Daniel J. Manella, Jr. Research Grant Progress Update

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Through the Fertility Preservation Program in Pittsburgh (https://fertilitypreservationpittsburgh.org/), we have cryopreserved testicular tissue (with informed consent) for 193 patients who were scheduled to initiate gonadotoxic therapy (chemotherapy or radiation). In addition to Pittsburgh (51 patients), we have expanded patient recruitment to coordinated centers in Washington DC (30), Cincinnati, OH (24), Chicago, IL (39), Milwaukee, WI (6), Los Angeles, CA (5), Rochester, MN (10) and Beer-Sheva Israel (28). Discussions are ongoing with several additional centers in the US and abroad. Combining the patient recruiting power and experiences from these top medical institutions has enabled us to optimize our testicular tissue freezing protocol and disseminate our current "best practices" to patients through all coordinated centers. Testis tissue is typically collected by incisional biopsy and allocated 75% for the patient's future use and 25% for research.

Xenografting of prepubertal human testicular tissue into rhesus macaques.

We have xenografted human prepubertal tissues into immune suppressed castrated Rhesus macaques. Patient tissues from 21 prepubertal boys were grafted either into the scrotum, or under the back skin. Each site received between 2 and 4 pieces of tissue. Unfortunately, the tissues did not survive grafting. Graft sizes slowly decreased over a period of a few months and the tissues disappeared by the end of 6 months. These results were in stark contrast to transplant of prepubertal Rhesus testicular tissues, which matured to produce complete spermatogenesis.

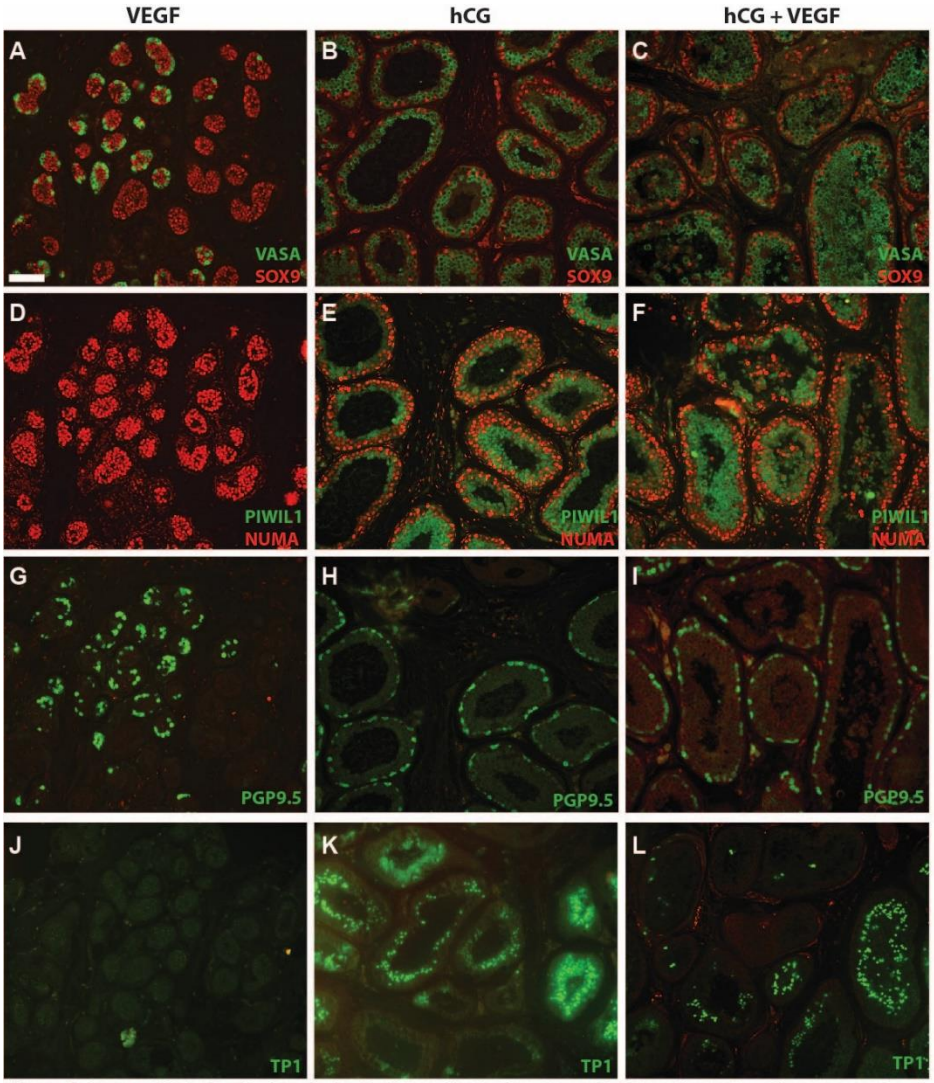

Figure 1: Immunohistochemical analysis of nHP grafts. Grafts recovered at 7 months post-grafting were analyzed by immunostaining for Sertoli cell and germ cell markers. **A.** Grafts obtained from VEGF treated mice had cells expressing VASA and SOX9, indicating the presence of germ cells and Sertoli cells respectively. **D, G, J.** Undifferentiated spermatogonia marked by PGP9.5 were also observed, however, expression of meiotic and post-meiotic markers, PIWIL1 and TP1 was absent. Grafts from hCG and hCG+VEGF mice had seminiferous tubules with enlarged lumen. **B, C**. VASA+ germ cells and SOX9+ Sertoli cells were observed. **H, I.** PGP9.5+ undifferentiated spermatogonia were observed on the basement membrane while PIWIL1+ meiotic and TP1+ post-meiotic germ cells marked were observed towards the lumen indicating the occurence of spermatogenesis in these grafts (**E, F, K, L**).

Xenografting of prepubertal human testicular tissue into immune deficient mice.

In addition to xenografting into Rhesus macaques, we also xenografted prepubertal human tissues into immune deficient mice. Previous studies have demonstrated that testicular tissue obtained from prepubertal Rhesus macaques can undergo complete spermatogenesis when grafted into recipient mice. It has also been shown that human chorionic gonadotropin (hCG) enhances maturation of monkey grafts, which is indicated by the

appearance of transition protein 1 (TP1, Fig. 1K and L). We hypothesized that treatment of recipient mice with hCG (stimulates testosterone production) along with vascular endothelial growth factor (VEGF – stimulates blood vessel formation) would promote maturation of human prepubertal testicular grafts. To test this hypothesis, we grafted prepubertal human testis tissue into castrated immunodeficient mice. Recipient mice received hCG and/or VEGF treatments and were compared with untreated mice. To compare the efficacy of these treatments in promoting maturation of human tissues with that observed in monkey tissues, we also grafted prepubertal monkey testicular tissue into mice under the same treatment groups. At 7 months post grafting recipients that received hCG had significantly larger grafts for both donor species. Monkey grafts obtained from hCG and hCG+VEGF treated mice showed tubular expansion with lumen formation. Germ cells expressing meiotic and post-meiotic markers, Piwi-like protein 1 (PIWIL1) and Transition protein 1 (TP1) were also observed. Monkey grafts from hCG and hCG+VEGF treated mice were also digested to isolate spermatozoa. These results demonstrate that treatment of recipients with monkey testicular grafts promoted maturation of prepuberal donor tissue. Mice with monkey grafts that received hCG and/or VEGF had significantly larger seminal vesicles (indicating the effects of testosterone from the grafts) compared to castrated mice that did not receive grafts. Similarly, mice with human grafts treated with hCG+VEGF (but not hCG or VEGF alone) had significantly larger seminal vesicles compared to castrated controls. In contrast to the monkey results, no germ cells (green VASA+ cells; Fig. 2) were observed in the human grafts. These results indicate that factors that promote prepubertal monkey graft maturation and spermatogenesis are not sufficient to maintain germ cells or promote spermatogenesis from human testicular grafts to the 7 month timepoint. In ongoing experiments, we are examining earlier timepoints to determine when human germ cells are lost. We are also testing additional factors, including Follicle Stimulating Hormone, to determine whether we can improve survival of germ cells and spermatogenesis from immature human testicular tissues.

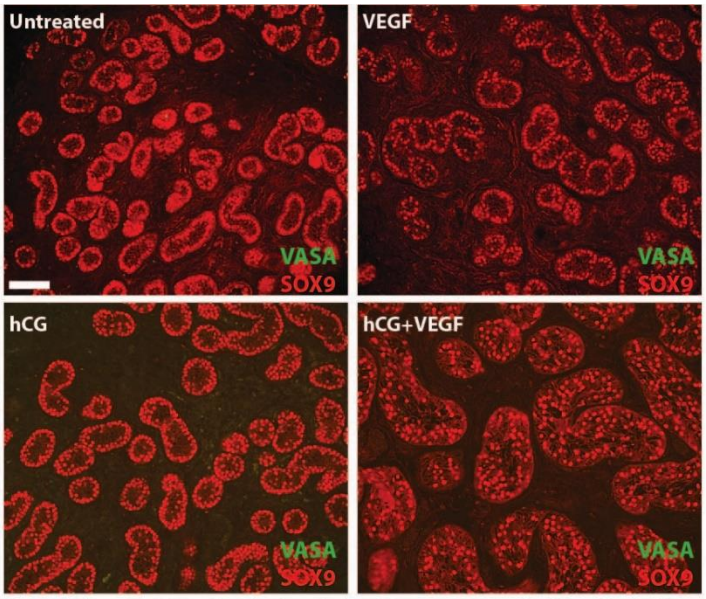

Figure 2: Immunohistochemical analysis of human gratfs. Grafts excised at 7 months post-grafting were stained with antibodies against pan germ cell marker, VASA and Setoli cell marker SOX9. Grafts retrieved from all treatment groups showed Sertoli Cell Only phenotype. Scale bar=100um.