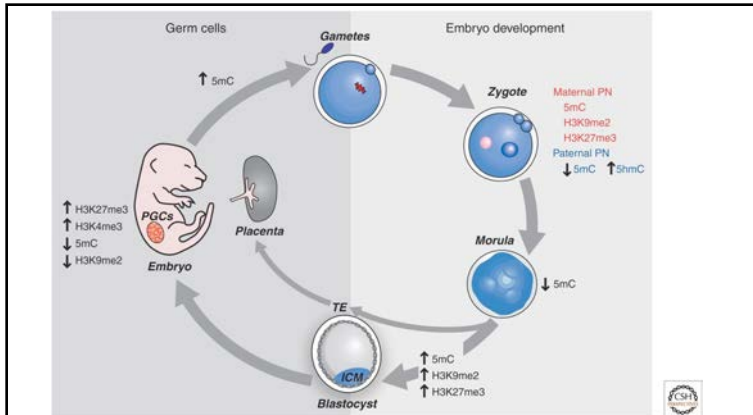
The epigenetic reprogramming cycle in mammalian development. Immediately after fertilization in the zygote, the paternal pronucleus (PN) is packaged with histones that lack H3K9me2 and H3K27me3, whereas the maternal chromatin contains these marks. The paternal PN also rapidly loses 5-methylcytosine (5mC) on a genome-wide scale, whereas the maternal does not. Passive loss of 5mC occurs during preimplantation development until the blastocyst stage when the ICM cells begin to acquire high levels of 5mC, H3K9me2, and H3K27me3. The placenta, which is largely derived from the TE of the blastocyst, remains relatively hypomethylated. PGCs undergo demethylation of 5mC and H3K9me2 progressively as they migrate into the gonads. De novo DNA methylation, including parent-specific imprinting, takes place during gametogenesis.
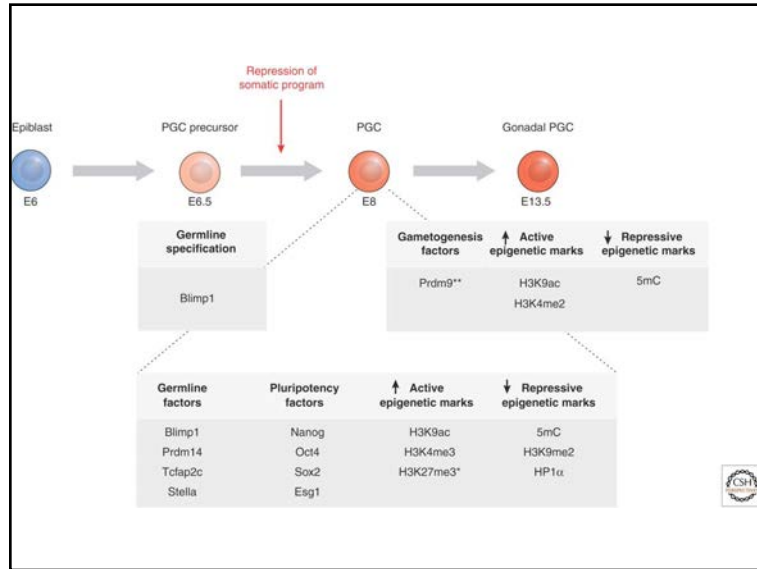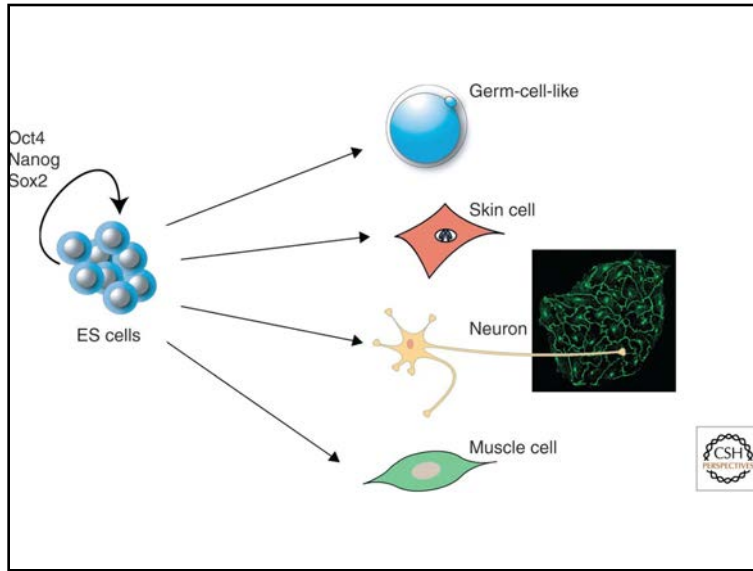
Naive pluripotency is associated with global DNA hypomethylation.
Leitch HG, et al. Nat Struct Mol Biol. 2013 Mar;20(3):311-6.
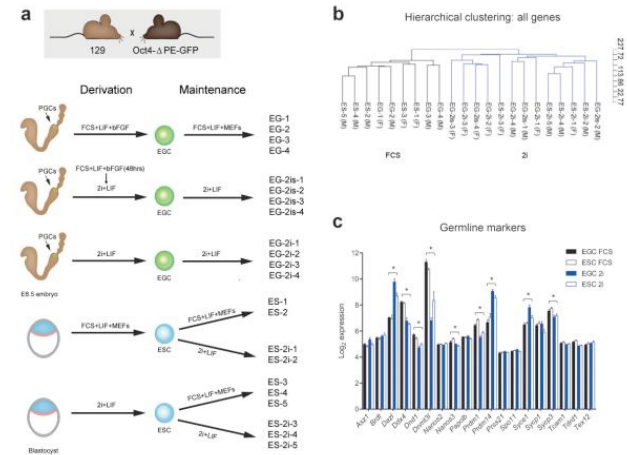
Heritable imprinting defect caused by epigenetic abnormalities in mouse spermatogonial stem cells.
Lee J, et al. Biol Reprod. 2009 Mar;80(3):518-27.
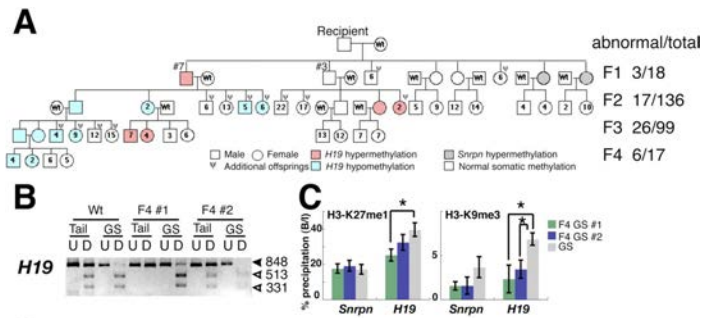
Germ-like cell differentiation from induced pluripotent stem cells (iPSCs).
Niu Z, et al. Cell Biochem Funct. 2013 Jan;31(1):12-9.
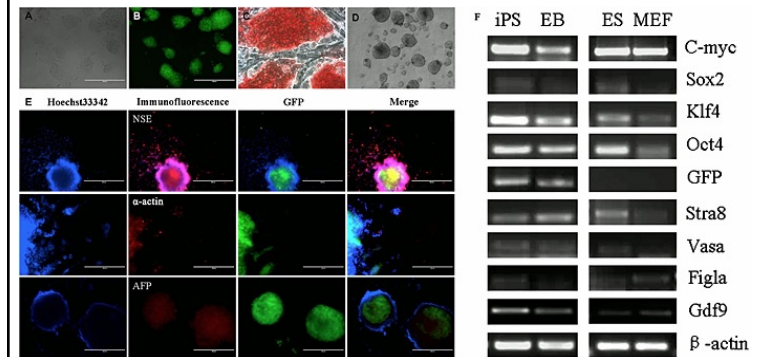
Characterization of iPSCs. A and B, iPSC colonies expressed GFP. C, iPSC colonies were positive for alkaline phosphatase (AP) staining. D, The iPSCs formed EBs after 3 days of suspension culture. E, Immunofluorescence analysis showed that EBs after 3 days of suspension in culture were positive for three germ layer specific markers: NSE (ectoderm marker), α-actin (mesoderm marker) and AFP (endoderm marker).
Bar = 200 μm. F, RT-PCR analysis compared the differences of the expression of pluripotent, germ cell markers in iPSCs, 3d EB-derived iPSCs, ES and MEF

Derivation of male germ cells from induced pluripotent stem cells in vitro and in reconstituted seminiferous tubules.
Yang S, et al. Cell Prolif. 2012 Apr;45(2):91-100.
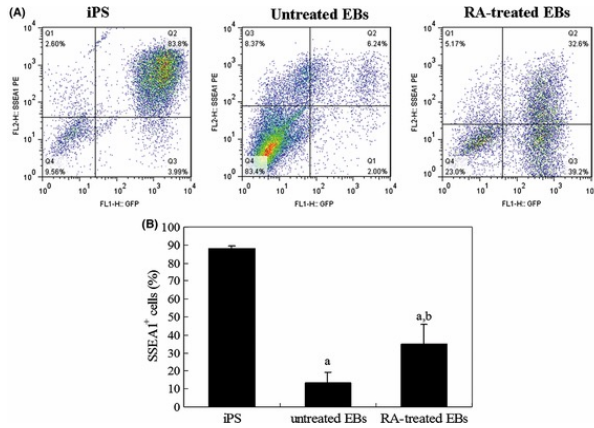
Flow cytometric analysis of SSEA1+cells, iPScells, untreated EBs and RA-treated EBs. (A) Example of flow cytometry of SSEA1+ cells. (B) Percentage SSEA1+ cells, iPS cells, untreated EBs and RA-treated EBs. [a]P < 0.05, compared to iPS cells; [b]P < 0.05, compared to untreated EBs.

Oocyte-like cells induced from mouse spermatogonial stem cells.
Wang L, et al. Cell Biosci. 2012 Aug 6;2(1):27.
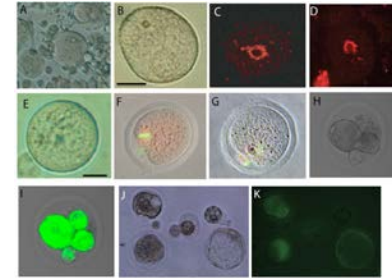
Characterization of mature oocytes and embryos derived from spermatogonial stem cells (SSCs) in culture.(A and B) Phase contrast microscopy of growing oocytes from the culture of SSCs (A), a SSCs-derived oocyte (SSC-Ooc) resembling that of germinal vesicle (GV) stage (B,scale bar = 30 μm). (C and D) Hoechst staining of a GV oocyte from a mouse ovary (C) and OG2-SSC-Ooc (D) showing a rim of chromatin around the nucleolus (the surrounded nucleolus). (E) Phase contrast microscopy of a fully-grown SSC-Ooc, scale bar = 30 μm. (F and G) YoYo1(green) and lamin B1 (red) staining of a control MII oocyte from a normal mouse ovary (F) and an oocyte with a polar body derived from SSC (G). (H and I) A 4-cell embryo was generated from SSC-oocyte by ICSI with sperm from an OG2 mouse, from which GFP gene was carried by sperm and was expressed in the resulting embryo (I). (J and K) Phase contrast microscopy of parthenogenetic embryos developed from OG2-SSC-oocytes in culture, Oct4/GFP from OG2 strain was expressed in the embryos (K). (L) Schematic representation of the reprogramming conditions from SSCs to oocyte-like cells.
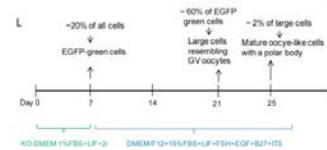
Offspring production of ovarian organoids derived from spermatogonial stem cells by defined factors with chromatin reorganization
Luo H, Li X, Tian GG, et al.
J Adv Res. 2021 Mar 17;33:81-98.
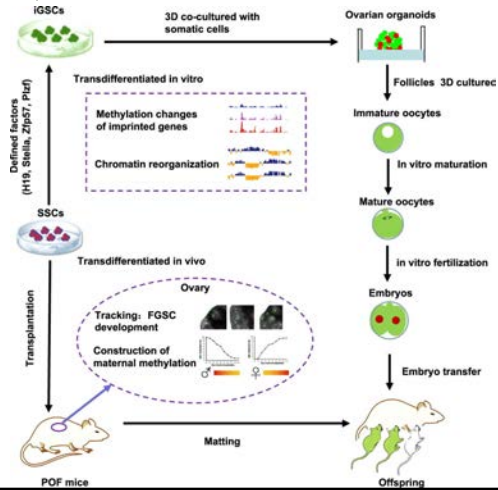
Conversion of adult mouse unipotent germline stem cells into pluripotent stem cells.
Ko K, et al. Nat Protoc. 2010 May;5(5):921-8.
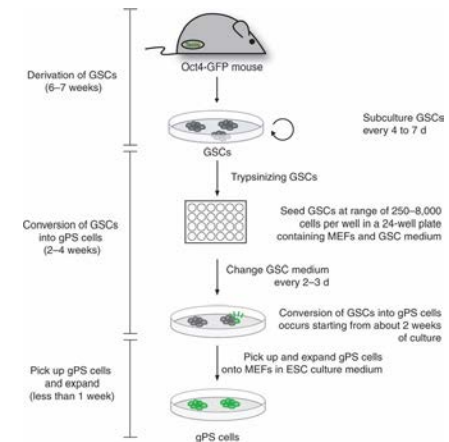
Schematic diagram depicting the establishment of germline stem cells (GSCs) from adult testis and the conversion of these GSCs into germline-derived pluripotent stem (gPS) cells.

Uniting germline and stem cells: the function of Piwi proteins and the piRNA pathway in diverse organisms.
Juliano C, Wang J, Lin H.
Annu Rev Genet. 2011;45:447-69.
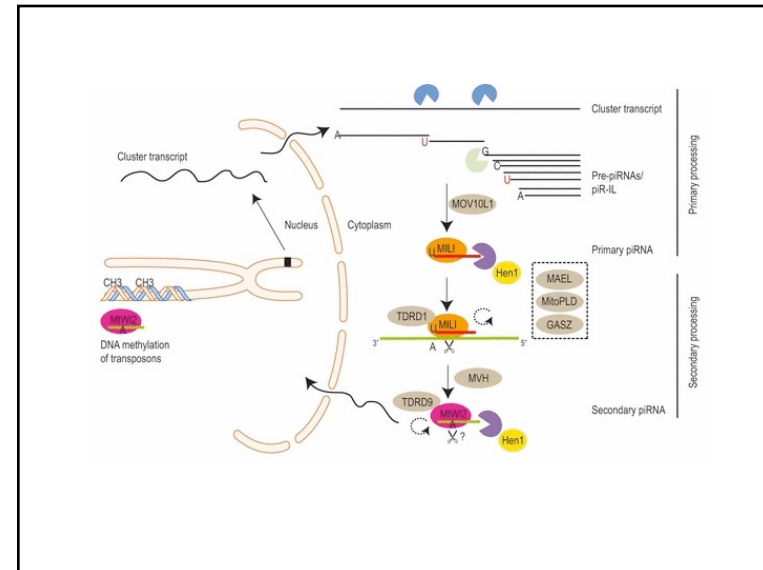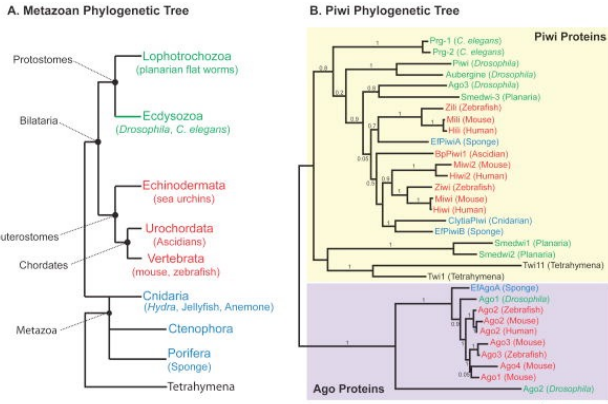
Emerging methods to generate artificial germ cells from stem cells.
Biol Reprod. 2015 Apr;92(4):89.
Zeng F, Huang F, Guo J, Hu X, Liu C, Wang H.
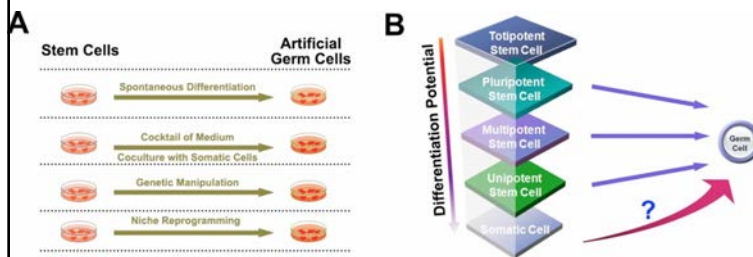
Different strategies for stem cell differentiation into germ cells. A) Methods of artificial germ cell generation currently under development include spontaneous differentiation, cocktail of differentiation induction factors and/or coculture enhancement, genetic manipulation, and niche reprogramming. B) Mammalian ESCs are considered to be totipotent and thus able to differentiate into almost all cell types, including germ cells. Artificial germ cells could be derived from different types of cells (pluripotent stem cells, multipotent stem cells, and unipotent stem cells); however, whether the in vitro-derived germ cells could be generated by somatic cell direct transdifferentiation with specific transcription factors similar to those of other cell lineage transdifferentiation processes is still unknown.
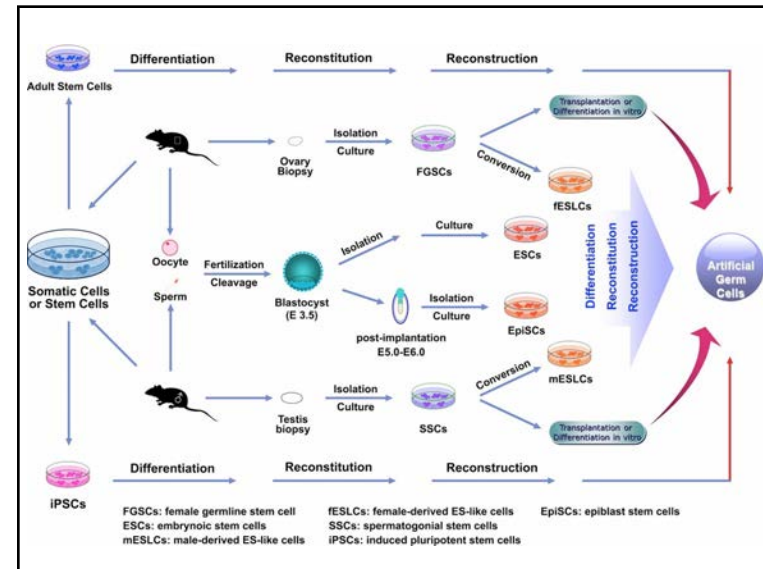
Making gametes from alternate sources of stem cells: past, present and future.
Reprod Biol Endocrinol. 2017 Nov 16;15(1):89.
Bhartiya D, Anand S, Patel H, Parte S.
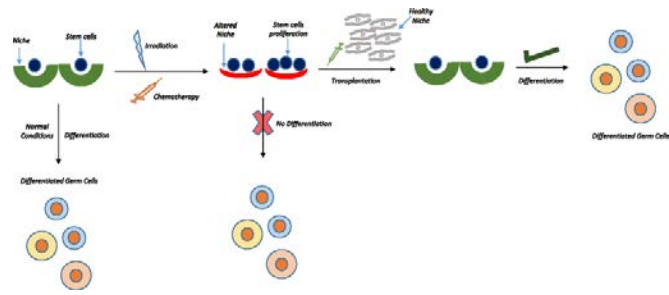
Human induced pluripotent stem cells and male infertility: an overview of current progress and perspectives.
Hum Reprod. 2018 Feb 1;33(2):188-195.
Fang F, Li Z, Zhao Q, Li H, Xiong C.
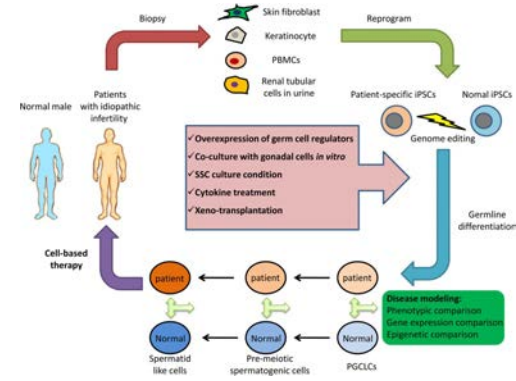
Generation of ovarian follicles from mouse pluripotent stem cells. Science
Yoshino T, Suzuki T, Nagamatsu G, et al.
2021 Jul 16;373(6552):eabe0237.
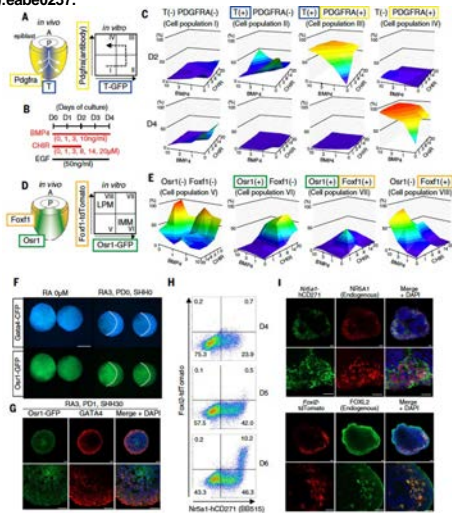
Generation of ovarian follicles from mouse pluripotent stem cells.
Yoshino T, Suzuki T, Nagamatsu G, et al.
Science. 2021 Jul 16;373(6552):eabe0237.
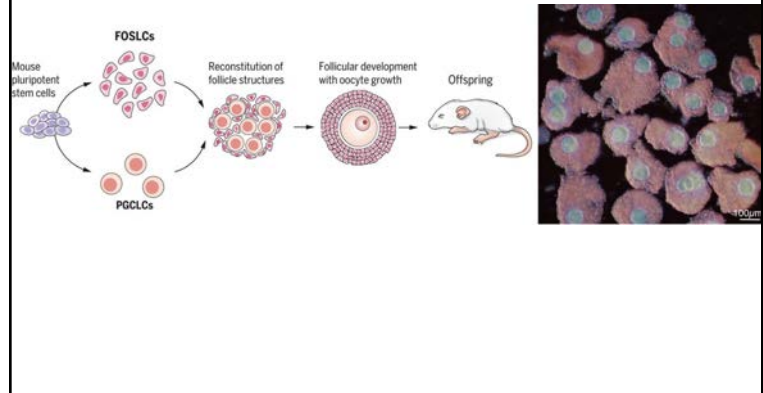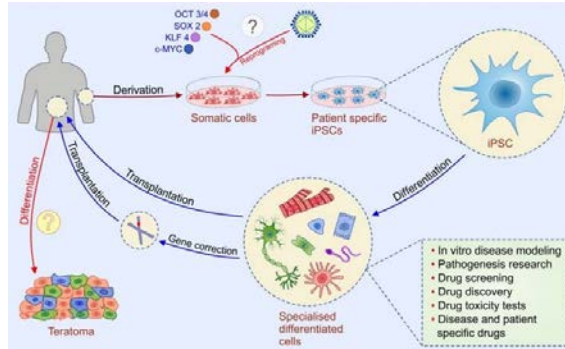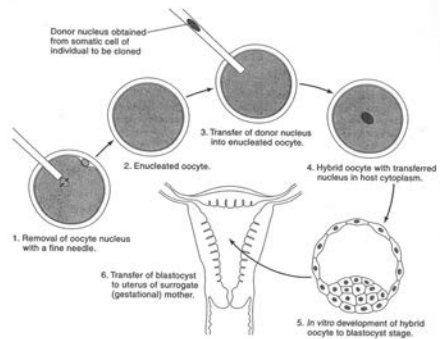
Cloning

Figure 20.1
Schematic illustrations of the somatic nuclear transfer protocol. This procedure has only been carried out in the mouse. In the reported sheep and cow cloning experiments, nuclear transfer is accomplished by fusing a somatic cell with an enucleated oocyte.
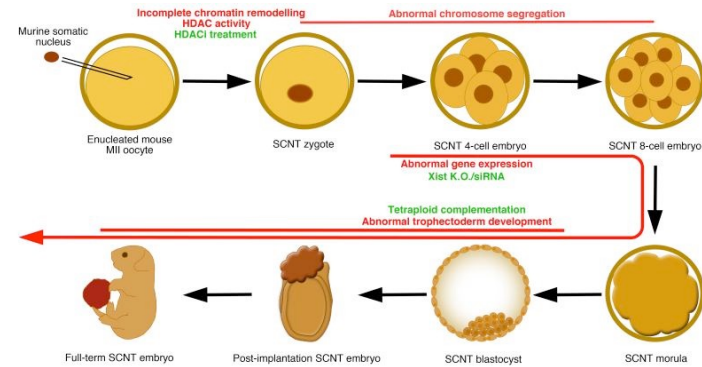
Recurrent reprogramming defects in SCNT embryos
In this example, a murine somatic cell nucleus is transferred into a mitotically-enucleated oocyte, containing cytoplasmic and nuclear reprogramming factors. After NT, the chromatin of the somatic nucleus is often not completely remodelled due to persistent histone deacetylase (HDAC) activity. This, and other reprogramming aspects, can be improved by HDAC inhibitor (HDACi) treatment. Abnormal chromosome segregation often occurs during the early cleavages and appears to be a major cause of developmental failure when it happens before the 8-cell stage. Following zygotic genome activation, abnormal gene expression, including Xist RNA from Xa and the subsequent under-expression of X-linked genes, further inhibits SCNT embryo development. This can be improved by removing Xist from Xa in donor nuclei, or injection of Xist siRNA in SCNT zygotes. Finally, incomplete reprogramming of the trophectoderm lineage, and the resulting defects in trophectoderm development are a major cause of the lethality of post-implantation stage SCNT embryos. This can be rescued by replacing the trophectoderm lineage with one generated from in vitro fertilized embryos through tetraploid complementation.

Cloning in action: can embryo splitting, induced pluripotency and somatic cell nuclear transfer contribute to endangered species conservation?
Swegen A, Appeltant R, Williams SA.
Biol Rev Camb Philos Soc. 2023 Aug;98(4):1225-1249.
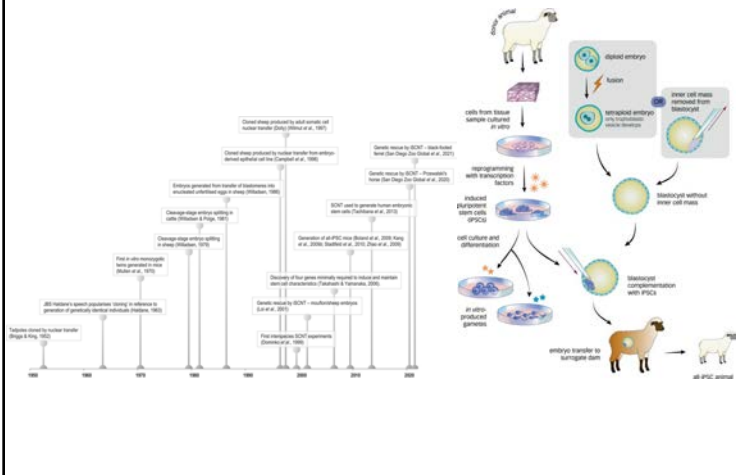
Table 1. Comparison of cloned embryo developmental potential with the initiation embryonic RNA synthesis.

species (reference)	cell type of donor nucleus	genome on	cloned offspring
pig (23)	embryonic; 4-cell stage	4-cell stage	yes
mouse (3)	embryonic; 2, 4 and 8-cell stage	2-cell stage	yes
mouse (33)	embryonic stem cells	2-cell stage	no
cattle (14)	embryonic; Inner cell mass (ICM)	8-cell stage	yes
cattle (26)	embryonic; cultured ICM cells	8-cell stage	yes
cattle (30)	embryonic; cell line derived from ICM	8-cell stage	no
sheep (2)	embryonic; cell line derived from ICM	8-cell stage	yes
sheep (37)	fetal and adult cells	8-cell stage	yes

Systems genetics implicates cytoskeletal genes in oocyte control of cloned embryo quality.
Cheng Y, et al. Genetics. 2013 Mar;193(3):877-96.
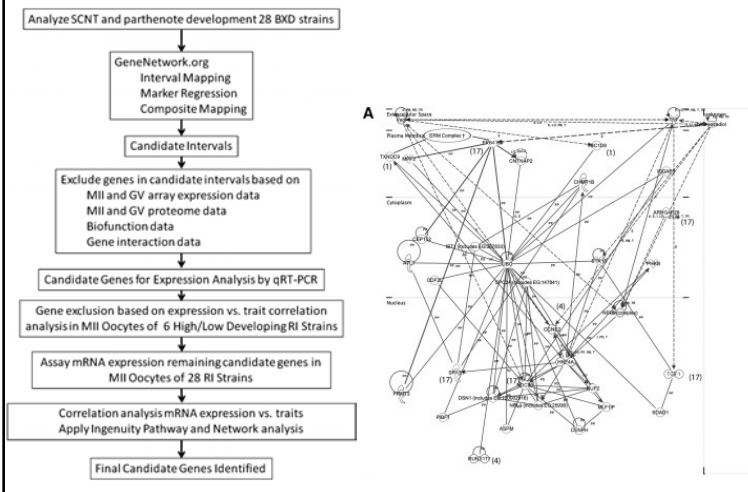
Early death of mice cloned from somatic cells

Published online: 11 February 2002; DOI: 10.1038/ng841

Human Cloning: Biology, Ethics, and Social Implications.
Bonetti G, Donato K, Medori MC, et al.
Clin Ter. 2023 Nov-Dec;174(Suppl 2(6)):230-235.

Culminating the analysis is a reiteration of the imperative to develop and govern human cloning technology judiciously and conscientiously. Finally, it discusses several ethical and practical issues, such as safety concerns, the possibility of exploitation, and the erosion of human dignity, and emphasizes the significance of carefully considering these issues.

"Systems Biology of Reproduction"

Spring 2024 (Even Years) – Course Syllabus
Biol 475/575 Undergraduate/Graduate (3 Credit)
SLN: (475) – 06763, (575) – 06764
Time - Tuesday and Thursday 10:35 am-11:50 am
Course Lectures in person and recorded on Canvas/Panopto and Discussion Sessions live in person and on WSU Zoom for all campuses (Hybrid Course)
Room – CUE 418
Course Director – Michael Skinner, Abelson Hall 507, 335-1524, skinner@wsu.edu
Co-Instructor – Eric Nilsson, Abelson Hall 507, 225-1835, nilsson@wsu.edu
Learning Objective -
Current literature based course on the Systems Biology of Reproduction. Learning Systems approaches to the biology of reproduction from a molecular to physiological level of understanding.
Schedule/Lecture Outline –

January	9 & 11	Week 1	Systems Biology Introduction
	16 & 18	Week 2	Molecular/ Cellular/ Reproduction Systems
	23 & 25	Week 3	Sex Determination Systems
Jan /Feb	30 & 1	Week 4	Male Reproductive Tract Development & Function
February	6 & 8	Week 5	Female Reproductive Tract Development & Function
	13 & 15	Week 6	Gonadal Developmental Systems Biology
	20 & 22	Week 7	Testis Systems Biology
	27 & 29	Week 8	Ovary Systems Biology
March	5 & 7	Week 9	Epigenetics and Transgenerational Gonadal Disease
	11 – 15	**Week 10**	**Spring Break**
	19 & 21	Week 11	Gametogenesis/ Stem Cells/ Cloning
	26 & 28	Week 12	Hypothalamus-Pituitary Development & Function
April	2 & 4	Week 13	Reproductive Endocrinology Systems
	9 & 11	Week 14	Fertilization & Implantation Systems
	16 & 18	Week 15	Fetal Development & Birth Systems
	23 & 25	Week 16	Assisted Reproduction/Contraception
Apr/May	30 & 2	Week 17	Exam or Grant Review