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Xenotransplantation exposes the etiology of *azoospermia factor* (*AZF*) induced male sterility

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Abstract

Ramathal et al. have employed an elegant xenotrans-plantation technique to study the fate of human induced pluripotent stem cells (hiPSCs) from fertile males and from males carrying Y chromosome deletions of the *azoospermia factor* (*AZF*) region. When placed in a mouse testis niche, hiPSCs from fertile males differentiate into germ cell-like cells (GCLCs). Highlighting the crucial role of cell autonomous factors in male sterility, hiPSCs derived from azoospermic males prove to be less successful under similar circumstances. Their studies argue that the agametic Sertoli cell only phenotype of two of the *AZF* deletions likely arises from a defect in the maintenance of germline stem cells (GSCs) rather than from a defect in their specification. These observations underscore the importance of the dialogue between the somatic niche and its inhabitant stem cells, and open up interesting questions concerning the functioning of the somatic niche and how it communicates to the GSCs.

Keywords

azoospermia factor; germ cells; hiPSCs; male sterility; niche; stem cells; testes

Introduction

Somatic niches that support germline stem cells (GSCs) have two central and seemingly opposite functions (for review, see [1–5]). The niche provides a microenvironment that establishes and maintains GSC identity. However, at the same time it also promotes differentiation by signaling the GSCs to divide asymmetrically and ensures that one, but not both, of the daughter cells commences subsequent development. These niche functions are realized by cell-cell signaling, via soluble growth factors and signaling ligands, by direct physical contact between somatic niche cells and GSCs, and by the 3-dimensional organization of the GSCs and the somatic niche cells. The ultimate outcome of this association results in maintenance of stem cell fate, which is limited by asymmetric cell

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division. Typically after a GSC division, the daughter cell proximal to the niche retains stem cell identity whereas the daughter cell distal from the niche undergoes differentiation.

Germline stem cell niches balance between differentiation and self-renewal

The balancing act between differentiation and stem cell self-renewal is crucial for maintaining tissue homeostasis. Excessive self-renewing mitotic divisions can lead to tumor formation (teratomas), while precocious stem cell differentiation can result in GSC depletion and sterility. In several well-documented cases this homeostatic balance is maintained by factors produced by the somatic niche cells. The best-studied examples of germline stem cell niches are the *Drosophila* ovary and testis (for review, see [4, 5]).

Drosophila ovary

The *Drosophila* ovary is organized into 16 ovarioles. The germarium, which contains the GSCs and their mitotically active daughter cells, is located at the anterior of the ovariole and it is followed by a string of egg chambers arranged in sequentially older developmental stages. The ovarian niche is located at the tip of the germarium and is composed of three somatic cell types: terminal filament cells, cap cells and escort cells (Fig. 1A). The terminal filament cells secrete Unpaired (Upd), which activates the JAK/STAT pathway in the cap and escort cells. This activation induces the production of Bone morphogenetic protein (BMP) ligands that function to maintain GSC identity by signaling to BMP receptor in GSCs [6–8]. BMP ligand expression by the escort cells is also regulated by Hedgehog (Hh) signaling from the cap cells [9]. The niche is arranged so that each GSC adheres directly to two to three cap cells, and there are a sufficient number of cap cells in the niche to support two to three GSCs per ovariole. The GSCs divide asymmetrically so that only one of the daughter cells retains adhesion to the cap cells, and thus is able to self-renew. The other daughter is positioned away from the niche and this physical arrangement ensures that it assumes a new “cystoblast” identity, and subsequently undergoes a series of mitotic divisions to create a germline cyst and ultimately an egg chamber. As is true for the maintenance of GSC identity, the differentiation of a mature egg from the cystoblast daughter cell is not an autonomous process, but instead requires an intricate set of signals back and forth between the developing germline cyst and the surrounding somatic cells.

Drosophila testis

As in the ovary, there is a stereotypical 3-dimensional arrangement of somatic and germline cells in the *Drosophila* testis niche (Fig. 1B). The niche is composed of somatic hub cells that adhere to a basement membrane and also directly contact 7 to 12 GSCs. Self-renewal of GSCs is mediated by JAK/STAT signals produced by hub cells and BMP signals produced by hub cells and somatic cyst progenitor cells [8, 10, 11]. During asymmetric GSC division, the mitotic spindle is oriented such that one daughter remains in contact with the hub while the other daughter is positioned away from the niche [12]. The daughter remaining in contact with the niche self renews, while the daughter distal to the niche initiates differentiation.

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Mouse testis

In contrast to the stereotypical 3-dimensional arrangement of cells in the ovary and testis niche of the fly, somatic niche cells and GSCs in the mouse testis appear to be loosely organized. The GSCs are located in the basal cell layer of the seminiferous tubules (Fig. 1C). The seminiferous tubules have a complex architecture in which germ cells contact somatic Sertoli cells throughout spermatogenesis. Tight junctions between adjacent Sertoli cells separate the seminiferous tubules into basal and adluminal compartments. The basal compartment is supported by a basement membrane, and GSCs contact both the basement membrane and Sertoli cells. Glial cell line derived neurotrophic factor (GDNF), related to transforming growth factor-β, is produced by Sertoli cells and is important for self-renewal of GSCs [13]. The niche may also include vasculature and interstitial cells underlying the basement membrane of the tubules, because undifferentiated germ cells are found in regions of the tubule adjacent to blood vessels and interstitial cells [14, 15]. Spermatogonia in contact with the basement membranes typically undergo several rounds of mitotic division with incomplete cytokinesis to produce a multicellular germ cell cyst. Germ cell cysts that enter meiosis are displaced from the basement membrane and move out of the basal compartment into the adluminal compartment, where they complete differentiation [16–18].

Experimental evidence for a niche

The regenerative potential of embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) has ignited interest in the mechanisms used to maintain stem cell identity and promote differentiation. While much research has focused on cell autonomous factors that confer ESC or iPSC identity, another important area of inquiry has focused on understanding the functioning of these cells in a native tissue context, where they can behave as stem cells and self-renew, and/or undergo differentiation. The analysis of the interaction between ESCs or iPSCs and the surrounding tissues has been instrumental in establishing operational criteria for identifying microenvironments that can function as niches.

One operational definition of a functional niche is the ability to induce stem cell behavior in an exogenously supplied pool of appropriately programmed cells. In a microenvironment that can function as a niche, an exogenously supplied pool of stem cells would be able to both maintain their stem cell fate and at the same time produce daughter cells that can enter and properly execute the appropriate differentiation pathway(s). This dual capacity for self-renewal and differentiation must be established independently of the presence of endogenous stem cells. A reconstitution assay of this nature has been used to define a niche in the mouse testis. When early mouse germ cells are transplanted into the seminiferous tubules of a host lacking endogenous germ cells, they can repopulate the tubules with GSCs that have the capacity to both self-renew and undergo differentiation into functional sperm [19, 20]. This transplantation assay demonstrates that the niche can exist independently of endogenous stem cells, and that exogenous stem cells can reconstitute a functional niche.

Transplantation experiments in *Drosophila* are not technically feasible. However, reconstitution of a GSC depleted niche is possible using genetic manipulations. When the

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ovarian niche is depleted, a GSC can divide symmetrically so that both daughters maintain contact with the cap cells and become GSCs [21]. If the niche is completely depleted, differentiating mitotic cyst cells that are exposed to niche signals can undergo dedifferentiation and replace missing GSCs [22]. Repopulation of a stem cell niche by breakdown of mitotic germline cysts and dedifferentiation is also observed in the *Drosophila* male germline [23, 24]. In the mouse seminiferous tubules, stochastic breakdown of germline cysts is used as a mechanism of stem cell replacement [25]. Therefore, reoccupation of the niche with cells that have not fully differentiated is a common mechanism to replenish the germline.

Another operational definition of a functional niche relies on molecular or genetic manipulations rather than transplantation experiments. In these experiments, loss or gain of function conditions either in the cells that constitute the somatic component of the niche or in the GSCs lead to reproducible and predictable outcomes. For example, Xie and Spradling [21] tested whether the GSC fate could be dictated by manipulating signaling pathways, either in the soma or in the germline. They showed that the BMP 2/4 homolog *decapentaplegic* (*dpp*) is the ligand that promotes GSC self-renewal in the fly ovary. Overexpression of *dpp* in somatic cells blocked differentiation and resulted in tumorous ovaries filled with many “stem cell-like” cells. Conversely, mutations in *dpp* caused a depletion of GSCs from the niche. Moreover, GSCs mutant for the Dpp receptor, *saxophone*, fail to self-renew and are depleted from the niche.

Xenotransplantation as a genetic tool

The genetic manipulations of the BMP pathway in the fly ovary illustrate the role of non-autonomous and cell autonomous factors in promoting GSC self-renewal or differentiation. Extracellular signals, emanating from the somatic cells of the niche, are received by GSCs, which then transduce the signal into an appropriate response, in this case self-renewal. Similar experiments aimed at defining non-autonomous and autonomous factors needed for GSC renewal and/or differentiation are technically challenging in mice, and impossible in humans. There are many genetic diseases that lead to sterility in humans; however, it is not readily possible to directly test their etiology. One way to overcome this limitation is the use of xenotransplantation. This is precisely the approach Ramathal et al. [26] employed to investigate the functioning of the human Y chromosome *azoospermia factor* (*AZF*) region in the process of spermatogenesis.

Generation of hiPSCs from infertile genotypes

Ramathal et al. [26] generated hiPSCs from dermal fibroblasts of fertile men and from infertile men with three different types of *AZF* deletions that disrupt spermatogenesis. The first deletes the *AZFa* region, the second removes sequences from the *AZFb* and *AZFc* regions, while the third deletes the *AZFc* region. The *AZF a* and *AZF c* donors presented with a “Sertoli cell only” phenotype with no germ cells. The *AZF bc* donor was oligospermic. He had germ cells that failed to differentiate resulting in very low sperm counts. The *AZF a* region contains several candidate genes including a ubiquitin specific protease (USP9Y) and a DEAD-box helicase, DBY, and the phenotypic defects likely arise

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from loss of several genes. Two of the genes in the *AZFb* region are RBMY and EIF1AY. RBMY encodes an RRM-type RNA binding protein, while E1F1AY encodes a Y specific eIF-1A isoform. It is not known whether either of these genes, or other unidentified genes in the *AZFb* interval, is important for spermatogenesis. Finally the *AZFc* region has four DAZ (Deleted in azoospermia) family genes. There are also two other autosomal members of this family, *DAZ-like* (*DAZL*) and *BOULE* [27–30]. The *DAZ* family genes encode RRM-type RNA-binding proteins that are conserved among metazoans. The *Drosophila DAZ* gene, *boule*, is expressed in the male germline and is required for meiosis and spermatid differentiation [31]. Several other genes in the *AZFc* deletion besides the *DAZ* family genes may contribute to the aberrant spermatogenesis.

hiPSCs from normal and infertile genotypes adopt GCL identity in vitro

Ramathal et al. [26] found that hiPSCs derived from both control and azoospermic dermal fibroblasts met the classic pluripotency criteria: all samples were capable of generating three germ layers in vitro and in vivo. Moreover, when the different hiPSC lines were subjected to a regimen that induces germ cell fate (BMP 4/8, retinoic acid and human recombinant leukemia inhibitory factor), all were able to adopt germ cell-like (GCL) identity as judged by the expression of a *VASA:GFP* reporter. However, hiPSCs derived from azoospermic donors gave rise to fewer numbers of germ cells than control hiPSCs following in vitro differentiation. Because the hiPSCs from azoospermic patients were able to differentiate into germ cells in vitro, the authors tested their fate after xenotransplantation into mouse seminiferous tubules.

hiPSCs xenotransplanted to mouse testes adopt GCL fate

Previous studies have shown that when human germ cells are transplanted into mouse seminiferous tubules they migrate toward the basement membrane and form long chains of spermatogonia; however, unlike mouse germ cells they do not progress through meiosis, nor do they undergo differentiation [32–34]. When Ramathal et al. [26] transplanted the hiPSCs into a mouse gonad (devoid of endogenous germ cells) they found that the behavior of the transplanted cells differed depending upon their location in the testis. Cells located away from the basement membrane underwent extensive proliferation and resembled embryonic carcinoma cells or yolk sac tumors. In contrast, transplanted hiPSCs associated with the basement membrane express *VASA*, indicating that they have acquired a germ cell-like (GCL) identity. This conclusion is supported by changes in the level of 5-methyl cytosine. In the mouse embryo, global demethylation occurs in the primordial germ cells (PGCs) while they migrate to the genital ridge [35]. Ramathal et al. found that irrespective of the genotype of transplanted human donor cells, demethylation was observed in the GCL cells (GCLCs) associated with the basement membrane.

As would be predicted if the genes deleted in the different AZF mutations function autonomously, *AZF*-deleted hiPSCs were not as competent as the control hiPSCs in the acquisition of a GCL fate in vivo. *AZF*-deleted lines gave rise to fewer GCLCs as compared to transplants of the control cells. Moreover, germ cell specific protein expression was weaker, and variable in GCLCs from *AZF*-deleted hiPSCs. This was also the case for in

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vitro derived primordial germ cells (iPGCs) from *AZF*-deleted hiPSCs, which resulted in variable numbers of cells that expressed germ cell specific markers such as *VASA*, *STELLA*, *IFITM3* and *NANOS3*. While *AZF*-deleted hiPSCs could acquire GCL fate in vivo and in vitro, the transplanted hiPSCs failed to express *DAZ*, *PLZF* and *UTF1* in vivo, which are thought to mark gonocyte-like cells [36–41]. Taken together these findings indicate that *AZF*-deleted cells are able to respond to signals from the mouse stem cell niche and execute the initial steps involved in the acquisition of a GCL identity, but are unable to complete critical downstream steps that hiPSCs derived from normal individuals are capable of completing. Moreover, it would appear that *VASA* expression and demethylation may be amongst the first markers of germ cell fate, and that the acquisition of GCL identity takes place in a sequential manner.

The niche and the specification of germ cell fate

The available evidence argues that the basement membrane of mouse seminiferous tubules constitutes the germline stem cell niche of the testis. As such it must produce self-renewal signals for the GSCs. However, undifferentiated human cells transplanted into this microenvironment express markers characteristic of a GCL fate, which argues that this microenvironment is competent to produce ligands that can initiate de novo a program that specifies germline identity. One plausible explanation for why the niche has these seemingly distinct activities is that key soma-germline signaling pathways are used reiteratively during development. The notion that the same signaling pathway is used at different steps is supported by studies on germline development in flies. In the adult ovaries and testes, BMP signals from the somatic niche play a central role in maintaining GSC-identity and ensuring that dividing GSCs self-renew. But this is not the only point in germline development where the BMP signaling pathway is deployed. PGC specification in blastoderm stage fly embryos is mediated by maternal factors deposited at the posterior pole that are incorporated into the PGCs when they cellularize. However, once PGCs begin their migration towards the somatic components of the embryonic gonad, BMP signaling from the surrounding soma is required for sustaining PGC identity [42, 43]. Unlike in flies, PGC specification in the mouse is a completely non-autonomous process, depending upon BMP signals from the extra-embryonic ectoderm to the epiblast [44]. Thus an obvious speculation is that the BMP signaling might be deployed yet again in the mouse testis to ensure that the germline stem cells self-renew. This could explain how basement membrane association is able to promote the acquisition of germline traits by transplanted hiPSCs.

The studies of Ramathal et al. [26] also demonstrate that the mouse testis niche is not sufficient to fully transform hiPSCs into germline cells. In addition to the fact that the hiPSCs cells don’t express the appropriate set of germline markers, their development also differs from that observed when human germ cells are transplanted into the mouse testis. When human fetal testis cell suspensions are transplanted into mouse testes, the human germline cells that associate with the basement membranes form small chains or clusters of interconnected cells [26]. Chains and clusters of spermatogonia are also observed when human spermatogonial stem cells are transplanted into the mouse testis [32–34]. The fact that hiPSCs fail to develop to this stage suggests that the basement membrane microenvironment does not produce critical determinants that are needed to further elaborate

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the specification process. This possibility is supported by studies showing that mRNA-based reprogramming of hiPSCs to a germline-like fate prior to their transplantation into the mouse seminiferous tubules increased the frequency of basement membrane localization of the transplanted cells [45]. Moreover, hiPSCs that expressed VASA prior to transplantation also differentiated further than control hiPSCs [45]. The notion that key determinants are not provided by the niche microenvironment in the mouse testis is also supported by studies showing that it is possible to reconstitute spermatogenesis or oogenesis by first differentiating mouse iPSCs in vitro to PGCLCs, and then transplanting them into the gonad [46–48]. Of course, in addition to missing germline determinants, it is likely that there are "secondary" incompatibilities (e.g. interactions of critical cell: cell adhesion proteins, or ligand: receptors) that stem from the evolutionary distance and interfere with communication between hiPSCs and the mouse testis niche. These secondary incompatibilities could interfere with the differentiation process even if mice and humans utilized identical pathways to mediate soma-germline communication and orchestrate germline development. Supporting this idea is the observation that human germ cells transplanted into the mouse testis do not enter meiosis or commence differentiation, whereas transplanted mouse germ cells can form a fully functional gonad.

Conclusions and outlook

Despite limitations of xenotransplantation, Ramathal et al. were able to use it to investigate the effects of different *AZF* deletions on germline development. They provide direct evidence that the phenotypic effects of the *AZF* deletions on male fertility arise from cell-autonomous germline defects, rather than somatic problems. Given the candidate genes implicated in *AZF* sterility, this is an expected result. However, it should be possible to determine whether defects in the functioning of the germline or the soma are responsible for male sterility in other less well-defined cases. It is also remarkable that hiPSCs generated from azoospermic AZFDa and AZFDc males that exhibit the "Sertoli cell only" phenotypes are able to form GCLCs upon xenotransplantation. This finding would argue that defects in the process of germline specification might not be responsible for the "Sertoli cell only" phenotypes of the *AZF a* and *AZF c* deletions. Instead, these lesions might compromise the maintenance of the germline stem cells after they are formed. The system used by Ramathal et al. [26]–transplantation of hiPSCs and the ability to assay GCL fate–lends itself to screens for genes required for germ cell differentiation, using specific mutations or gene expression knockdown using RNA interference (RNAi). For example, transplanting hiPSCs or miPSCs that fail to express specific ligand receptors could be used as a method to determine what signals from the niche specify GCL fate or self-renewal. In fact, knockdown experiments of E-cadherin and Stat3 indicate that these molecules are not important for GSC self-renewal [49, 50].

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Abbreviations

AZF	azoospermia factor
BMP	bone morphogenetic protein
GCLC	germ cell-like cell
GSC	germline stem cell
hiPSC	human induced pluripotent stem cell

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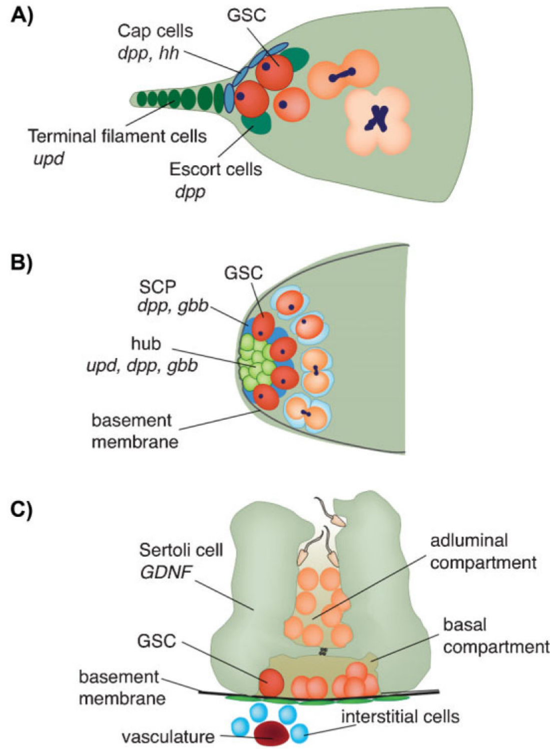

Figure 1.

Germline stem cell niches in the *Drosophila* ovary, testis and the mouse testis. **A:** The *Drosophila* ovary niche. GSCs (dark orange) are maintained by three somatic cell types: terminal filament cells, which secrete Upd, cap cells, which secrete Dpp and Hh, and escort cells, which secrete Dpp. GSCs directly adhere to cap cells. Differentiating daughter cells (light orange) move away from the niche and form interconnected cysts. **B:** The *Drosophila* testis niche. GSCs (dark orange) and somatic cyst progenitor cells (SCPCs -blue) adhere to the hub cells (green) that produce Upd. BMP ligands Dpp and Gbb produced by hub cells and SCPCs maintain GSC fate. Following asymmetric cell division daughter cells positioned away from the hub (light orange) differentiate. **C:** The mouse testis niche. GSCs reside in the basal compartment in direct contact with the basement membrane and Sertoli cells, which produce GDNF, and GSCs are positioned near to underlying vasculature and interstitial cells. Differentiating cells (light orange) form cysts and clusters, and ultimately lose contact with the basement membrane and enter the adluminal compartment.