LEYDIG CELL SURVIVAL AND REGENERATION IN AUTOGRAFTED ADULT MICE TESTIS

DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE AWARD OF DEGREE OF

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SUBMITTED BY

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This is to certify that the dissertation entitled "**Leydig cell survival and regeneration in autografted adult mice testis**" being submitted by **Ms. Surabhi Sonam** in partial fulfillment of the requirement for the award of degree of **Master of Science in Biotechnology** to the **Department of Biotechnology and Environmental Sciences, Thapar University, Patiala, Punjab.** This work is carried out at **Laboratory for the Conservation of Endangered Species (LaCONES), Centre for Cellular and Molecular Biology Annex-1, Hyderabad,** under my guidance and supervision. It is further certified that no part of this dissertation has been submitted for the award of any other degree or diploma.

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CANDIDATE'S DECLARATION

I **Surabhi Sonam** hereby declare that the work which is being described in the dissertation report entitled " **Leydig cell survival and regeneration in autografted adult mice testis** " has been carried out by me under the guidance of Dr. Sandeep Goel, Senior Scientist, Laboratory for Conservation of Endangered species, Centre for Cellular and Molecular Biology, Hyderabad. It is an authentic record to my own work during a period of 6 months from January 2012 to June 2012. This work is original and has not been submitted either in part or full for the award of any degree or diploma in any university or institute.

Surabhi Sonam

Hyderabad SURABHI SONAM

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This is to certify that the above statement given by the candidate is correct and true to the best of my knowledge.

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SURABHI SONAM

INDEX

LIST OF ABBREVIATIONS

ALC	-	Adult Leydig Cell
AMH	-	Anti-Mullerian Hormone
ATP	-	Adenosine Tri Phosphate
BSA	-	Bovine Serum Albumin
cAMP	-	Cyclic Adenosine Monophosphate
cM	-	Centimorgan
cDNA	-	Complementary DNA
CYP11A1	-	Cytochrome P450, family 11, subfamily A, polypeptide 1
CYP17A1	-	Cytochrome P450, family 17, subfamily A, polypeptide 1
DAB	-	3,3´-Diaminobenzidine tetrahydrochloride
DDX4	-	DEAD [Asp-Glu-Ala-Asp] box polypeptide 4
DHEA	-	Dihydroepiandrosterone
DHT	-	Dihydrotestosterone
DNA	-	Deoxyribonucleic Acid
EDS	-	Ethanedimethane sulfonate
FBS	-	Fetal Bovine Serum
FLC	-	Fetal Leydig Cell
FSH	-	Follicle-stimulating hormone
GnRH	-	Gonadotropin-Releasing Hormone
hCG	-	Human Chorionic Gonadotropin Hormone
HRP	-	Horse Radish Peroxidase
3βHSD	-	3-beta–hydroxysteroid dehydrogenase
HSD3B	-	Hydroxy-delta-5-steroid- dehydrogenase, 3-beta and steroid delta isomerase
HSD17B	-	Hydroxysteroid (17-beta) dehydrogenase

IgG	-	Immunoglobulin G
ILC	-	Immature Leydig cell
LH	-	Luteinizing Hormone
LHCGR	-	Luteinizing Hormone/Choriogonadotropin Receptor
MIS	-	Mullerian Inhibiting Substance
mRNA	-	Messenger Ribonucleic Acid
NAD	-	Nicotinamide adenine dinucleotide
NADPH	-	Nicotinamide Adenine dinucleotide phosphate dehyrogenase
P450scc	-	Cytochrome P450 side chain cleavage enzyme
PBR	-	Peripheral-type benzodiazepine receptor
PBS	-	Phosphate Buffered Saline
PCR	-	Polymerase Chain Reaction
PDGFA	-	Platelet-derived growth factor alpha polypeptide
PDGFRA	-	Platelet-derived growth factor receptor, alpha polypeptide
PLC	-	Progenitor Leydig Cell
PKA	-	Protein Kinase
PGC	-	Primordial germ cell
PMC	-	Peritubular Myoid Cell
RNA	-	Ribonucleic Acid
RT-PCR	-	Reverse Transcriptase Polymerase Chain Reaction
SCID	-	Severe Combined Immune Deficiency
SEM	-	Standard Error Mean
SLC	-	Stem Leydig cell
Sry	-	Sex determining region Y
STAR	-	Steroidogenic Acute Regulatory Protein
WT-1	-	Wilms tumor 1

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ABSTRACT

***Background*:** The ectopic autografting of testis tissue is a promising approach towards studying testicular development, male germline preservation and restoration of male fertility. However the grafted adult testis is severely affected by ischemia and does not support spermatogenesis. The present study was a novel attempt to study the fate of testicular cells in adult testis subjected to autografting.

***Methodology*:** The testis of adult inbred Balb/c mice (6- weeks old) was autografted subcutaneously under the dorsal skin. These grafts were analyzed 1-, 2-, 4- and 8- week after grafting. The expression of cell-specific proteins was examined in testes by immunohistology. Seminal vesicle weight was determined to assess the functionality of Leydig cells.

***Results*:** Although the tubular architecture was progressively destroyed in autografts, the Leydig cell regenerated and their number progressively increased in grafts as indicated by CYP11A1, LHCGR and HSD3B immunohistochemically. Restoration of seminal vesicle weight of recipients exhibited Leydig cell functionality. Expression of PDGFRA, a stem Leydig cell (SLCs) marker, in 1- and 2- week grafts further confirmed Leydig cell regeneration. Absence of DDX4 staining from grafts at all collection time points suggested complete loss of germ cell population. Presence of Sertoli cells in grafts was confirmed by WT-1 immunohistology.

***Conclusions*:** These present study strongly indicate that Leydig cells regenerated *de-novo* in testis autografts however, germ cell population in grafts were unable to survive. The success of adult testis autografting in mouse containing functional Leydig cells can application to other vertebrates with testosterone deficiency and Leydig cell dysfunction.

1. INTRODUCTION

The fascination towards testis grafting gained significant momentum since the time John Hunter performed first testicular grafting in 18th century in chicken (Palmer 1837). Testis transplantation is achieved by grafting the tissue to an ectopic site, usually subcutaneously under the skin or orthotopically into the scrotal sac, where once revascularized the graft can generate sperm. The factors like temperature at grafting site, size of tissue being grafted, resistance against period of hypoxia and angiogenesis play a combined effect in success of grafting (Luetjens *et al*., 2008). The grafting of testicular tissues can be performed xenologously between individuals of different species, heterologously (between two different individuals of the same species) and autologously (within an individual functioning simultaneously as donor and host). The ectopic grafting of immature testis has emerged as a promising approach to investigate testicular development and preserve fertility in mammals. It is an invaluable tool for the conservation of fertility even from immature gonads. It also opens avenues to preserve the genetic materials from rare or endangered animals that die before puberty.

Testis grafting as an experimental endeavour has seen a long history in reproductive biology (Goldstein *et al*., 1983; Johnson *et al*., 1996a, b). Grafting of testis tissue was developed as a tool for androgen substitution in the 1950s (Deanesly 1954) and has subsequently been applied to study steroidogenesis and the functions of Sertoli or Leydig cells (Kuopio *et al*., 1989; Wilker and Johnson 1995; Wilker *et al*., 1995; Johnson *et al*., 1996a, b). In recent years, testicular tissue from the pigs and goats, hamsters , rabbits, bulls, monkeys, cats, horses and human have been transplanted to an ectopic location on mice (Honaramooz *et al*., 2002; Schlatt *et al*., 2002a, b; Shinohara *et al*., 2002; Oatley *et al*., 2004; Snedaker *et al*., 2004; Dobrinski 2005; Rathi *et al*., 2006) with the spermatogenesis having been maintained. These xenografting experiments revealed that adult testis has poor surviving ability compared to immature testis. The proposed reasons for this include lack of proliferation of Sertoli cells, increased sensitivity to ischemia, and a decreased angiogenic ability of adult tissue (Schlatt *et al*., 2002b; Arregui *et al*., 2008a,b).

The mouse testis is extremely sensitive to ischemia (Attaran and Hodges 1966; Goldstein *et al*., 1983) and therefore is considered less for autologous transplantation model. Autologous transplantation of testis in dog (Attaran and Hodges 1966), *Callithrix jacchus*, marmoset (Wistuba *et al*., 2006), Wistar rats (Miragem *et al*., 2009) and chicken (Song *et al*., 2010) has already been reported. A solitary study on mouse testis autografting is present where; Leydig cell survival was reported based on recovery of recipient seminal weight (Boyle *et al*., 1975). However, it still remains elusive if the Leydig cells survived or regenerated *de-novo* in the autografts.

The objective of the present study was to investigate the Leydig cell regeneration in adult mice testis autografts. The grafts were analysed at 1-, 2-, 4- and 8- week post-grafting immunohistochemically for Leydig cell and stem Leydig cell (SLC) specific proteins. Further, existence of other cells such as germ and somatic cells were also determined by immunohistology.

2. REVIEW OF LITERATURE

2.1 GRAFTING

Grafting refers to a surgical procedure to move tissue from one site to another on the body, or from another person, without bringing its own blood supply with it. Instead, a new blood supply grows in the tissue after it is placed. Grafting can be considered as a specialized form of tissue culture in which the ectopic grafting site acts as a bioincubator for the grafted tissue (Orwig and Schlatt 2005). Grafting can be divided into 4 major categories depending upon involvement of host and donor: (1) Autografting- tissue transfer within same individual (2) Isografting- tissue transfer between genetically identical individuals (3) Allografting- tissue transfer between genetically different members of the same species and (4) Xenografting- transfer of tissue between different species. The immunologically privileged sites like eyes, placenta, skin and testes survive better as grafts for extended periods of time without rejection.

2.2 TESTIS: *Structure and Physiology*

The testis is the male gonad that produces steroid hormones and male gametes. The testis consists of two compartments, the highly convoluted seminiferous tubules and the interstitial space (Russell *et al*., 1990). The interstitium includes Leydig cells, blood vessels, macrophages, lymphocytes, connective tissue and lymphatics vessels (Fawcett *et al*., 1973). The Leydig cells within the interstitial space produce testosterone, which is important to maintain spermatogenesis (Lipsett *et al*., 1966; Bartlett *et al*., 1989) and to maintain the male secondary sex characteristics (Walsh *et al*., 1934). The seminiferous epithelium is the site of spermatogenesis and includes the Sertoli cells and germ cells (Russell *et al*., 1990). This seminiferous epithelium is surrounded by one or more layers (depending on the species) of peritubular myoid cells (Maeda *et al*., 2007). The Sertoli cells extend from the basement membrane to the lumen surrounding the germ cells and are responsible for providing structural and functional support to the developing germ cells (Russell 1990). Sertoli cells provide various necessary factors for the germline cell types and support the proliferation and differentiation of immature undifferentiated germ cells to mature spermatozoa (Griswold 1993).

2.3 TESTIS GRAFTING

At first glance, the testis did not appear very promising for grafting because of its tough capsule, complex vascular architecture and sensitivity to ischaemia. But the testis is regarded as an immunologically privileged organ (Barker and Billingham 1977) and this made it suitable for grafting. The immune privileged nature of testis is not only attributed to a single factor such as the sequestration of neo-antigens from the immune system behind the blood–testis barrier, but is based on a complex multifaceted interplay between cells and factors that are essential for the reproductive function of the testis and the testicular immune system (Fijak *et al*., 2011). This status of the testis has been appreciated for many years from studies of allogeneic (between genetically different individuals of the same species) and xenogeneic (between individuals of different species) transplantation of tissues, which survived for various lengths of time following engraftment into the testes of small and large animals (Mital *et al*., 2010).

The grafting of testis within and between individual animals is better in comparison with germ cell culture and spermatogonial transplantation as this approach can maintain microenvironment integrity and provide the accessibility that is essential for studying the function of testes (Ma *et al*., 2004)

2.4 HISTORY OF TESTIS GRAFTING

The first recorded testis transplantation is attributed to the 18th century Scottish anatomist and surgeon John Hunter, who grafted chicken testis and ovaries (Palmer 1837); however, it is the Gottingen biologist, Berthold, who should be credited with the first successful testicular transplants because, by luck or perspicacity, he used autografts and avoided the problems of rejection. After replacing the testes of capons in their own body cavity he found that the growth of comb, plumage and courting behaviour, all of which are androgen-dependent, was maintained. There followed numerous attempts during the early part of the 20th century aimed at testicular transplantation to boost male hormone levels, the lack of which were felt to be an underlying cause of ageing.

Deansley (1954) reported that grafting of the prepubertal rat testis tissue to other rats was successful and that the tissue was able to grow and differentiate as homo-grafts after freezing and thawing. Chan *et al.* (1969) demonstrated that grafting of testis tissue from normal or pseudohermaphroditic male rats to the ear of normal or pseudohermaphroditic

adult rats maintained the ability for spermatogenesis. This work confirmed that the hypothalamo-pituitary-gonadal axis is functional in these animal models.

Xenografting of human fetal testis tissue into the abdominal wall of adult nude mice was applied in 1974, but did not result in spermatogenic development (Skakkebaek *et al.,* 1974). Gosden and Aubard (1996a, b) reviewed the reports on autologous and homologous testis tissue grafting up to that time-point.

Immunodeficient lines of mice allowed xenotransplantation of testicular tissues (Paris *et al*., 2004), thus making it possible to transplant tissue from large animals into mouse hosts (Dobrinski 2005). The complete cross species spermatogenesis was first reported in 2002 (Honaramooz *et al*., 2002b). Grafting of testis tissue from sexually immature males to immunodeficient mice resulted in germ cell differentiation and production of sperm from different mammalian species like hamsters (Schlatt *et al.,* 2002), rabbits (Shinohara *et al*., 2002), bulls (Oatley *et al*., 2004), monkeys (Honaramooz *et al*., 2004), cats (Snedaker *et al*., 2004) and horses (Rathi *et al*., 2006).

2.5 AUTOGRAFTING OF TESTIS

It is the self-transfer of testis tissue from one body site to another in the same individual. Prior to the 21st century; testis tissue grafting was largely restricted to homologous and autologous grafts. In 1950, Williams used a transparent chamber technique in the ear of a 6 month old rabbit to perform an autologous grafting of testis tissue to study and describe the interaction between seminiferous tubules and the interstitial cells and their daily changes. This experiment demonstrated that seminiferous tubules needed interstitial cells to grow likely because of the testosterone secretion, but the interstitial cells were not dependent on the tubules to grow or maintain their function.

In 1975, autografting of testicular tissue was performed in inbred mice to subcutaneous trunk, ear lobe and intraperitoneal sites and the results were assessed after 3 months by determining the weights of seminal vesicle and penis and by the histological appearances of the grafts and seminal vesicles. Spermatogenic tissue atrophied in all cases but interstitial cells were shown to survive and function 3 months after subcutaneous grafting (Boyle *et al*., 1975).

Autologous ectopic transplantation of immature testicular fragments under the back skin in newborn marmoset monkeys has also been reported. Immunohistochemistry revealed normal maturation of Sertoli cells, Leydig cells, and peritubular cells but the serum testosterone values were not restored to the normal range. It was concluded that autotransplants in the marmoset can mature up to meiosis but the normal serum testosterone levels are not restored (Wistuba *et al*., 2006). To overcome meiotic arrest, the immature testis was autologously grafted into the scrotal position in marmoset and it was seen that spermatogenesis was completed. The study also showed that while in the adults, ectopic grafts degenerated, in the immature animals, grafts survived at the spermatogonial level. The findings indicated that transplantation site and developmental age of the tissue play a role more important than the endocrine milieu (Luetjens *et al*., 2008).

More recently, subcutaneous autologous transplantation of total and sliced testicle has been reported in Wistar rats. Sexual behaviour, serum testosterone levels, and body weight were measured. The study demonstrated that testicles transplanted without vascular anastomosis maintain their hormone secretion (Miragem *et al*., 2009).

2.6 FACTORS AFFECTING THE OUTCOMES OF ECTOPIC TESTIS TISSUE AUTOGRAFTING

2.6.1 Age and developmental stage of testis

The age or developmental stage of the testis tissue affects graft survival and development. Survival of graft decreases with the degree of maturity of the donor tissue. Tissue from adult donors shows poor survival and a marked tendency to degenerate making it unsuitable for transplantation (Schlatt *et al*., 2002b, Geens *et al*., 2006, Kim *et al*., 2007, Arregui *et al*., 2008b). In xenografting experiments it was seen that only xenografting of immature donor testicular tissue has resulted in successful progression of grafts to full spermatogenesis. All attempts to xenograft adult testicular tissue failed, as these grafts did not contain differentiating male germ cells (Schlatt *et al*., 2006; Geens *et al*., 2006; Arregui *et al*., 2008b). The failure of adult testicular tissue to establish functional xenograft has been attributed to the high demand for oxygen in adult testicular tissue with ongoing spermatogenesis. The high metabolic activity of seminiferous epithelium leads to a prolonged and fatal period of hypoxia between dissection and

reconnection of the xenografted tissue to the circulatory system of the host (Schlatt *et al*., 2006; Arregui *et al*., 2008b). Thus after xenografting of fully adult testicular tissues, only degenerated and fully hyalinised testicular tissue has been recovered. Other reasons for poor survival of adult tissue include lack of proliferation of Sertoli cells, increased sensitivity to ischemia, and a decreased angiogenic ability of the adult tissue (Schlatt *et al*., 2002b, Arregui *et al*., 2008a,b). Degeneration of adult testis xenografts seems to occur faster in the species with higher spermatogenic activity and is more severe in mature adult donors than in sub adult donors (Arregui *et al.*, 2008b).

2.6.2 Transplantation site

Turner (1938) demonstrated that the anterior chamber of the recipient rat´s eye was a better site for homologous grafting of testis tissue as compared with the subcutaneous, intra-peritoneal, scrotal, or intra-muscular sites. He cited the lower temperature and more vascularisation in the eye as likely reasons for these observations. Low temperature and high potential for vascularisation are still considered to be key factors for making a site suitable for grafting. In the first report of successful testicular maturation and completion of spermatogenesis following testis xenografting from diverse species into mice (Honaramooz *et al.* 2002), the tissue fragments were sutured subcutaneously under the back skin of immunodeficient nude mice. Since the nude mice do not have a fur coat, one might assume that grafts developing under the skin are exposed to a relatively lower temperature than those in mice with fur. However, Snedaker *et al.* (2004) suggested that there is no difference in spermatogenic efficiency between nude and SCID mice (which have complete fur). Shinohara *et al.* (2002) also reported successful xenografting of neonatal testis tissue under the tunica albuginea of the recipient mouse testes. However, most of the other studies utilizing xenografting have shown that the subcutaneous space under the back skin of recipient mice as the ideal site for grafting testis tissue fragments, since it provides a low temperature, is highly vascularised, and easily accessible for growth and development of the xenograft.

2.7 LEYDIG CELL

Leydig cells are the steroidogenic cells of the testes that produce testosterone needed for spermatogenesis and male function. The cytology of Leydig cells was first described in 1850 by the German histologist Franz Leydig. In the mouse, much information on the ontogeny of Leydig cells is available (Dong *et al*., 2007). These are post-mitotic cells with neuroendocrine characteristics (Davidoff *et al*., 2004). In all mammalian species, Leydig cells are found within the interstitial tissue of the testes within the seminiferous tubules, and are characterized as round or ovoid cells with extensive smooth endoplasmic reticulum, mitochondria with tubular cristae, variable number of lipid droplets, and a large round centrally located nucleus with dense peripheral nuclear heterochromatin and a prominent nucleolus (De Kretser 1967; Christensen and Gillim 1969). The cytoskeleton of the Leydig cell comprises microtubules, actin microfilaments, and vimentin intermediates filaments which are characteristic of cells of mesenchymal origin (Van Vorstenboch *et al*., 1984; Russel *et al*., 1987).

2.7.1 Leydig cell lineage

In rats and mice, two generations of Leydig cells are formed: fetal Leydig cells (FLCs), by day 12.5 of gestation, and adult Leydig cells (ALCs), by day 56 postpartum. The two Leydig-cell generations exist in distinctly different hormonal milieus and can be distinguished from one another by cytological and physiological criteria. The testosterone production from FLCs is necessary for the masculinisation of the fetus whereas ALCs are required for the progression and maintenance of spermatogenesis. The first generation develops *in utero*, from undifferentiated stem Leydig cells (SLCs) that differentiate into fetal Leydig cells (FLCs). After birth, SLCs that may differ from the fetal SLCs undergo lineage-specific commitment and give rise to adult Leydig cells (ALCs). The intermediates of ALCs first become apparent by day 11 postpartum. These first-appearing intermediates, progenitor Leydig cells (PLCs), are spindle shaped and identifiable as steroidogenic because they express Luteinizing Hormone/Choriogonadotropin Receptor (LHCGR) and Hydroxy-delta-5-steroid- dehydrogenase, 3-beta and steroid delta isomerase (HSD3B). The next step in the transition of PLCs to ALCs is the appearance of the immature Leydig cells (ILCs), most commonly seen in the testis during days 28 to 56 postpartum. ILCs undergo a final division before ALC steroidogenic function matures

by postnatal day 56. ALCs mark the point of maximum differentiation, and at this stage, the Leydig cell secretes testosterone at the highest rate (Dong *et al*., 2007). Developmental changes in the expression of Leydig cell-specific mRNA measured by RT- PCR have partially characterized developmental phenotype of cell in the mouse and identified markers of adult Leydig cell differentiation (O´Shaughnessy *et al*., 2002).

Adult Leydig cells do not normally proliferate (Keeney *et al*., 1988), but can be regenerated if destroyed. The adult population of Leydig cells is completely regenerated within 7 weeks of its destruction by Ethanedimethane sulfonate (EDS), an agent specifically lethal to adult Leydig cells (Sharpe *et al*., 1990). PLCs and ILCs are not commonly observed in the testis immediately following EDS administration. So, it is regarded that Leydig cell regeneration probably results from commitment of Leydig stem cells, which persist in the testis throughout adult life (Benton *et al*., 1995).

Five main cell types are present during Adult Leydig cell differentiation in postnatal testis, namely the mesenchymal precursor cells, progenitor cells, newly formed adult Leydig cells, immature Leydig cells, and mature Leydig cells. Peritubular mesenchymal cells are the precursors to Leydig cells at the onset of Leydig cell differentiation in the prepubertal rat as well as in the adult rat during postnatal development (Figure1). The onset of precursor cell differentiation into progenitor cells is independent of LH; however, LH is essential for the later stages in the Leydig cell lineage to induce cell proliferation (Mendis-Handagama and Ariyaratne 2001)

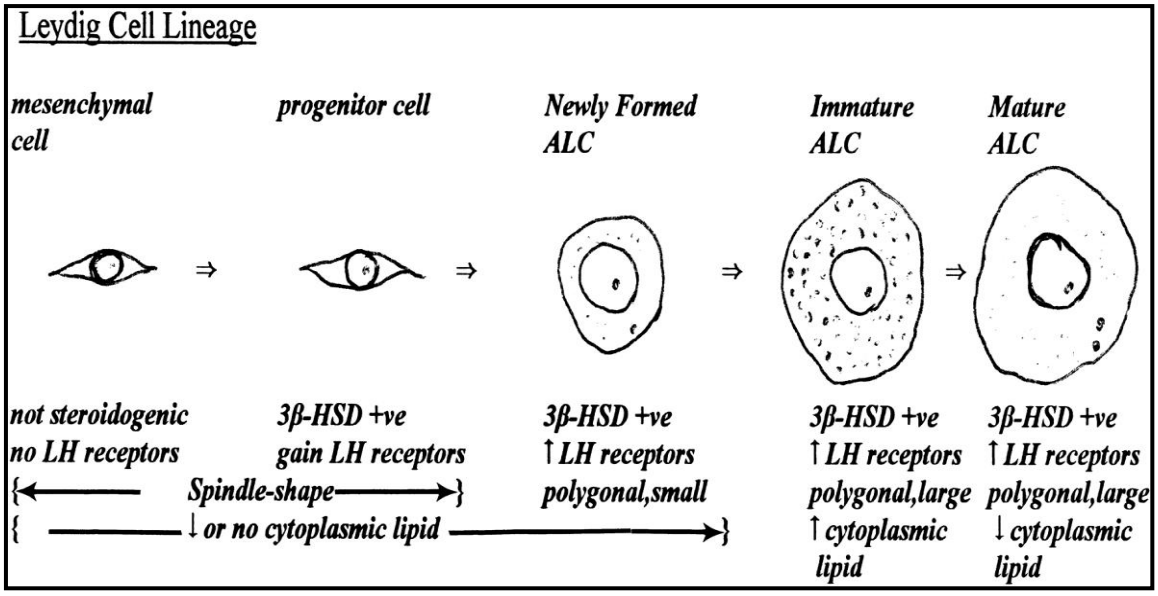

Figure 1: ***Postnatal Leydig cell differentiation in rat.*** *The postnatal differentiation of Leydig cells is reported to take place around the second postnatal week (Lording and de Krester 1972).This developmental process consists of multiple steps: proliferation of precursor cells, differentiation of precursor cells to Leydig cell progenitors, progenitors into newly formed adult Leydig cells, newly formed adult Leydig cells into immature adult Leydig cells, and finally maturation of the immature adult Leydig cells to mature adult Leydig cells* [**Source**: Mendis-Handagama and Ariyaratne 2001]

2.7.2 Steroidogenic activity of Leydig cells

Within the testis, steroidogenesis occurs in the Leydig cell. Inside the Leydig cell, the steroidogenic pathway begins in the cytoplasm and includes chemical reactions that occur in the mitochondria and smooth endoplasmic reticulum, where the final end-product hormone, i.e., testosterone, is produced (Chen *et al*., 1996). Steroidogenesis in Leydig cells is regulated mainly by Luteinizing Hormone (LH), a glycoprotein produced by the anterior pituitary. Binding of Luteinizing Hormone (LH) to its 7-transmembrane G-protein coupled receptor stimulates adenylate cyclase activity, results in increased cyclic adenosine monophosphate (cAMP) formation (Dufau 1998). cAMP stimulates the cAMP-dependent protein kinase (PKA), which phosphorylates serine and threonine residues on specific protein substrates (Hansson *et al*., 1999). cAMP activates steroidogenesis by temporally distinct manners; acutely (minutes) or chronically (hours). The cholesterol transport mechanism is a complex process, involving an interaction between the Steroidogenic acute regulatory protein (STAR) and the Peripheral-type benzodiazepine receptor (PBR) (Stocco 2000; Papadopoulos 2004). STAR is required for

the transfer of cholesterol from the outer to the inner mitochondrial membrane, the first and rate-limiting step in steroid hormone biosynthesis (Stocco 1999).

2.7.3 Testosterone biosynthesis pathway in mice

Once cholesterol reaches the inner mitochondrial membrane, it is immediately converted into pregnenolone. The reaction for the conversion of C_{27} cholesterol to C_{21} steroid, pregnenolone is catalysed by the Cytochrome P450 11a1 (Cyp11a1) and specific electron transferring proteins, localized at the inner mitochondrial membrane (Payne and Hales 2004). Pregnenolone diffuses across the mitochondrial membranes and is further metabolized by enzymes associated with the smooth endoplasmic reticulum. In the mouse Leydig cell, pregnenolone is converted to progesterone by the action of Hsd3b. Progesterone is a substrate for the Cytochrome P450 17a (Cyp17a). This is a single protein that catalyzes two distinct reactions, the hydroxylation of progesterone at C_{17} followed by the cleavage of the two-carbon side-chain to yield the C_{19} steroid, androstenedione, the immediate precursor of testosterone. The final reaction is the reduction of the 17-ketone of androstenedione by hydroxysteroid (17-beta) dehydrogenase (Hsd17b) to yield testosterone (Payne and Youngblood 1995). Figure 2 provides an illustration of the steps involved in testosterone biosynthesis.

Testosterone is required for sexual development and testis descent during fetal period (Huhtaniemi *et al*., 1992), the production of sperm in the seminiferous tubules (Awoniyi *et al*., 1989) and the maintenance of accessory sex organs (Fuji 1977) and sexual behaviour (Wilson *et al*., 2001) at adulthood.

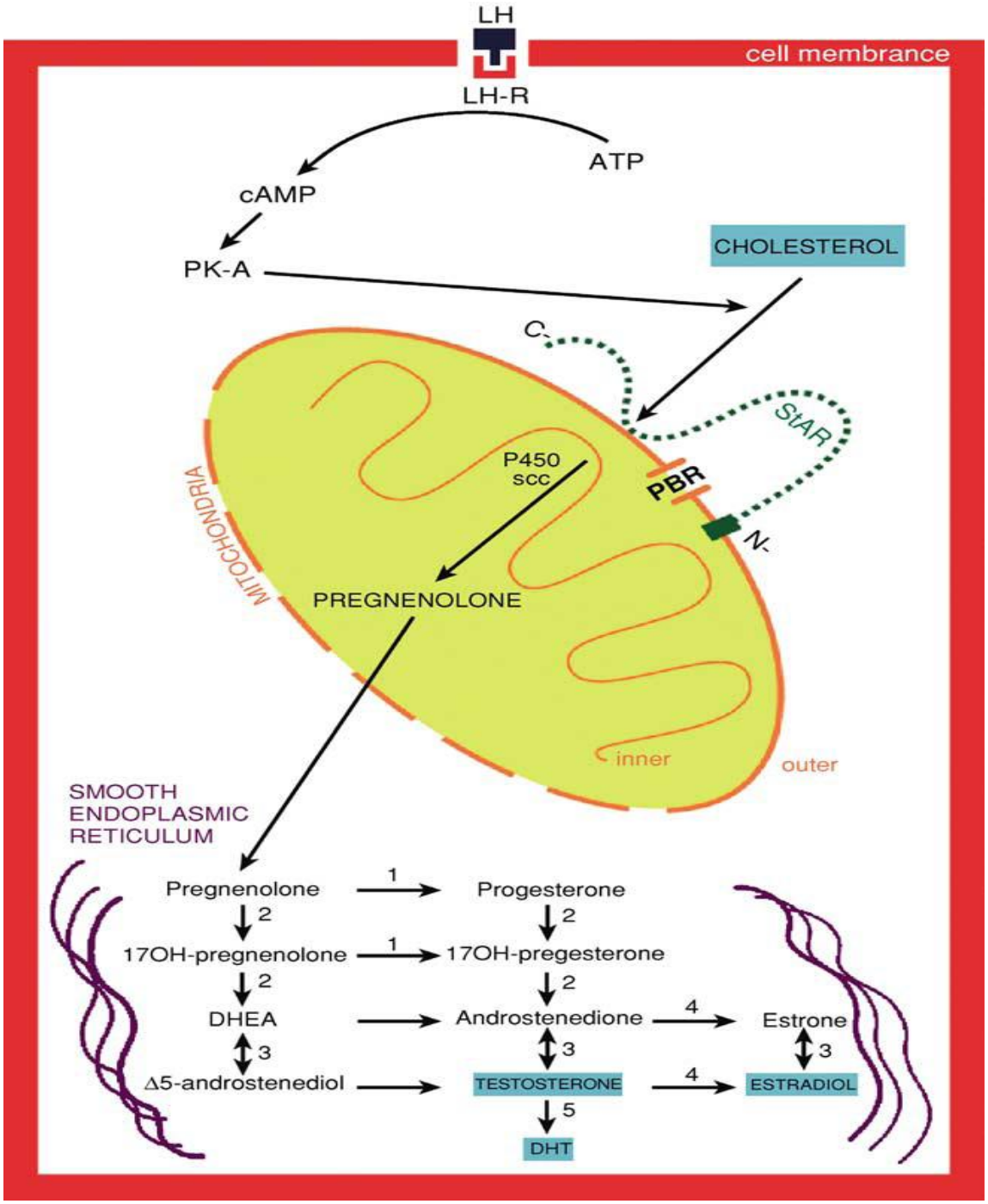

Figure 2: ***Steps for testosterone biosynthesis in Leydig cells***. *Luteinizing hormone (LH), on binding with the receptor (LHCGR), induces the synthesis of cyclic AMP (cAMP) from ATP. cAMP catalyzes the synthesis of protein kinase A (PKA), which is needed for the transport of cholesterol from the cytoplasmic pool to mitochondria. Steroidogenic acute regulatory protein (STAR) and peripheral benzodiazepine receptor (PBR) transfer cholesterol from the outer membrane to the inner mitochondrial membrane, where the enzyme CYP11A1 resides. It converts cholesterol into pregnenolone, which is ultimately transferred to smooth endoplasmic reticulum, where the synthesis of testosterone takes place. DHEA, Dihydroepiandrosterone; DHT, dihydrotestosterone. Reaction 1, HSD3B; reaction 2, CYP17; reaction 3, HSD17B; reaction 4, CYP19/Aromatase; reaction 5, 5alpha-reductase.* [**Source**- Haider 1995]

2.8 LEYDIG CELL-SPECIFIC MARKERS

Various growth factors and enzymes that modulate Leydig cell differentiation, regeneration and steroidogenic capacity are known to be specific for Leydig cell and are used to identify these cell types in testes. These are designated as Leydig cell-specific markers.

2.8.1 Cytochrome P450, family 11, subfamily A, polypeptide 1 (CYP11A1)

Cytochrome P450 11A1 (CYP11A1), which was previously referred to as P450 cholesterol side-chain cleavage enzyme (P450SCC) catalyzes the first and rate-limiting enzymatic step in the biosynthesis of all steroid hormones. It is encoded by gene *Cyp11a1* which in mouse is located on chromosome 9 at 31 cM. (Youngblood *et al*., 1989). It belongs to Cytochrome P450, which is heme-containing super family of enzyme found in bacteria, fungi, plants and animals (Nelson *et al*., 1996). The only site of expression of Cyp11a1 in the testis is the Leydig cell (Payne and Youngblood 1995) and it is located on inner mitochondrial membrane. *In situ* hybridization in mouse has demonstrated its expression as early as 12.5 days post conception (Ikeda *et al*., 1994). Postnatally, in rodents (Ge *et al*., 1996), testicular expression of CYP11A1 decreases due to the disappearance of the fetal Leydig cell population. As development of the adult population of Leydig cells occurs after postnatal day 10 in the rodents, there is a sharp increase in the expression of CYP11A reaching adult levels in mouse by postnatal day 25 (Chemes 1996).

The enzyme resides on inner mitochondrial membrane, facing the matrix and catalyzes three sequential oxidation reactions of cholesterol with each reaction requiring one molecule of oxygen and one molecule of NADPH (Payne and Hales 2004). *Cyp11a1* mRNA is expressed at high levels basally and increases in response to cAMP, LH or hCG (Clark *et al*., 1997; Payne and Youngblood 1995).

2.8.2 Hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta isomerase (HSD3B)

Previously known as 3β-hydroxysteroid dehydrogenase (3β-HSD), HSD3B is an enzyme to catalyze the conversion of pregnenolone to progesterone in the presence of cofactor NAD. HSD3B catalysis has two steps, catalyzing dehydrogenation and

isomerisation of a double bond in the steroid molecule, with the first dehydrogenase step being rate-limiting (Ye *et al*., 2011). HSD3B are membrane-bound enzymes. Mouse *Hsd3b* genes are located in a cluster on mouse chromosome 3 (Bain *et al*., 1993; Clarke *et al*., 1996). *In situ* hybridization in mouse testicular sections demonstrated the exclusive expression of Hsd3b in the Leydig cells of the testis. According to studies by Pelletier *et al.* (2001), immunostaining for HSD3B was found exclusively in the mitochondria of the Leydig cell. To date, six distinct cDNA encoding murine members of the Hsd3b family has been cloned (Abbaszade *et al*., 1995). The isoforms of Hsd3b are expressed in a cell- and tissue-specific manner. Mouse type I and type VI Hsd3b is expressed in adult testes, and RT-PCR analysis has showed that type I was expressed from embryonic day 13 until adulthood, whereas type VI was first detected at day 11 postnatally and was the predominant isoform in the adult animal. Thus, while Hsd3b type I isoform is expressed at significant levels in fetal-type Leydig cells, the expression of type VI coincides with development of the adult Leydig cell population. The limited expression of the type VI isoform means that it will be a useful marker in studies of adult Leydig cell development (Greco and Payne 1994; Baker *et al*., 1999). In the adult mouse, Hsd3b type VI is expressed in Leydig cells of the testis to a lesser extent than Hsd3b type I (Simard *et al*., 2005). Investigations using gonadotropin-deficient mice to study the role of LH/hCG in regulating the expression of Hsd3b type I and VI mRNA in the adult Leydig cell lineage showed that the expression of Hsd3b type I was independent of LH stimulation (Baker *et al*., 2003). In contrast, the expression of Hsd3b type VI mRNA was highly dependent on LH/hCG stimulation.

2.8.3 Luteinizing hormone/choriogonadotropin receptor (LHCGR)

Leydig cells are the primary target for Luteinizing Hormone (LH) action in the testis (Wing *et al*., 1984). LH is the master control factor for completing and maintaining fully differentiated structure and testosterone biosynthetic function in Leydig cells (Cooke *et al*., 1996). Previously known as LH receptor (LHR), LHCGR is located on the Leydig cell surface within the plasma membrane and binding of LH to its receptor leads to aggregation and rapid internalization of the ligand receptor complex (Dufau 1988). The LH receptor belongs to the seven-transmembrane helix-G protein complex coupled receptor family and possess a very long (64kDa) extracellular domain which comprises the LH binding site (McFarland *et al*., 1989). The action of this hormone involves activation of membrane G protein complex coupled to adenyl cyclase and protein kinase

A-mediated protein phosphorylation (Cooke *et al*., 1996). Luteinizing hormone levels in normal mice are low throughout pregnancy and after birth. Luteinizing hormone levels rise sharply with the onset of puberty between Days 20 and 25 of postnatal life, peaking around Day 30 and then declining slowly thereafter (O'Shaughnessy *et al*., 2009).

LH also plays a critical role in the development of Leydig cells. In mice, which are deficient in circulating LH, Leydig cell numbers are about 10% of control (Baker and O'Shaughnessy 2001). Mice in which the *Lhcgr* is knocked out show reduced testicular size, reduced testosterone and elevated serum LH (Griffin *et al*., 2010). Leydig cells are severely hypoplastic in *Lhcgr* knockout mice. Their development does not progress to the adult stage and the gene expression and steroid synthesis are affected (Lei *et al*., 2001; Zhang *et al*., 2001). The observation that LH is essential for the differentiation of precursor cells into Leydig cell progenitors is based on study in adult rats. After the destruction of the existing Leydig cell population by the cytotoxic drug Ethanedimethane sulphonate (EDS), regeneration of a new adult-type Leydig cell population occurred only in the presence of LH (Molenaar *et al*., 1986; Tena-Sempere *et al*., 1997; Teerds *et al*. 1999). This regeneration process shows many similarities with the development of the adult-type Leydig cell population in the pre-pubertal and pubertal testis (Teerds 1996).

The onset of the process of adult Leydig cell differentiation is independent of LH but is required for the later stages in the lineage, i.e., proliferation of progenitors and their differentiation to newly formed adult Leydig cells, newly formed adult Leydig cells to undergo cell hypertrophy and differentiate into immature and mature adult Leydig cells, as well as for the mitosis of Leydig cells following their differentiation (Mendis-Handagama and Ariyaratne 2001).

2.8.4 Platelet-derived growth factor receptor, alpha polypeptide (PDGFRA)

Platelet-derived growth factor A (PDGFA) is secreted by Sertoli cells and acts on Leydig precursor cells, which express the receptor PDGFRA, triggering their differentiation into steroidogenically active Leydig cells (Bergeron *et al*., 2011). PDGFA is a major mitogen and chemotactic factor for mesenchymal cells (O'Shaughnessy *et al*., 2008). In mice, Pdgfra is expressed at low levels in the mesenchyme of the mesonephros at day 11.5 post conception in both sexes. It is also highly expressed in the coelomic epithelium and at the gonad-mesonephros border at 11.5 days in both sexes. By day 12.5,

expression is confined to the male and is very strong in the interstitial cells of the fetal testis, near the coelomic epithelium (Brennan *et al*., 2003). In neonatal testis, Pdgfra is located in the interstitial areas near peritubular cells (Thuillier *et al*., 2003) and in adult testes; Pdgfra is localized exclusively in Leydig cell. This supports the hypothesis that Pdgfra can be used as a Leydig cell lineage marker. Disruption of the *Pdgfa* or *Pdgfra* gene in mice (Gnessi *et al*., 2000; Brennan *et al*.,2003; Schmahl *et al*., 2008) was shown to impair the differentiation of Leydig cells (Basciani *et al*., 2010). These male mice were also found to develop progressive reduction of testicular size, Leydig cells loss, and spermatogenic arrest (Gnessi *et al*., 2000). Despite the importance of PDGFRA in Leydig cell differentiation, there is no information regarding the molecular mechanisms that regulate its expression in Leydig cells (Bergeron *et al*., 2011). In mice, paracrine Pdgfa/Pdgfra signalling also constitutes a critical part of the epithelial-mesenchymal interaction, essential for adult Leydig cells development (Gnessi *et al*., 2000)

A recent study in rat has reported the isolation of stem cells from the pre-pubertal testis, which gives rise to Leydig cells in vivo following transplantation. These stem Leydig cells express receptors for PDGFA (Ge *et al*., 2006) and proliferate in the presence of the mitogen.This suggests a central role for PDGFA in the development of the adult Leydig cell cohort.

2.9 SERTOLI CELLS

Sertoli cells are the first somatic cell type to differentiate in the testis and express *Sry*, the male sex-determining gene. Thereby, they play a crucial role in directing testis development (Karl and Capel 1998). They are located within the seminiferous tubules and are Christmas-tree-shaped. Laterally they are present in direct contact with one another and with the germ cells. Sertoli cells undergo a maturation process during post-natal testicular development that leads to the adult-type Sertoli cell, which is required for spermatogenesis (Gassei *et al*., 2010). These cells transduce signals from testosterone and FSH into the production of factors that are required by germ cells as they mature into spermatozoa (Walker and Cheng 2005) and provide nourishment to these spermatozoa. The number of Sertoli cells determines testicular size, germ cell numbers per testis and spermatozoa output (Orth *et al*., 1988). In addition, it is likely that adult Leydig cell activity, function, and survival are dependent on the continued presence of Sertoli cells (Baker *et al*., 2003; Russell *et al*., 2001). It is clear, therefore, that regulation of Sertoli cell proliferation and activity during

development and in the adult animal is crucial for normal adult fertility. Sertoli cells proliferate in the mouse through fetal and neonatal development to reach a maximum about 15–20 day postpartum (Baker and O' Shaughnessy 2001; Vergouwen *et al*., 1993).

2.9.1 Wilms tumor 1 (WT-1)

The Wilms tumor 1 (WT-1) is an early expressed transcription factor, which plays a major role in urogenital development and function in mammals (Kreidberg *et al*., 1993). It belongs to the zinc finger family of transcription factors with four DNA/RNA binding zinc fingers at its carboxy-terminal end (Call *et al*., 1990; Gessler *et al*., 1990). It is one of the key regulators of early male genital development (Kohler *et al*., 2007). *WT-1* expression persists after birth in Sertoli nurse cells in the testis (Armstrong *et al.*, 1993), but the functional significance of this expression is not known, as *Wt-1* null mice die during embryogenesis (Little *et al*., 1999; Park and Jameson 2005). Mice depleted of Wt-1 in Sertoli nurse cells suffered from disruption of developing seminiferous tubules, increased germ cell apoptosis, loss of adherens junctions, disregulation of adherence junction-associated genes, and impaired fertility. Thus, WT-1 expression in Sertoli cells is crucial for the survival and development of the adjacent germ cells. This postnatal and adult role is distinct from its previously defined embryonic role in promoting the development of the gonad (Gao *et al*., 2006). The continued expression of *Wt1* in the mature mice testes suggests a role for *Wt1* at later stages of testicular development, as have *in vitro* studies implicating *Wt1* in the up-regulation of anti-Mullerian hormone, AMH (also known as Mullerian inhibiting substance, MIS) in the differentiated testes (Nachtigal *et al*., 1998). Wt-1 expression has not been detected in Leydig cells of adult mice testis, suggesting that Wt-1 is not directly involved in regulating testosterone production. But an indirect effect during embryogenesis by Sertoli cells on Leydig cell function and differentiation cannot be excluded (Pelletier *et al*., 1991).

The polyclonal WT-1 antibody, sc-192, is a WT-1 specific rabbit antibody raised against a peptide corresponding to the 9 amino acid at the carboxyl terminus of WT-1 (Silberstein *et al*., 1997). Therefore, it enables us to employ this widely used marker of Sertoli cells to assess Sertoli cell number and morphology.

2.10 GERM CELLS

Germ cells are unique cells, which are capable of transmitting genetic information from generation to generation. Most primitive germ cells are primordial germ cells (PGCs) which later turn into gonocytes (Capel 2000). Gonocytes are germ cells that reside in the seminiferous tubules of neonatal testes and further differentiate to spermatogonia, thereby initiating spermatogenesis in postnatal testis. Therefore, gonocytes are the first cells committed to male germline development and are the only germ cells in the neonatal testis (Goel *et al*., 2008). In the adult male, germ cell differentiation takes place in the seminiferous tubules of the testis by a complex, highly organized and very efficient process. A population of diploid stem-cell spermatogonia that lie on the basement membrane of the tubule continuously undergoes self-renewal and produces progeny cells, which initiate the process of cellular differentiation to generate mature spermatozoa (Ogawa *et al*., 1997).

2.10.1 DEAD [Asp-Glu-Ala-Asp] box polypeptide 4 (DDX4)

DDX4 (DEAD [Asp-Glu-Ala-Asp] box polypeptide 4) or *VASA* is a member of the DEAD-box family of genes and encodes an ATP-dependent RNA helicase. *DDX4* has been characterized as a marker of primordial germ cells and as such is usually studied in the early stages of germ cell differentiation. It was first identified in *Drosophila*, where it was shown to be essential for female germ cell development (Lasko and Ashburner 1988). In mice, Ddx4 protein is exclusively expressed in primordial germ cells (PGCs) just after their colonization to embryonic gonads and in germ cells undergoing gametogenic processes until the post-meiotic stage in both males and females (Toyooka *et al*. 2000). However, Ddx4 protein is not detected in pluripotent embryonic cells such as stem and germ cells (Fujiwara *et al*. 1994). DDX4 protein is known to be localized to spermatogenic germ cells such as spermatocytes and round spermatids, but not to elongated spermatids and spermatozoa in the testis of several mammalian species including pig (Toyooka *et al*. 2000). Lee *et al*. (2005) showed that DDX4 protein is expressed by spermatocytes and weakly by spermatogonia in adult pig testis.

2.11 APPLICATION OF TESTIS TISSUE AUTOGRAFTING

Autografting of testis can be an important alternative means of gonadal preservation for oncological patients undergoing oncological therapies. For preservation of fertility, autografts avoid both the immunological problems of allograft and the ethical dilemmas when using donor tissue (Nugent *et al*., 1997). In males, low testosterone production leads to abnormalities in spermatogenesis, undescended testes, ambiguous genitalia, and infertility (Habert *et al*., 2001). Autografting of testis can be of therapeutic aid to overcome testosterone insufficiency. Maintenance of endogenous testosterone secretion post autografting may avoid Leydig cell insufficiency symptoms and long-term testosterone replacement adverse effects in severe cases.

3. MATERIALS AND METHODOLOGY

3.1 TESTIS AUTOGRAFTING

Balb/c mice were maintained and bred in the Animal Housing Facilities of Centre for Cellular And Molecular Biology(CCMB) under controlled conditions. The adult mice were used as the source of testis for autografting. All the experimental procedures involving the animals were performed in compliance with guidelines of the Institute´s Bio-Ethical Committee.

- Adult inbred Balb/c mice (6-week old) were taken for autografting. A total of 17 mice species were considered for the experimental purpose. The mice were kept under specific pathogen-free conditions. The food, water and bedding were autoclaved before use.
- They experimental mice were anaesthetized by intraperitoneal injections of ketamine hydrochloride and xylazine hydrochloride (0.1 ml/10 grams of body weight).
- The mice were castrated; the testis was taken out and cut into two equal halves.
- The four testis fragments from each mouse were dipped into 2% sodium alginate and then into 1.5% calcium chloride to embed the testis fragments into beads for grafting. So one graft becomes equivalent to half a testis.
- The same mouse then received four transverse linear incisions on the dorsal skin, two on each side of the midline. A small subcutaneous pouch was made in each incision, a testis tissue fragment bead was inserted (auto grafted) and the incision was closed with a single suture.
- The mice were categorised into 4 groups. First group included 4 mice, second group had 5 mice, third group had 4 and fourth group also included 4 mice.
- Throughout the experimental period, the animals were kept in their family groups, with normal diet and water available.
- A total of 17 mice were considered as age-match control. For the first group, 4 mice each 5- week old were regarded as age-match, for second group 4 mice each 6- week old, for third group 5 mice each 8- week old and for fourth group 4 mice each 12-week old formed the age-match controls.

3.1.1 Sample retrieval

- The grafted testis fragments were retrieved after 1 week from first group animals, after 2 week from second group, after 4 week from third group and at 8 week from fourth group. For graft retrieval, the mice were killed by exsanguinations under deep anaesthesia.
- The dorsal skin of each mouse was removed, and grafts were located on the interior surface of the skin. The grafts were distinguished as flat to round patches of soft tissue attached to the inner side of the dorsal skin, most prominently along dorsal blood vessels. The number of encountered grafts and weight of grafts were determined in each animal.
- The weight of the unemptied seminal vesicles was assessed as an indicator of bioactive testosterone produced by the graft. Average weight of seminal vesicle from age-matched control mice was also taken.
- Grafts were fixed in Davidson fixative overnight at -4°C temperature in the cold room. Fixation of tissue is essential to ensure the preservation of tissue architecture and cell morphology. It is the most important step in the process of histology. The purpose of fixative is to stabilize the protein in the tissue. The fixed testis tissues were analysed immunohistochemically using a battery of cell-specific antibodies. The composition of Davidson fixative is as:

Formalin (37%)	20 ml
Absolute alcohol	30 ml
Glacial acetic acid	10 ml
Tap water	40 ml

3.2 IMMUNOHISTOCHEMISTRY

Immunohistochemical technique exploits the specific interaction of an antibody with its antigen to locate or to determine the distribution of the antigen *in situ* in tissues. The antibody is incubated with thin sections of solid tissue mounted on glass slides. The antibody is conjugated with an enzyme label that gives an intense signal to allow visualization when the sections are examined using microscopy. The location of the label reveals the site of the antibody-antigen interaction which can be localized to particular cell type. All immunohistochemical procedures need stringent positive and negative control antibodies for comparison with the test antibody to ensure that the immunostaining is specific.

3.2.1 Dehydration and embedding of tissue samples

- The fixed tissues were processed in 50% ethanol and 70% ethanol for 5 minutes each at room temperature.
- The tissues were subsequently processed in 70% ethanol, 80% ethanol, 90% ethanol, 100% ethanol twice on a rotor for one hour each respectively in cold room. This was done in order to dehydrate out the water content from the tissues.
- The tissues were then processed in Xylene-I, Xylene-II, Xylene-III in cold room on a shaker for one hour each respectively.
- The tissues were then transferred to Xylene: Paraffin (1:1) for one hour at 65°C.
- The tissues were sequentially transferred to paraffin-I, paraffin-II and paraffin-III for one hour each at 65°C respectively.
- The tissues were embedded in paraffin blocks.

3.2.2 Sectioning

- 5-7 μm sections were prepared from the paraffin embedded tissues blocks with the help of microtome (Lieca Microtome, www.leica-microsystems.com) and affixed onto the charged slide (Fisher Scientific, www.fishersci.com).
- The slides were then dried to remove any water that may have been trapped under the sections. This was done by incubating the slide at 60°C in oven for 45minutes.

3.2.3 Histochemical Staining Protocol

- The fixed testicular tissues on the slides were deparaffinised by washing them in Xylene-I, Xylene-II and Xylene-III taken in coplin jars for 5 minutes each.
- The sections were then rehydrated in 100% ethanol-I, 100% ethanol-II, 90% ethanol, 80% ethanol, 70% ethanol and 50% ethanol for 1 minute each respectively.
- Sections were rinsed with MilliQ water twice.
- The sections were thereafter rinsed with PBS thrice.

- The tissue sections on the slides were marked with hydrophobic pen (abcam PAP hydrophobic barrier pen).
- Next the sections were incubated with 3% hydrogen peroxide(H_2O_2) for 10 minutes in a moist chamber to block the endogenous peroxidase activity present in the tissues.
- The sections were then rinsed twice with PBS.
- The sections were blocked with 15% Fetal Bovine Serum (Gibco, www.invitrogen.com) in PBS or with 15% rabbit serum (for HSD3B) for 30 minutes in a moist chamber at room temperature. Blocking is carried out to eliminate non-specific background staining caused by non-immunological binding of secondary antibody.
- After incubation the blocking solution was removed.
- Sections were then incubated overnight with primary antibody at 4°C in a humified chamber. The primary antibody was diluted in 1% BSA (Sigma, www.sigmaaldrich.com) prepared in PBS. Table 1 gives a detailed list of primary antibodies which were employed for the purpose of detecting the various cell types in the grafted tissues.
- An excess primary antibody was washed off the tissue sections using PBS on the next day.
- The sections were then incubated with secondary antibody at 37°C for 1 hour in a moist chamber. Table 1 gives a description of the secondary antibodies specific for each primary antibody being used in the study.
- The sections were again washed with PBS thrice.
- The sections were incubated with chromogen mix containing 3, 3'-diaminobenzidine tetra hydrochloride (DAB), 3% H_2O_2 and PBS for 3-5 minutes. DAB was in the concentration of 10mg/ml. Positive staining of the cells with DAB resulted in brown colour development which was visualized easily.
- Sections were washed thoroughly with distilled water and dehydrated by sequential washing with 50%, 70%, 80%, 90% and 100% ethanol for 1 minute each and finally washing with Xylene thrice for 5 minutes each. For observing the cellular morphology and counting the number of positive Leydig cells in sections, the sections were counterstained with haematoxylin.

- Sections were permanently mounted in Vecta-Mount (Vector Laboratories, www.vectorlabs.com).
- Finally the sections were observed under a Zeiss Axioplan 2 microscope (Carl Zeiss AG, www.zeiss.de).
- In negative controls, the primary antibody was omitted and instead the sections were incubated with 1% BSA in PBS.

To observe the histology of the graft tissues, the sections after being dewaxed and rehydrated were stained with haematoxylin for 30 seconds. The sections were rinsed with MilliQ water to remove the stain and were counterstained with eosin for 5-7 minutes. After thorough washing with MilliQ water and PBS, the sections were mounted in Vectamount and observed under microscope.

Table 1: *List of primary antibodies and corresponding secondary antibodies along with description of host in which it was raised, dilution used and specificity for cell types.*

Primary Antibody	**Manufacturer (Catalogue no)**	**Testis Target Cell**	**Antigen Source**	**Host**	**Dilution**	**Secondary Antibody (Manufacturer)**
CYP11A1	Millipore AB1244	Adult and fetal Leydig cell	Rat	Rabbit	1:100	Peroxidase conjugated Goat Anti-Rabbit IgG (Calbiochem; 401393)
HSD3B	Santa Cruz sc-30820	Adult Leydig cell	Human	Goat	1:30	HRP conjugated Rabbit Anti-Goat IgG (Calbiochem; 401515)
LHCGR	Santa Cruz sc-25828	Adult and fetal Leydig cell	Human	Rabbit	1:10	Peroxidase conjugated Goat Anti-Rabbit IgG (Calbiochem; 401393)
PDGFRA	Santa Cruz sc-7958	Stem Leydig cell (SLC)	Human	Rabbit	1:5	Peroxidase conjugated Goat Anti-Rabbit IgG (Calbiochem; 401393)
WT-1	Santa Cruz (C-19) sc-192	Sertoli cell	Human	Rabbit	1:4	Peroxidase conjugated Goat Anti-Rabbit IgG (Calbiochem; 401393)
DDX4	Millipore AB4330	Germ cell	Mouse	Rabbit	1:10	Peroxidase conjugated Goat Anti-Rabbit IgG (Calbiochem; 401393)

4. RESULTS

4.1 GRAFT RECOVERY, GRAFT WEIGHT AND SEMINAL VESICLE WEIGHT

The autografted testicular tissue in mice was identified and recovered from the skin. The recovery of grafts was 100% during the 1- and 2- week collection time points. At 4- week collection time point, the recovery of grafts was not significantly different from that of 1- and 2- week. However, the number of grafts recovered was significantly lower at 8- week collection time point ($P < 0.05$; Table 2).

Table 2: *Testis weight, weight of accessory sex glands, and survival and development of grafts*

Time after grafting (weeks)	Number of mice grafted and analysed	Number of graft recovered (%)	Average recovered graft weight (mg)	Seminal vesicle weight of recipients (mg)	Age-matched control testis weight (mg)	Age-matched control seminal vesicle weight (mg)
1	4	100 (16/16)	139	10	175	128
2	5	100 (20/20)	134	92.5	186	143
4	4	93.75 (15/16)	78	93	198	170
8	4	87.5 (14/16)	58	140	251	193

Average testis graft weight showed a decrease in a time-dependent manner (Table 2). The average weight of grafts from autografted animal was not significantly different from the average testis weight of age-matched control mice during first 1- and 2- weeks. However, there was a significant difference between the average graft weight and age-matched mice testis weight at 4- and 8- week collection time points ($P < 0.05$; Figure 3).

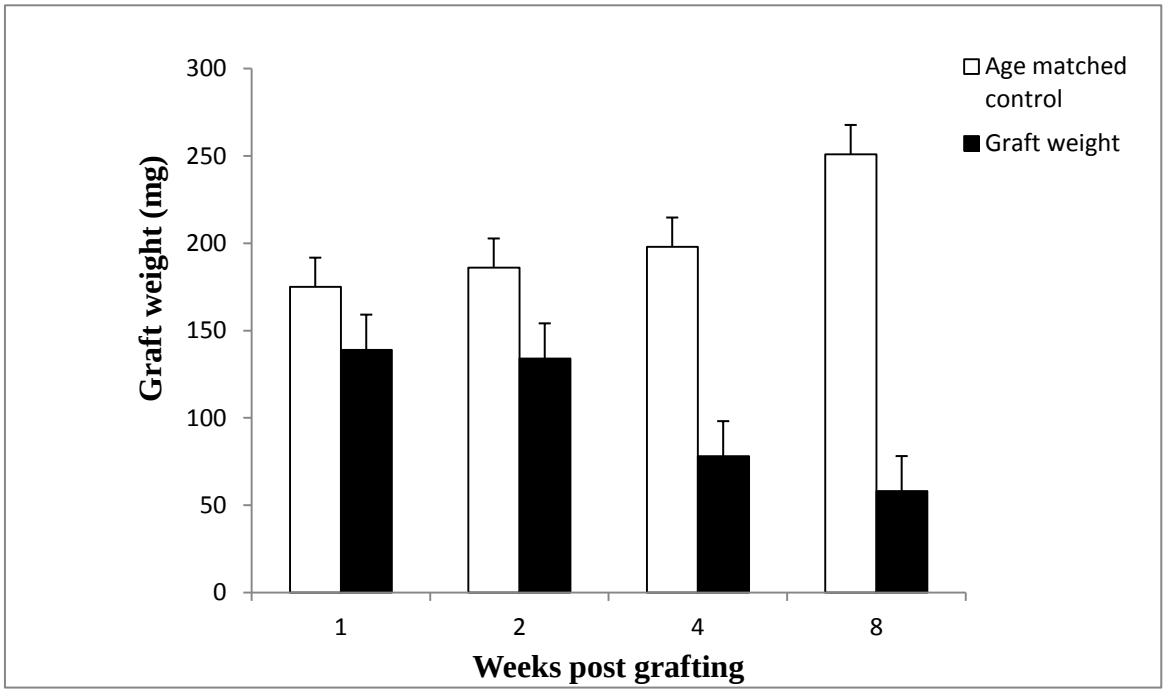

Figure 3: *Autografted testis weight from mice collected after 1-, 2-, 4- and 8- weeks as compared to the age-matched control testis weight. Data represented as* mean ± S.E.M.

The seminal vesicle weight recovered from grafted mice was collected at all time-points. Seminal vesicle weight is an indicator of bio-active testosterone secretion from the grafted testis. The average weight of seminal vesicle obtained from autografted mice increased with the progression of grafting period; starting from 10 mg in 1- week to 140 mg in 8- week. The average seminal vesicle weight recovered from autografted mice when compared to age-matched control mice seminal vesicle weight was significantly different up to the 4- week. However, at 8- week the average vesicle weight recovered to a value of 140mg which was comparable to a vesicle weight of 193mg from age-match controls (Table 2 and Figure 4).

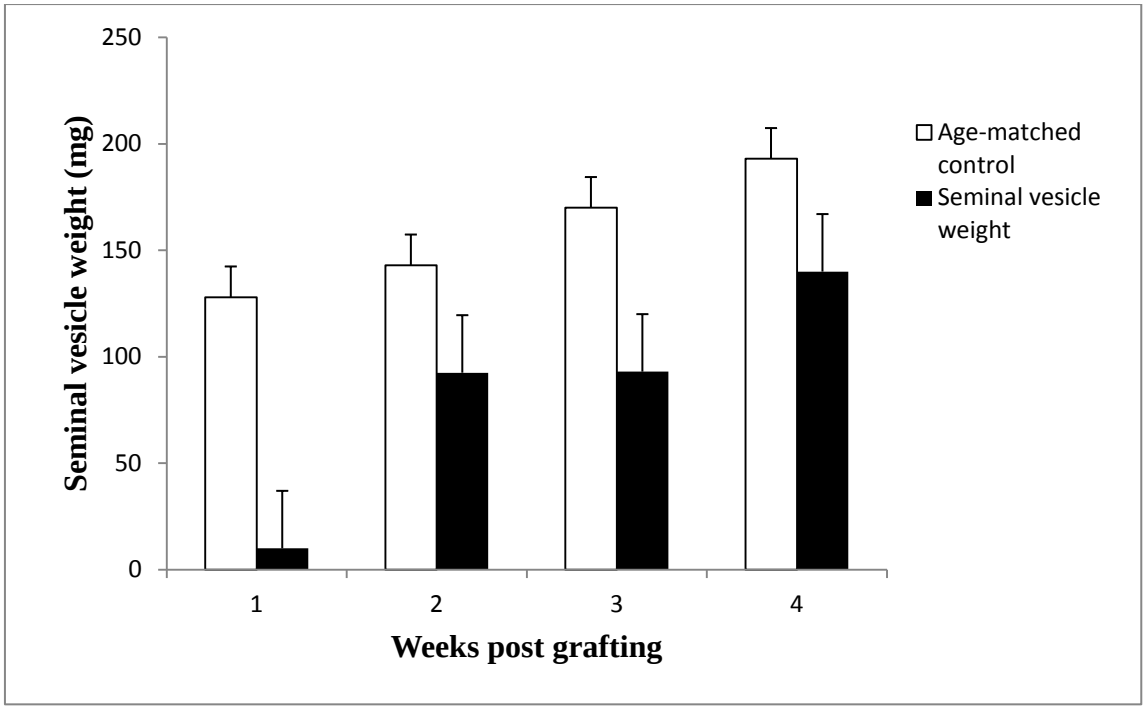

Figure 4: *Seminal vesicle weights from autografted mice collected at 1-, 2-, 4- and 8- weeks after grafting and compared against seminal vesicle weight from their age-match controls. Data represented as* mean ± S.E.M.

4.2 HISTOLOGICAL ANALYSIS OF AUTOGRAFTED TESTIS

The adult mouse testis, before grafting, showed the presence of intact seminiferous tubules with interstitial cells, when stained with haematoxylin and eosin (Figure 5A). A prominent lumen was present in seminiferous tubules and most advanced germ cells such as sperm were visible. Autografted testis tissue showed varying degree of tubular degeneration during the grafting period. Grafts harvested after 1- week showed tubular disintegration with only few persisting tubules (Figure 5B). In the grafts recovered at 2- and 4- week the tubules started exhibiting progressively higher degeneration (Figure 5C and 5D). The tubular architecture was completely lost in the autograft testis tissue by the 8- week (Figure 5E) and no intact tubules were detectable.

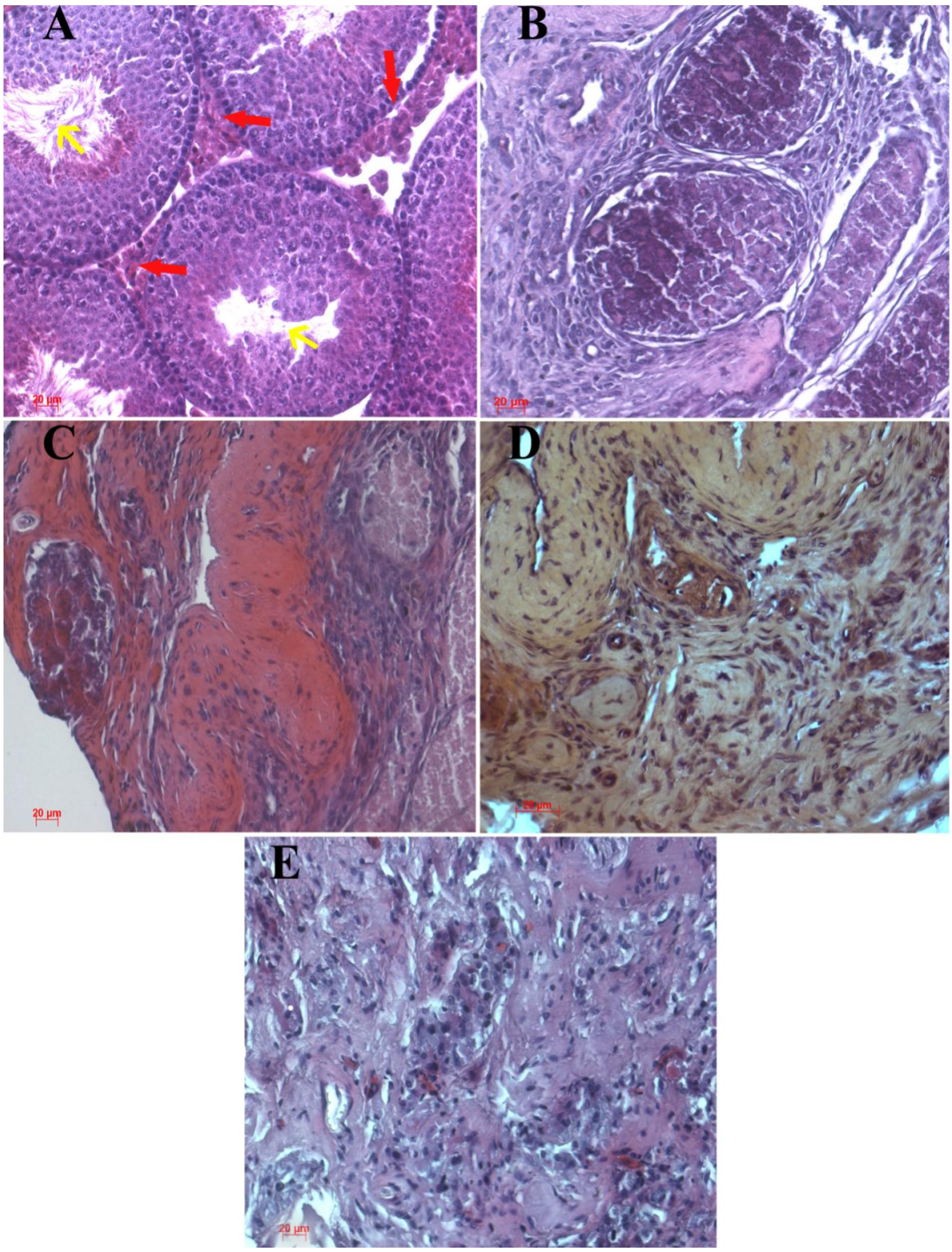

Figure 5: ***Histological photomicrographs of adult mouse testis before and after autografting (haematoxylin and eosin stain)*** (A) 6- week old adult mouse testis with well organised seminiferous (lumen of tubule indicated by yellow arrows) and interstitial space containing Leydig cells (indicated by red arrows) (B) Testes tissues recovered at 1- week post grafting contains few seminiferous tubules (C) At 2- week after grafting the tubular architecture started degenerating (D) Testes sections at 4-week after grafting (E) In autograft recovered at 8- week no tubules were left .Scale bar = 20µm.

4.3 IMMUNOHISTOCHEMICAL ANALYSIS

Immunohistochemical analysis for Leydig cell-specific markers, CYP11A1, HSD3B and LHCGR revealed that there was a significant increase in the number of Leydig cells with the progression in grafting period (Figure 6, Figure 7 and Figure 8).

The steroidogenic enzymes CYP11A1 and HSD3B have a restricted immunolocalization in the Leydig cells present as well-defined clusters in the interstitial space of the adult mouse control testis. The Leydig cells staining was cytoplasmic with CYP11A1 (Figure 6A) and HSD3B (Figure 7A). The Leydig cells, which were positive for both CYP11A1 and HSD3B antibodies were scattered throughout the testis sections in the autografts (Figure 6B-6E and 7B-7E respectively). The number of cells which are positive for CYP11A1 and HSD3B were significantly fewer in the grafts collected at 1-week (Figure 6B and 7B). However, the grafts retrieved after 2-, 4- and 8- weeks had a substantially higher number of CYP11A1- positive (Figure 6C, 6D and 6E) and HSD3B-positive (Figure 7C, 7D and 7E) cells. The staining intensity for CYP11A1 and HSD3B also increased progressively, reaching to a maximum during 8- week collection time point (Figure 6E and 7E). Interestingly, the staining intensity for CYP11A1 was higher than that of HSD3B in the grafts collected at all time points.

The LHCGR staining was also localized in the cytoplasm of Leydig cells present in interstitial spaces of adult mouse testis (Figure 8A). No staining was observed in Sertoli cell, peritubular myoid cells or germ cells of the adult testis. The number of LHCGR positive cells was fewer in the grafts collected at 1- and 2- weeks (Figure 8B and 8C). However, the number of positive cells increased progressively at 4- and 8- week collection points (Figure 8D and 8E).

PDGFRA staining was observed in Leydig cells in the 7- day old mouse testis (Figure 9A).The staining was present in the cytoplasm of stained cells. PDGFRA staining was not seen in adult testis sections (data not shown). PDGFRA- positive cells were observed in autografts collected at 1- week (Figure 9B) and 2- week (Figure 9C). The positive cells were located in close proximity to peritubular myoid cells (PMCs) that surrounds the periphery of persisting seminiferous tubule in these autografts. The grafts collected at 4- and 8- week did not show positive staining with PDGFRA (Figure 9D and 9E).

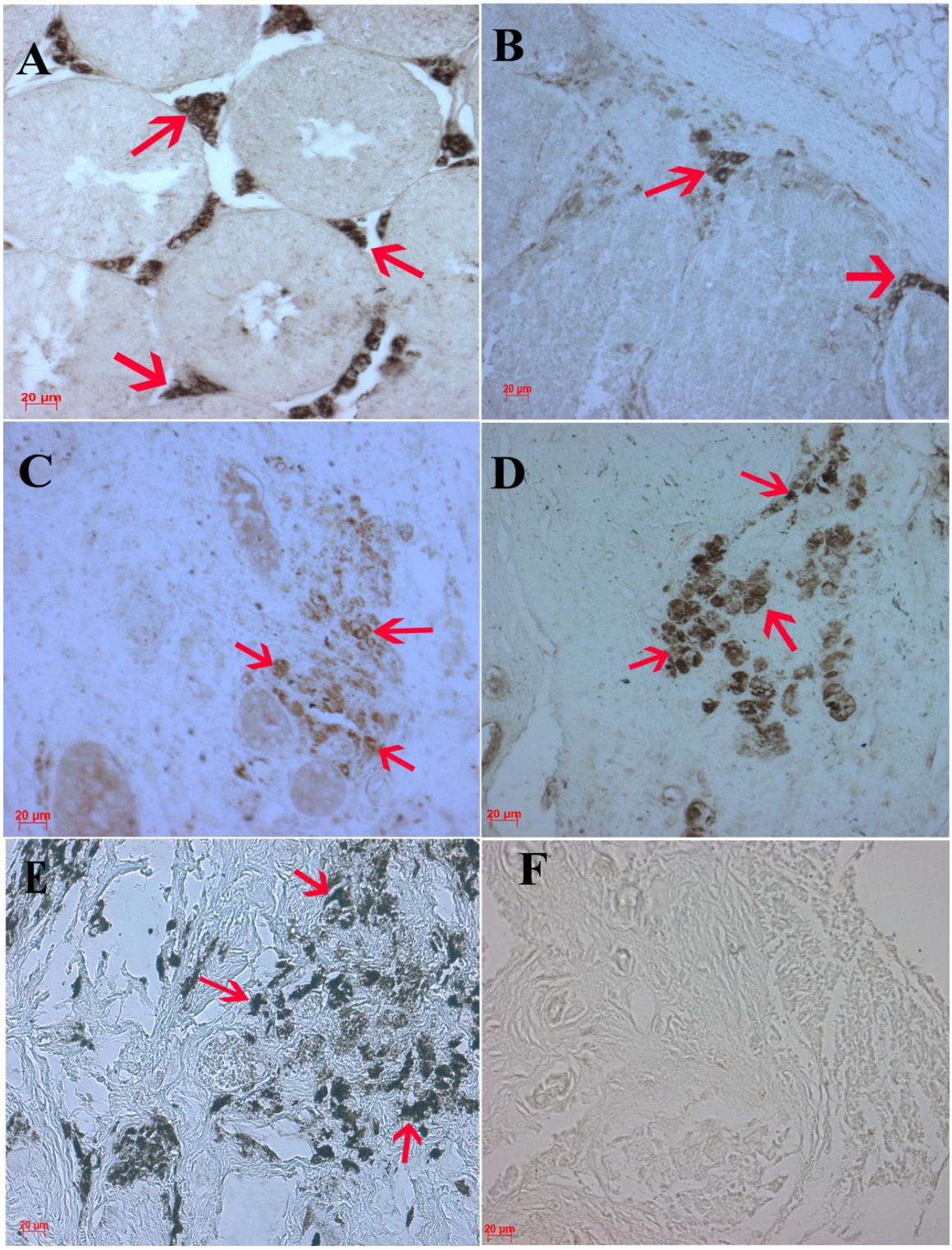

Figure 6: ***Expression and immunolocalization of*** **CYP11A1.**The positive cells are indicated by arrows. (A) Testicular cross-section from adult mouse shows specific staining of Leydig cells in the interstitial compartment (B) Small groups of CYP11A1 positive cells were observed in autografts after 1- week (C) Leydig cells become more abundant in autografts after 2- week (D) Progressive development in number of CYP11A1 positive Leydig cells after 4- week of autografting (E) Most

CYP11A1 positive cells are located in testicular cross-section recovered at 8- week (F) In a negative control where primary antibody, no positive cells are present. Scale bar =20µm.

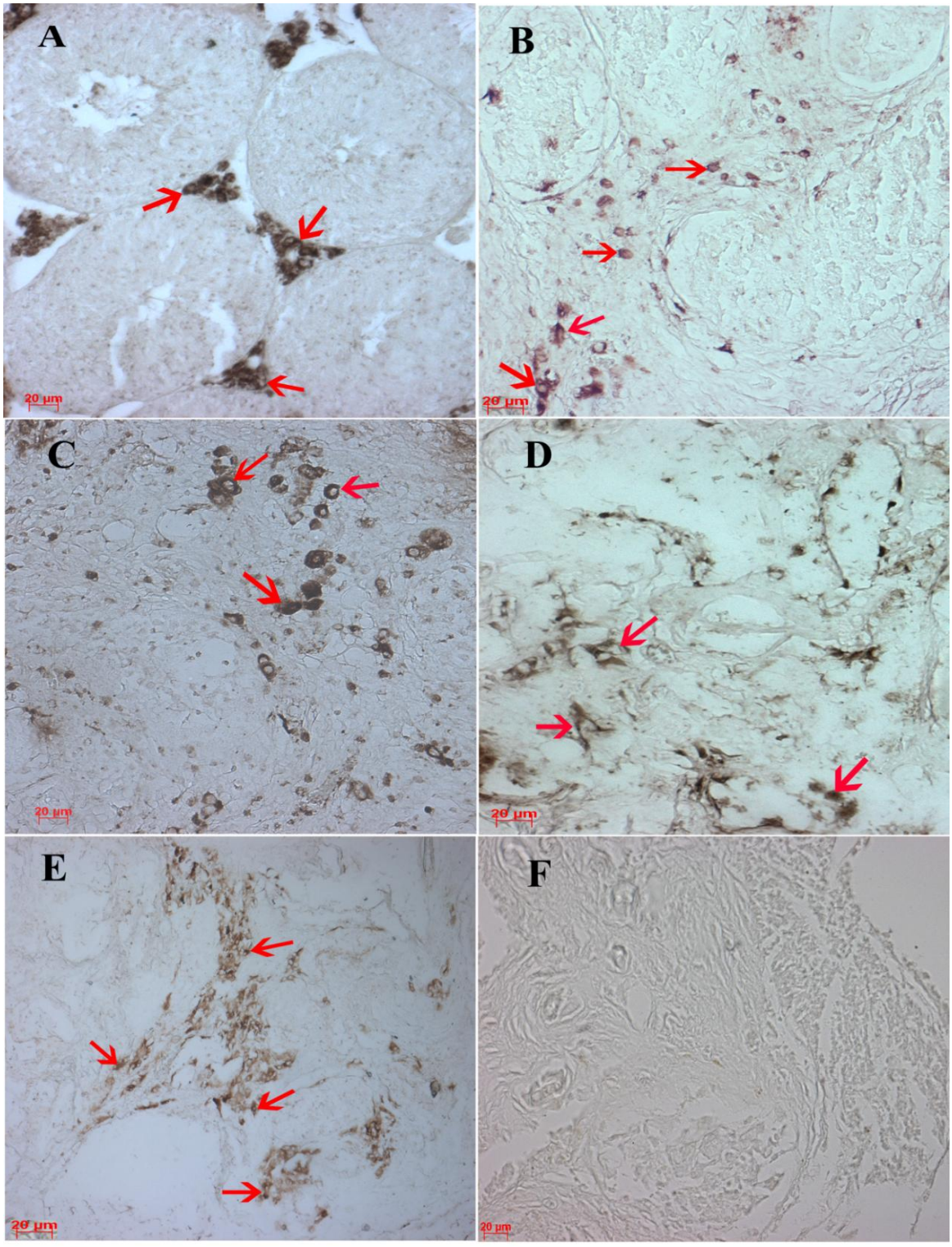

Figure 7: ***Expression and immunolocalization of*** **HSD3B.** The positive cells are indicated by arrows. (A) 6- week old adult testis tissue with strong HSD3B expression in interstitial Leydig cells. HSD3B stains the cytoplasm of Leydig cell (B) Testes autograft recovered from mouse after 1 week shows the existence of some HSD3B positive cells. Autograft testes retrieved after (C) 2-week and (D) 4-week revealed an increase in number of HSD3B positive cells. (E) Graft from

autografted animal recovered at 8- week showed significantly strong HSD3B expression in Leydig cell cytoplasm. The number of positive cells was maximum (F) Negative control without primary antibody being added. Scale bar =20µm.

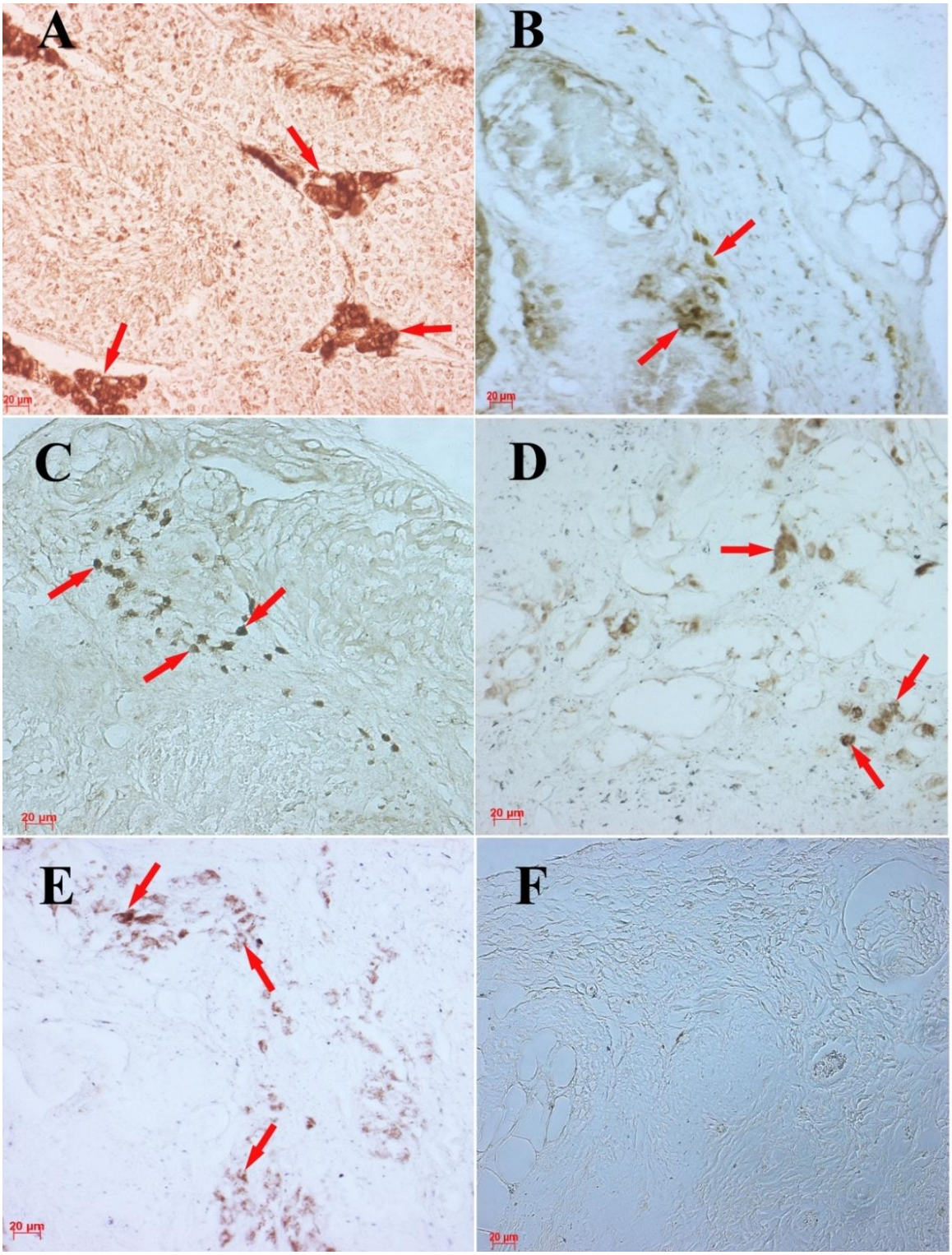

Figure 8: ***Expression and immunolocalization of*** **LHCGR.** The positive cells are indicated by arrows. (A) In adult mouse testis LHCGR expression was localized to interstitial Leydig cells. Autografted testes collected after (B) 1- week and (C) 2- week of grafting showed few LHCGR positive Leydig cells. The number of LHCGR positive cells increased at (D) 4- week, and was highest in graft harvested at (E) 8- week after grafting. (F) Negative control in which primary antibody was omitted had no positive cells. Scale bar = 20µm.

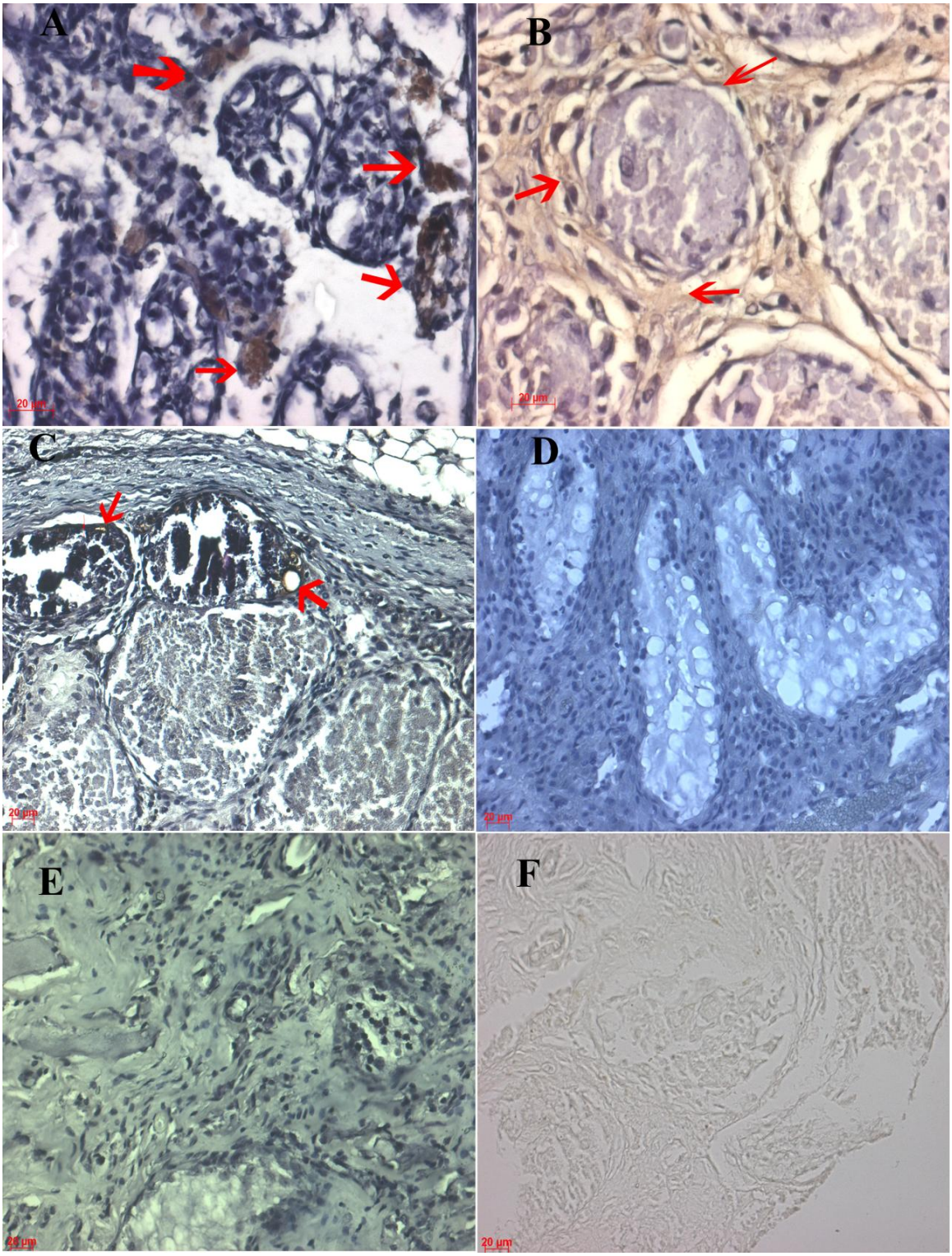

Figure 9: ***Expression and immunolocalization of*** **PDGFRA** (A) 7-day control mouse testis when stained with PDGFRA antibody showed cytoplasmic staining in interstitial Leydig cells (B) In autografted testis, positively stained cells were detected at 1- week after grafting, lying in close association to peritubular myoid cells (C) Few PDGFRA positive cells were also present during 2-week post grafting period. The graft recovered at (D) 4- week and (E) 8- week after grafting displayed no positive staining (F) Negative control with omission of primary antibody did not show any positive cells. Scale bar = 20µm.

The Sertoli cell-specific marker, WT-1, immunostaining was present in autografted testes. Testis tissue autografts from mice recovered at 1- week showed staining for WT-1 in cells present inside degenerating seminiferous tubules (Figure 10A). The number of WT-1 positive cells was considerably high in number at 4- week (Figure 10C) and then showed a marked decline in 8- week (Figure 10D). Interestingly, the reduction in number of WT-1 positive cells corroborates with the increase in degeneration of seminiferous tubules.

To look into the presence of germ cells in these grafts, DDX4 protein was taken as the marker of choice. DDX4 localizes in the cytoplasm of germ cells. In the adult mice testis, the staining was seen in the germ cell present inside the tubule (Figure 11A). However, DDX4 immunoreactivity was not detected in any of the grafts collected at 1-, 2-, 4- and 8- weeks (Figure 11B, 11C, 11D and 11E respectively); clearly indicating absence of germ cells in autografts.

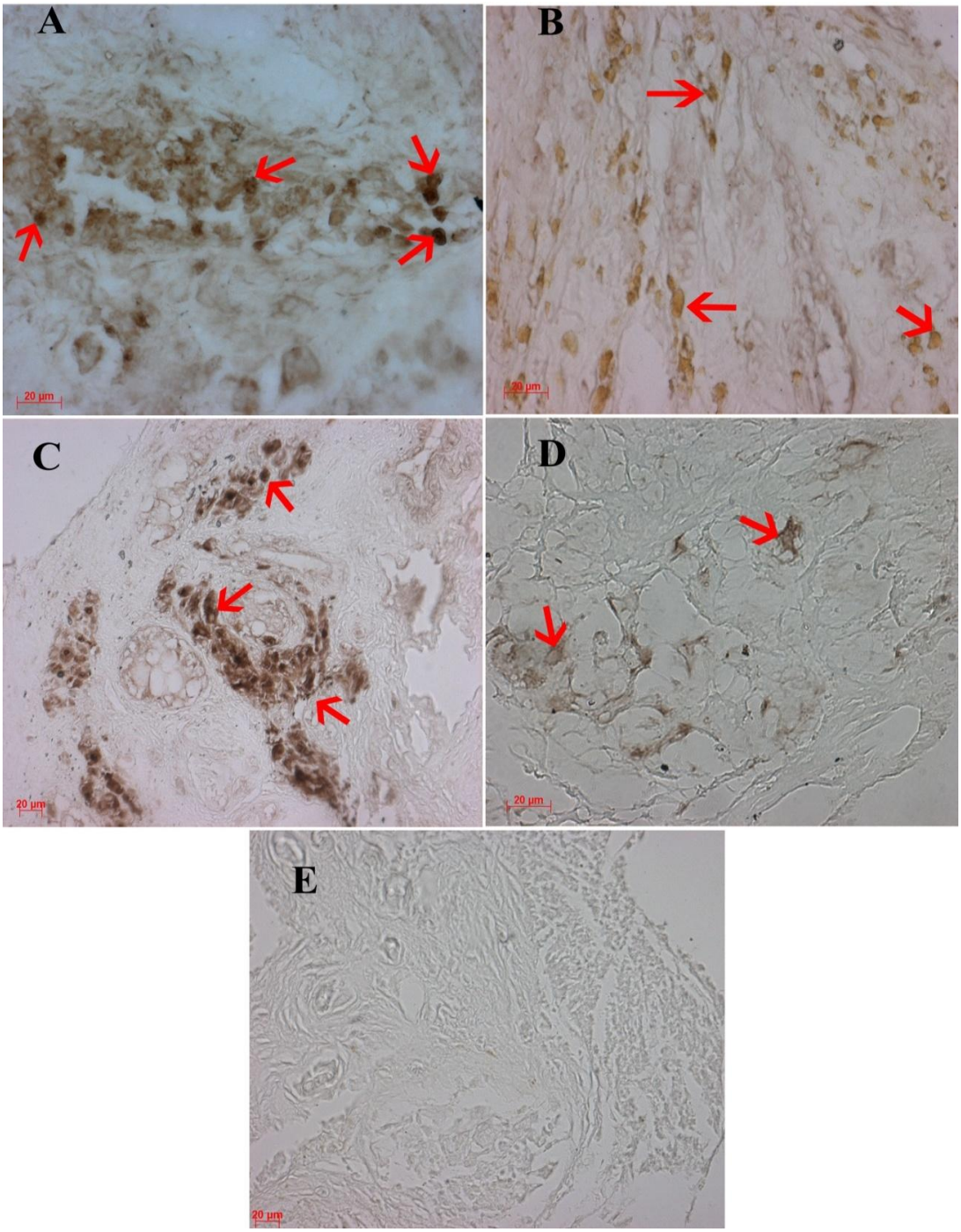

Figure 10: ***Expression and immunolocalization of*** **WT-1**. The positive cells are indicated by arrows. (A) WT-1 positive Sertoli cells are located inside seminiferous tubules in which degeneration has been initiated at 1- week after grafting. The number of WT-1 positive cells was high in number during (B) 2- week and (C) 4- week post grafting. (D) Number of WT-1 positive Sertoli cells declined significantly in graft collected at 8- week (E) Negative control in which primary antibody was not added. Scale bar = 20µm.

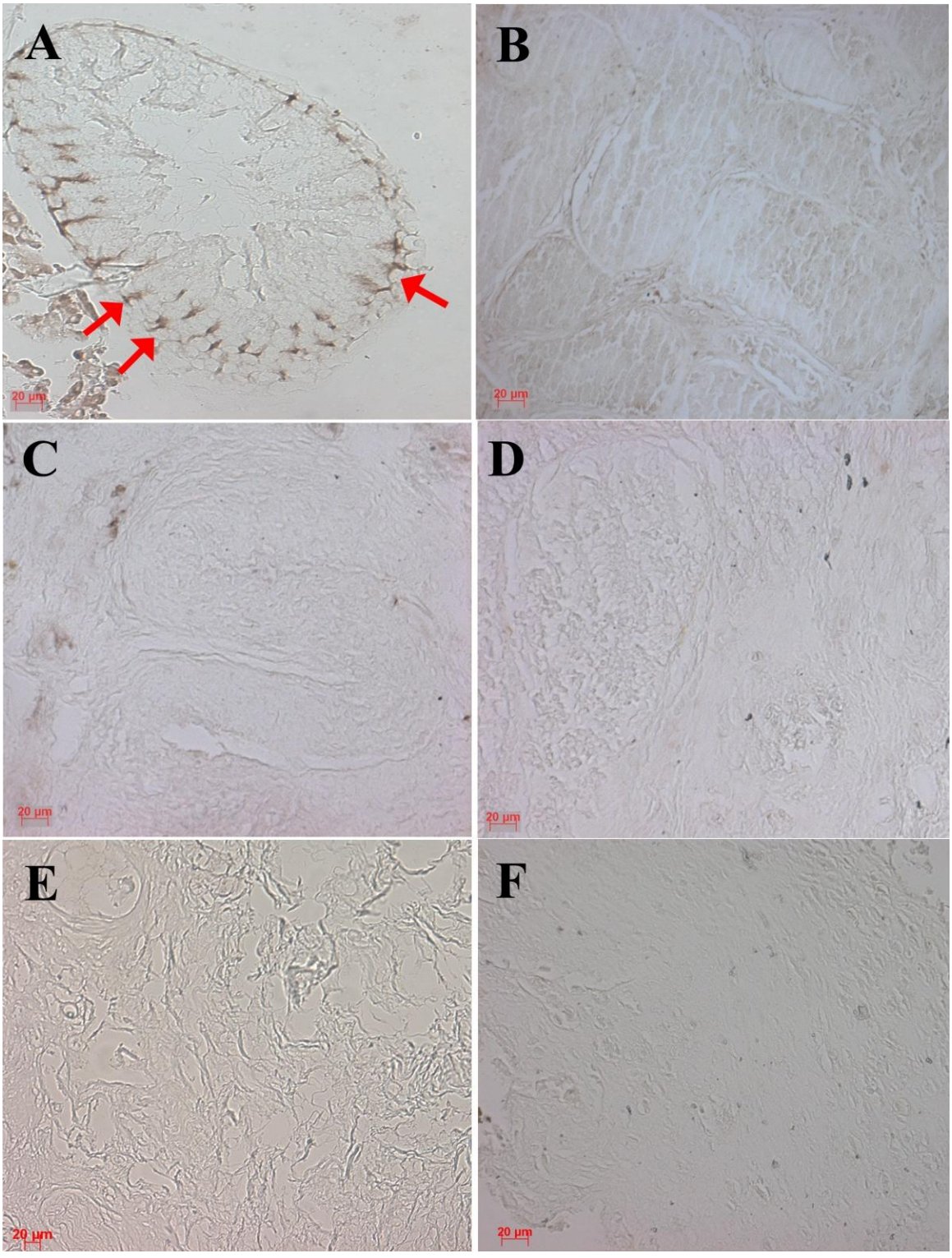

Figure 11: ***Expression and immunolocalization of*** **DDX4.** The positive cells are indicated by arrows. (A) Somatic germ cells are localised inside the seminiferous tubules as detected by DDX4 staining. No germ cell population was present in adult auto grafted testis tissues during (B) 1 week (C) 2 week (D) 4 week and (E) 8 week. (F) Negative control in which primary antibody was omitted. Scale =20µm.

5. DISCUSSION

The current work attempts to analyse the survival and regeneration of Leydig cell population in adult mice testis tissue subjected to autografting. Their presence has already been indicated in autologous testis transplantation studies in rodents such as mice (Boyle *et al*., 1975) and rats (Miragem *et al*., 2009). These studies suggested that the Leydig cells survive in autografts. This observation was based on recovery of seminal vesicle weight (Boyle *et al*., 1975) and serum testosterone level (Miragem *et al*., 2009). However, there is no present report which specifically demonstrates their survival in autografted testis. Further, it remains unclear in these studies whether the Leydig cell population survived the graft or regenerated *de-novo*.

Histological appearance of retrieved grafts showed a progressive disintegration of the testicular architecture with extensive degeneration of seminiferous tubules with the advancement in graft recovery time. The ectopic location suffers from disadvantages like absence of natural scrotal environment and exposure to hyperthermic conditions which would have possibly contributed towards testis degeneration (Saen *et al*., 2011). The testis is an organ with a high sensitivity to ischemia (Nugent *et al*., 1997). The high cell proliferation rate makes the adult testis dependent on high blood supply and, consequently very susceptible to the period of ischemia during the grafting period (Arregui *et al*., 2012). The adult testis also has high demand for oxygen with ongoing spermatogenesis (Schlatt *et al*., 2010). The higher metabolic demands to support differentiation during spermatogenesis in mature tissue have been proposed to be make the tissue more sensitive to hypoxia as compared to immature testis during grafting (Arregui *et al*., 2008b). Thus the ischemic damage and hypoxic environment being created possibly contributed to degeneration of tubules in these tissues; ultimately leading to progressive reduction of graft weight. This accedes to previous reports of xenografting for adult testis tissue where the transplanted tissue either degenerated or had surviving tubules with Sertoli cells only (Schlatt *et al*., 2006; Geens *et al*., 2006; Kim *et al*., 2007; Arregui *et al*., 2008b).

The existence of Leydig cells in the grafts was confirmed by the use of Leydig cell-specific markers in the present study. Steroidogenic specific enzyme, Cytochrome P450 11A1 (CYP11A1) and Hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta

isomerase (HSD3B) were considered as the markers of choice. CYP11A1 is expressed in steroidogenic cell types, including both fetal and adult Leydig cells. The Hsd3b enzyme which is involved in pregnenolone to progesterone conversion (Payne and Youngblood 1995) and has six different isoforms in mouse. While Hsd3b1 is expressed by both adult and fetal Leydig cells (Dupont *et al*., 1991), Hsd3b6 is a marker specific for adult Leydig cell differentiation (Baker *et al*., 1999; O'Shaughnessy *et al*., 2002). Both CYP11A1 and HSD3B stained cells were rare and the staining intensity was weak in 1- and 2- week grafts which gradually increased with increasing collection time points. This suggests loss of original population of Leydig cells from autografted testis and their regeneration thereafter. The immunolocalization of CYP11A1 in Leydig cell has already been reported in rat (Rouiller *et al*., 1990; Gassei *et al*., 2010), mouse (Davidoff *et al*., 2004) and several other species. Similarly, HSD3B has reportedly been reported as Leydig cell-specific marker in rat (Mueller *et al*., 1998; Majdic *et al*., 1998), monkey (Liang *et al*., 1999). The staining intensity of the CYP11A1 protein was stronger in autografts compared to that of HSD3B in the present study. This could be due to the fact that CYP11A1 is the first and rate-limiting enzyme during testosterone biosynthesis (Payne and Youngblood 1995). Therefore, CYP11A1 abundant expression is required for effective steroidogenesis in Leydig cells.

To assess the functionality of the Leydig cells in grafted testes, seminal vesicle weight was measured. The first reported testis autografting in mice had employed seminal vesicle weight assessment as a parameter to examine the functional ability of Leydig cells (Boyle *et al*., 1975). Seminal vesicle weight measurement has been used as indicator of bio-active testosterone in several xenografting works reported (Arregui *et al*., 2008; Goel *et al*., 2011).The autografted mice showed seminal vesicle weight as low as 10mg during first collection. This is significantly lower than that in control mice having seminal vesicle weight in range of 128mg to 193mg. This is indicative that testosterone secretion was reduced during initial period of grafting due to loss of Leydig cells in autografted tissues. However, the rise in vesicle weight confirms of increase in Leydig cell number by 8- week.

An up-regulated immunohistochemical staining pattern was also detected with LHCGR antibody. LHCGR stained cells were rare in 1- and 2- week grafts but the number increased progressively in 4- and 8- week grafts collected. Luteinizing hormone

(LH) stimulates testosterone synthesis in Leydig cells by binding to specific, high-affinity receptors (LHCGR) present on the surface of Leydig cells (Dufau 1988). LH receptor signalling is essential for Leydig cell differentiation (Teerds *et al*., 2007). Leydig cell progenitors are known to possess very few LH receptors (Shan and Hardy 1992). These findings are suggestive of the presence of Leydig progenitor/stem Leydig cell (SLC) population in autografts of 1-` and 2- week.

The positive immunostaining with PDGFRA antibody was detected in grafts collected at 1- and 2- week only. Platelet-derived growth factor A (PDGFA) is secreted by Sertoli cells and acts on Leydig precursor cells expressing the receptor PDGFRA. This triggers their differentiation into steroidogenically active Leydig cells (Bergeron *et al*., 2011). Gnessi *et al*. (2000) in his study on *Pdgfra* knockout mice has concluded that Pdgfa is an essential factor for adult Leydig cells development whereas it does not influence fetal generation of Leydig cells in pre-pubertal animals. These null males showed absence of adult Leydig cell population in testis. PDGFRA is reported to be expressed in stem Leydig cell (SLC) population of fetal and neonatal rat testis (Ge *et al*., 2006). The presence of Pdgfra expression in autografts is further suggestive of presence of SLC/progenitor population that may contribute towards Leydig cell regeneration in a manner similar to that reported in rats (Kerr *et al.,* 1985, 1986, 1987; Molenaar *et al*., 1985; Morris *et al.,* 1986; Teerds *et al*., 2007).This finding corroborate with previous finding in this study where, LHCGR expressing cells were absent or very few in 1- and 2-week grafts. However, further investigations of these issues are needed.

The presence of Sertoli cells in the grafts was examined immunohistologically by the use of WT-1 antibody. The transcription factor, Wilms tumor 1 (WT-1), is switched on in Sertoli cells early in fetal life (Mackay 2000) and thereafter, continues to be expressed in Sertoli cells, localized primarily to the nucleus, throughout all phases of life. Thus, it is a stable marker of Sertoli cells (Sharpe *et al*., 2003). Gao *et al*. (2006) has shown that *Wt-1* knockout in mice resulted in disruption of developing seminiferous tubules and progressive loss of Sertoli and germ cells such that postnatal mutant testes were completely devoid of these cell-types and were severely hypoplastic. Therefore, it was conclusive that *Wt-1* is essential for the maintenance of Sertoli cells and seminiferous tubules in the developing testes. Immunohistochemically, WT-1 has been localized to Sertoli cells in both embryonic (Gao *et al*., 2006) and adult mice (Sridharan *et al*., 2007).

WT-1-positive cells were higher in number in 1- to 4- week grafts. This indicates that Sertoli cells are initially resilient to ischemic damage induced due to grafting and survive due to persisting seminiferous tubular structure. In the graft collected at 8- week, Sertoli cell number reduces considerably due to damage of tubules. It was concluded in the present study that Sertoli cells could survive in autografted testis, despite severe destruction of seminiferous tubule structure. It however remains elusive if the Sertoli cells survived or originated *de-novo* like Leydig cells in the autografts, which needs further validation.

To examine the presence of germ cell population in retrieved autograft testes, we looked into the protein expression of DDX4 immunohistochemically. DDX4 *(DEAD* [Asp-Glu-Ala-Asp] box polypeptide 4), previously known as *VASA* is a member of DEAD-box family of genes and encodes an ATP-dependent RNA helicase. It is a specific marker of the germ cell lineage in both sexes in mouse (Fujiwara *et al*., 1994). In mice, Ddx4 protein is exclusively expressed in primordial germ cells (PGCs) just after their colonization to embryonic gonads and in germ cells undergoing gametogenic processes until the post-meiotic stage in both males and females (Toyooka *et al*., 2000). The targeted loss of function causes a deficiency in the proliferation and differentiation of male germ cells, leading to absence of sperm in the mouse testis (Tanaka *et al*., 2000). Immunohistochemically, DDX4 has been localized in cytoplasm of spermatocytes and round spermatids of adult mice testis (Fujiwara *et al*., 1994) and maturing gonocytes in fetal human testis (Anderson *et al*., 2007). DDX4 has been used to detect germ cells in immature sheep testis (Arregui *et al*., 2008) and pre-pubertal and pubertal cat testis (Mota *et al*., 2012) post xenografting. The absence of DDX4 expression in all the grafts collected clearly indicates that germ cells were absent in the testes.

In conclusion, this study describes a novel model system for studying Leydig cell regeneration in mice. The results from the present study suggests *de-novo* regeneration of Leydig cell population from stem Leydig cells/progenitor cells. This model system can be used for investigating Leydig cell steroidogenesis and regulation of the intrinsic factors involved in Leydig cell development. It may also provide an insight in overcoming testosterone deficiency and Leydig cell dysfunction in adult vertebrate species. The success of this rodent model can be put to therapeutic applications in adult human males undergoing oncological treatments.

REFERENCES

- Abbaszade IG, Clarke TR, Park CH, Payne AH (1995) The mouse 3β-hydroxysteroid dehydrogenase multigene family includes two functionally distinct groups of proteins. Mol Endocrinology 9: 1214–1222.
- Anderson RA, Fulton N, Cowan G, Coutts S, Saunders PT (2007) Conserved and divergent patterns of expression of DAZL, VASA and OCT4 in the germ cells of the human fetal ovary and testis.BMC Dev Biol 7: 136.
- Armstrong JF, Pritchard-Jones K, Bickmore WA, Hastie ND, Bard JB (1993) The expression of the Wilms' tumour gene, WT1, in the developing mammalian embryo. Mech Dev. 40: 85–97.
- Arregui L, Rathi R, Megee SO, Honaramooz A, Gomendio M, Roldan ER, Dobrinski I (2008a) Xenografting of sheep testis tissue and isolated cells as a model for preservation of genetic material from endangered ungulates. Reproduction 136: 85–93.
- Arregui L, Rathi R, Zeng W, Honaramooz A, Gomendio M, Roldan ER, Dobrinski I (2008b) Xenografting of adult mammalian testis tissue. Anim Reprod Sci 106: 65–76.
- Arregui L, Rathi R, Modelski M, Zeng W, Roldan ER, Dobrinski I (2012) Suppression of spermatogenesis before grafting increases survival and supports resurgence of spermatogenesis in adult mouse testis. Fertil Steril 97(6): 1422-14299.
- Attaran S, Hodges CV (1966) Technique for testicular transplants. Investig Urol 3: 390–394.
- Awoniyi CA, Santulli R, Sprando RL, Ewing LL, Zirkin BR (1989) Restoration of advanced spermatogenic cells in the experimentally regressed rat testis: Quantitative relationship to testosterone concentration within the testis. Endocrinology 124: 1217-1223.
- Bain PA, Meisler MH, Taylor BA, Payne AH (1993) The genes encoding gonadal and nongonadal forms of 3β-hydroxysteroid dehydrogenase/Δ_5-Δ_4 isomerase are closely linked on mouse chromosome 3. Genomics 16: 219–223.

- Baker PJ, O'Shaughnessy PJ (2001) Role of gonadotrophins in regulatingnumbers of Leydig and Sertoli cells during fetal and postnatal development in mice. Reproduction 122:227–234.
- Baker PJ, Pakarinen P, Huhtaniemi IT, Abel MH, Charlton HM, Kumar TR, O'Shaughnessy PJ (2003) Failure of normal leydig cell development in folliclestimulating hormone (FSH) receptor-deficient mice, but not FSHβ-deficient mice: role for constitutive FSH receptor activity. Endocrinology 144: 138–145.
- Baker PJ, Sha JA, McBride MW, Peng L, Payne AH, O'Shaughnessy PJ (1999) Expression of 3β-hydroxysteroid dehydrogenase type I and type VI isoforms in the mouse testis during development. Eur J Biochem 260: 911–917.
- Bartlett JM, Weinbauer GF, Nieschlag E (1989) Differential effects of FSH and testosterone on the maintenance of spermatogenesis in the adult hypophysectomized rat. J Endocrinol 121: 49-58.
- Basciani S, Mariani S, Spera G, Gnessi L (2010) Role of platelet derived growth factors in the testis. Endocr Rev 31: 916–939.
- Benton L, Shan LX, Hardy MP (1995) Differentiation of adult Leydig cells. J Steroid Biochem Mol Biol 53(1-6):61-68.
- Bergeron F, Bagu ET, Tremblay JJ (2011) Transcription of platelet-derived growth factor receptor α in Leydig cells involves specificity protein 1 and 3. J Mol Endocrinol 46(2): 125-138.
- Boyle PF, Fox M, Slater D (1975) Transplantation of interstitial cells of the testis: effect of implant site, graft mass and ischaemia Br J Urol 47(7): 891-8.
- Brennan J, Tilmann C, Capel B (2003) Pdgfr-a mediates testis cord organization and fetal Leydig cell development in the XY gonad. Gene Dev 17: 800–810.
- Call KM, Glaser T, Ito CY, Buckler AJ, Pelletier J, Haber DA, Rose EA, Kral A, Yeger H, Lewis WH, Jones C, Housman DE (1990) Isolation and characterization of a zinc finger polypeptide gene at the human chromosome 11 Wilms' tumor locus. Cell 60: 509–520.
- Capel (2000) The battle of the sexes. Mech Dev 92: 89-103.
- Chan F, Allison JE, Stanley AJ, Gumbreck LG (1969) Reciprocal transplantation of testes between normal and pseudohermaphroditic male rats. Fertil Steril 20(3): 482-494.

- Chemes H (1996) Leydig cell development in humans. In: Payne AH, Hardy MP, Russell LD, eds. The Leydig cell. Vienna, IL: Cache River Press; 175–202.
- Chen H, Luo L, and Zirkin BR (1996). Leydig cell structure and function during aging. In:The Leydig Cell (eds. Payne, A. H., Hardy, M. P., and Russel, L. D.), Cache River Press,Vienna, IL.
- Christensen AK, Gillim S (1969) The correlation of fine structure and function in steroid-secreting cells, with emphasis on those of the gonads. In: The Gonads. Ed K McKerns. New York: Appleton–Century–Crofts; 415-488.
- Clark BJ, Combs R, Hales KH, Hales DB, Stocco DM (1997) Inhibition of transcription affects synthesis of steroidogenic acute regulatory protein and steroidogenesis in MA-10 mouse Leydig tumor cells. Endocrinology 138: 4893–4901.
- Clarke TR, Bain PA, Burmeister M, Payne AH (1996) Isolation and characterization of several members of the murine Hsd3b gene family. DNA Cell Biol 15; 387–399.
- Cooke BA (1996) Transduction of the luteinizing hormone signal within the Leydig cell. In: Lussell D (ed.), The Leydig Cell. Vienna, IL: Cache River Press; 351–364.
- Davidoff MS, Middendorff R , Enikolopov G, Riethmacher D, Holstein AF, Muller D (2004) Progenitor cells of the testosterone-producing Leydig cells revealed. J Cell Biol 167(5): 935–944.
- De Kretser DM (1967) The fine structure of the testicular interstitial cells in men of normal androgenic status. Z Zellforsch 80: 594-609.
- Deanesly R (1954) Spermatogenesis and endocrine activity in grafts of frozen and thawed rat testis.J Endocrinol 11: 201–206.
- Dobrinski I (2005) Germ cell transplantation and testis tissue xenografting in domestic animals. Anim Reprod Sci 89(1-4): 137-145.
- Dong L, Jelinsky SA, Finger JN, Johnston DS, Kopf GS, Sottas CM, Hardy MP, Ge RS (2007) Gene expression during development of fetal and adult Leydig cells. Ann N Y Acad Sci 1120: 16-35.
- Dufau ML (1988) Endocrine regulation and communicating functions of the Leydig cell. Annu Rev Physiol 50: 483-508.

- Dufau ML (1998). The luteinizing hormone receptor. Annu Rev Physiol 60: 461–496.
- Dupont E, Luu-The V, Labrie F, Pelletier G (1991) Ontogeny of 3β-hydroxysteroid dehydrogenase/Δ_5-Δ_4 isomerase (3β-HSD) in human testis as studied by immunocytochemistry. J Androl 12: 161–164.
- Fawcett DW, Neaves WB, Flores MN (1973) Comparative observations on intertubular lymphatics and the organization of the interstitial tissue of the mammalian testis. Biol Reprod 9: 500-532.
- Fijak M, Bhushan S, Meinhardt A (2011) Immunoprivileged sites: the testis. Methods Mol Biol 677: 459-70.
- Fujii T (1977) Roles of age and androgen in the regulation of sex accessory organs. Adv Sex Horm Res 3: 103-137.
- Fujiwara Y, Komiya T, Kawabata H, Sato M, Fujimoto H, Furusawa M, Noce T (1994) Isolation of a DEAD-family protein gene that encodes a murine homolog of *Drosophila* vasa and its specific expression in germ cell lineage. Proc Natl Acad Sci U S A 91: 12258-12262.
- Gao F, Maiti S, Alam N, Zhang Z, Deng JM, Behringer RR, Lecureuil C, Guillou F, Huff V (2006) The Wilms tumor gene, Wt1, is required for Sox9 expression and maintenance of tubular architecture in the developing testis. Proc Natl Acad Sci U S A 103(32): 11987-11992.
- Gassei K, Ehmcke J, Wood MA, Walker WH, Schlatt S (2010) Immature rat seminiferous tubules reconstructed in vitro express markers of Sertoli cell maturation after xenografting into nude mouse hosts. Mol Hum Reprod 16(2): 97-110.
- Ge R, Shan L, HardyM (1996) Pubertal development of Leydig cells. In: Payne AH, Hardy MP, Russell LD, eds. The Leydig cell. Vienna, IL: Cache River Press; 159–174.
- Ge RS, Dong Q, Sottas CM, Papadopoulos V, Zirkin BR, Hardy MP (2006) In search of rat stem Leydig cells: identification, isolation, and lineage-specific development. Proc Natl Acad Sci U S A. 103(8): 2719-2724.
- Geens M, de Block G, Goossens E, Frederickx V, van Steirteghem A, Tournaye H (2006) Spermatogonial survival after grafting human testicular tissue to immunodeficient mice. Hum Reprod 21: 390–396.

- Gessler M, Poustka A, Cavenee W, Neve RL, Orkin SH, Bruns GA (1990) Homozygous deletion in Wilms tumours of a zinc-finger gene identified by chromosome jumping. Nature 343: 774–778.
- Gnessi L, Basciani S,Mariani S, Arizzi M, Spera G,Wang C, Bondjers C, Karlsson L, Betsholtz C (2000) Leydig cell loss and spermatogenic arrest in platelet-derived growth factor (PDGF)-A-deficient mice. J Cell Biol 149: 1019–1026.
- Goel S, Fujihara M, Minami N, Yamada M, Imai H (2008) Expression of NANOG, but not POU5F1, points to the stem cell potential of primitive germ cells in neonatal pig testis. Reproduction 135: 785–95.
- Goldstein M, Phillips DM, Sundaram K, Young GP, Gunsalus GL, Thau R, Bardin CW (1983) Microsurgical transplantation of testes in isogenic rats: method and function. Biol Reprod 28: 971–982.
- Gosden RG, Aubard Y (1996b) Transplantation of testicular tissue. In Transplantation of Ovarian and Testicular Tissues. Eds RG Gosden & Y Aubard. Texas: Landes Company and Chapman & Hall; 89-97.
- Gosden RG, Aubard Y (1996a) Why transplant gonadal tissue? In Transplantation of Ovarian and Testicular Tissues. Eds RG Gosden & Y Aubard. Texas: Landes Company and Chapman & Hall;1-15.
- Greco TL, Payne AH (1994) Ontogeny of expression of the genes for steroidogenic enzymes P450 side-chain cleavage, 3β-hydroxysteroid dehydrogenase, P45017α hydroxylase/C17–20 lyase, and P450 aromatase in fetal mouse gonads. Endocrinology 135: 262–268.
- Griffin DK, Ellis PJ, Dunmore B, Bauer J, Abel MH, Affara NA (2010) Transcriptional profiling of luteinizing hormone receptor-deficient mice before and after testosterone treatment provides insight into the hormonal control of postnatal testicular development and Leydig cell differentiation. Biol Reprod. 82(6):1139-50.
- Griswold MD (1993) Protein secretion by Sertoli cells: General considerations. In: Russell L.D. and Griswold MD (Eds.) The Sertoli cell. Cache River Press, Clearwater FL, USA; 195-200.
- Habert R, Lejeune H, Saez JM (2001) Origin, differentiation and regulation of fetal and adult Leydig cells. Mol Cell Endocrinol 179: 47–74.

- Haider SG (1995) Cell biology of Leydig cells in the testis. International Review of Cytology 233.
- Hansson V, Skalhegg BS, Tasken K (1999) Cyclic-AMPdependent protein kinase (PKA) in testicular cells. Cell specific expression, differential regulation and targeting of subunits of PKA. J Steroid Biochem 69(1–6): 367–378.
- Honaramooz A, Li MW, Penedo MCT, Meyers S, Dobrinski I (2004) Accelerated maturation of primate testis by xenografting into mice. Biol Reprod 70: 1500–1503.
- Honaramooz A, Snedaker A, Boiani M, Scholer H, Dobrinski I, Schlatt S (2002) Sperm from neonatal mammalian testes grafted in mice. Nature 418: 778-781.
- Huhtaniemi I, Pelliniemi LJ (1992) Fetal Leydig cells: Cellular origin, morphology, life span, and special functional features. Proc Soc Exp Biol Med 201: 125-140.
- Ikeda Y, Shen WH, Ingraham HA, Parker KL (1994) Developmental expression of mouse steroidogenic factor-1, an essential regulator of the steroid hydroxylases. Mol Endocrinol 8: 654-662.
- Johnson L, Suggs LC, Norton YM , Zeh WC (1996a) Effect of developmental age or time after transplantation on Sertoli cell number and testicular size in inbred Fischer rats. Biol Reprod 54: 948–959.
- Johnson L, Suggs LC, Norton YM, Welsh TH Jr, Wilker CE (1996b) Effect of hypophysectomy, sex of host, and/or number of transplanted testes on Sertoli cell number and testicular size of syngeneic testicular grafts in Fischer rats. Biol Reprod 54: 960–969.
- Karl J, Capel B (1998) Sertoli Cells of the mouse testis originate from the coelomic epithelium. Dev Biol 203(2): 323-333.
- Keeney DS, Mendis-Handagama SM, Zirkin BR, Ewing LL (1988) Effect of long term deprivation of luteinizing hormone on Leydig cell volume, Leydig cell number, and steroidogenic capacity of the rat testis. Endocrinology 123(6): 2906-2915.
- Kerr JB, Bartlett JMS, Donachie K (1986) Acute response of testicular interstitial tissue in rats to the cytotoxic drug ethane dimethane sulfonate. Cell Tissue Res 243: 405-414.

- Kerr JB, Bartlett JMS, Donnachie K, Sharpe RM (1987) Origin of regenerating Leydig cells in the testis of the adult rat. Cell Tissue Res 249: 367-377.
- Kerr JB, Donachie KL, Rommerts FFG (1985) Selective destruction and regeneration of rat Leydig cells *in vivo*. A new method for the study of seminiferous tubular-interstitial tissue interaction. Cell Tissue Res 243: 405-414.
- Kim Y, Selvaraj V, Pukazhenthi B, Travis AJ (2007) Effect of donor age on success of spermatogenesis in feline testis xenograft. Reprod Fertil Dev 19: 869–876.
- Kreidberg JA, Sariola H, Loring JM, Maeda M, Pelletier J, Housman D and Jaenisch R(1993) WT-1 is required for early kidney development.Cell 74: 679–691.
- Kuopio T, Savouras PO, Pelliniemi LJ, Huhtaniemi IT (1989) Transplantation of newborn rat testis under the kidney capsule of adult host as a model to study the structure and function of Leydig cells. J Androl 10: 335–345.
- Lasko PF, Ashburner (1988) The product of the Drosophila gene vasa is very similar to eukaryotic initiation factor-4A. Nature 335(6191): 611-617.
- Lee GS, Kim HS, Lee SH, Kang MS, Kim DY, Lee CK, Kang SK, Lee BC, Hwang WS (2005) Characterization of pig vasa homolog gene and specific expression in germ cell lineage. Mol Reprod Dev 72: 320–8.
- Lei ZM, Mishra S, Zou W, Xu B, Foltz M, Li X, Rao CV (2001) Targeted disruption of luteinizing hormone/human chorionic gonadotropin receptor gene. Mol Endocrinol 15: 184–200.
- Liang JH, Sankai T, Yoshida T, Cho F, Yoshikawa Y (1999) Localization of immunoreactive testosterone and 3beta-hydroxysteroid dehydrogenase/ Δ_5-Δ_4 isomerase in cynomolgus monkey (*Macaca fascicularis*) testis during postnatal development. J Med Primatol 28: 62–66.
- Lipsett MB, Wilson H, Kirschner MA, Korenman SG, Fishman LM, Sarfaty GA, Bardin CW (1966) Studies on Leydig cell physiology and pathology: secretion and metabolism of testosterone. Recent Prog Horm Res 22: 245-281.
- Little M, Holmes G, Walsh P (1999) WT1: What has the last decade told us? Bioessays 21: 191–202.

- Lording DW, de Kretser DM (1972) Comparative ultrastructural and histochemical studies of the interstitial cells of the rat testis during fetal and postnatal development. J Reprod Fertil 29: 261–269.
- Luetjens CM, Stukenborg JB, Nieschlag E, Simoni M, Wistuba J (2008) Complete spermatogenesis in orthotopic but not in ectopic transplants of autologously grafted marmoset testicular tissue. Endocrinology 149(4): 1736-47.
- Ma P, Ge Y, Wang S, Ma J, Xue S, Han D (2004) Spermatogenesis following syngeneic testicular transplantation in Balb/c mice. Reproduction 128(2): 163-70.
- Mackay S (2000) Gonadal development in mammals at the cellular and molecular levels. Int Rev Cytol 200: 47–99.
- Maeda T, Goto A, Kobayashi D, Tamai I (2007) Transport of organic cations across the blood-testis barrier. Mol Pharm 4: 600-607.
- Majdic G, Saunders PT, Teerds KJ (1998) Immunoexpression of the steroidogenic enzymes 3-beta hydroxysteroid dehydrogenase and 17 alpha-hydroxylase, C17,20 lyase and the receptor for luteinizing hormone (LH) in the fetal rat testis suggests that the onset of Leydig cell steroid production is independent of LH action. Biol Reprod 58(2): 520-525.
- McFarland KC, Spengel R, Philips HS, Kohlet M, Rosemblit N, Nikolics K, Segaloff DL, Seeburg PH (1989) Lutropin–choriogonadotropin receptor an unusual member of the G proteincoupled receptor family. Science 245: 494-499.
- Mendis-Handagama SM, Ariyaratne HB (2001) Differentiation of the adult Leydig cell population in the postnatal testis. Biol Reprod. 65(3): 660-71.
- Miragem A, Neto BS, Reche M, Kliemann LM, Capp E, vo**n** Eye Corleta H (2009) Subcutaneous autologous testicle transplantation in Wistar rats. Int Urol Nephrol 41(2): 313-318.
- Mital P, Kaur G, Dufour JM (2010) Immunoprotective sertoli cells: making allogeneic and xenogeneic transplantation feasible. Reproduction 139(3): 495-504.
- Molenaar R, de Rooij DG, Rommerts FF, van der Molen HJ (1985) Repopulation of Leydig cells in mature rats after selective destruction of the existent Leydig cells with ethylene dimethane sulfonate is dependent on luteinizing hormone and not follicle-stimulating hormone. Endocrinology 118: 2546–2554.

- Morris ID, Philips DM, Bardwin CW (1986) Ethane dimethanesulphonate destroys Leydig cells in the rat testis. Endocrinology 118: 709-719.
- Mota PC, Ehmcke J, Westernstroer B, Gassei K, Ramalho-Santos J, Schlatt S (2012) Effects of different storage protocols on cat testis tissue potential for xenografting and recovery of spermatogenesis. Theriogenology 77(2): 299-310.
- Mueller A, Hermo L, Robaire B (1998) The effects of aging on the expression of glutathione S-transferases in the testis and epididymis of the Brown Norway rat. J Androl 19(4): 450-465.
- Nachtigal MW, Hirokawa Y, Enyeart-VanHouten DL, Flanagan JN, Hammer GD, Ingraham HA (1998) Wilms' tumor 1 and Dax-1 modulate the orphan nuclear receptor SF-1 in sex-specific gene expression. Cell 93(3): 445-454.
- Nelson DR, Koymans L, Kamataki T, Stegeman JJ, Feyereisen R, Waxman D, Waterman MR, Gotoh O, Coon MJ, Estabrook RW, Gunsulus IC, Nebert DW (1996) P450 superfamily: update on new sequences, gene mapping, accession numbers and nomenclature.Pharmacogenetics 6: 1–42.
- Nugent D, Meirow D, Brook PF, Aubard Y, Gosden RG (1997) Transplantation in reproductive medicine: previous experience, present knowledge and future prospects. Hum Reprod Update. 3(3): 267-80.
- O'Shaughnessy PJ, Morris ID, Baker PJ (2008) Leydig cell re-generation and expression of cell signalling molecules in the germ cell-free testis. Reproduction 135: 851-858.
- O'Shaughnessy PJ, Morris ID, Huhtaniemi I, Baker PJ, Abel MH (2009) Role of androgen and gonadotrophins in the development and function of the Sertoli cells and Leydig cells: data from mutant and genetically modified mice. Mol Cell Endocrinol 306: 2–8.
- O' Shaughnessy PJ, Willerton L, Baker PJ (2002) Changes in Leydig cell gene expression during development in the mouse. Biol Reprod. 66(4): 966-975.
- Oatley JM, de Avila DM, Reeves JJ, McLean DJ (2004) Spermatogenesis and germ cell transgene expression in xenografted bovine testicular tissue. Biol Reprod 71: 494–501.
- Ogawa T, Arechaga JM, Avarbock MR, Brinster RL (1997) Transplantation of testis germinal cells into mouse seminiferous tubules. Int J Dev Biol 41(1): 111-122.

- Orth JM, Gunsalus GL , Lamperti AA (1988) Evidence from Sertoli cell-depleted rats indicates that spermatid number in adults depends on numbers of Sertoli cells produced during perinatal development. Endocrinology 122: 787–794.
- Orwig KE, Schlatt S (2005) Cryopreservation and transplantation of spermatogonia and testicular tissue for preservation of male fertility. J Natl Cancer Inst Monogr 34: 51-56.
- Palmer JF (1837) The Works of John Hunter, F.R.S., with notes, vol. III. Longman, Rees, Orme, Brown, Green and Longman, London.
- Papadopoulos V (2004) In search of the function of the peripheral type benzodiazepine receptor. Endocr Res 30(4): 677–684.
- Paris MCJ, Snow M, Cox S, Shaw JM (2004) Xenotransplantation: a tool for reproductive biology and animal conservation? Theriogenology 61: 277–291.
- Park SY, Jameson JL (2005) Minireview: Transcriptional regulation of gonadal development and differentiation. Endocrinology 146:1035–1042.
- Payne AH, Hales DB (2004) Overview of steroidogenic enzymes in the pathway from cholesterol to active steroid hormones. Endocr Rev 25(6): 947–970.
- Payne AH, Youngblood GL (1995) Regulation of expression of steroidogenic enzymes in Leydig cells. Biol Reprod 52(2): 217-25.
- Pelletier J, Schalling M, Buckler AJ, Rogers A, Haber DA, Housman D (1991) Expression of the Wilms' tumor gene WT1 in the murine urogenital system. Genes Dev 5(8): 1345-1356.
- Rathi R, Honaramooz A, Zeng W, Turner R, Dobrinski I (2006) Germ cell development in equine testis tissue xenografted into mice. Reproduction 131: 1–9.
- Rouiller V, Gangnerau MN, Vayssiere JL, Picon R (1990) Cholesterol side chain cleavage activity in rat fetal gonads: a limiting step for ovarian steroidogenesis. Mol Cell Endocrinol 72: 111-120.
- Russell LD (1990) Form, dimensions, and cytology of mammalian Sertoli cells. In: Russell LD, Griswold MD (eds), The Sertoli Cell. Clearwater, FL: Cache River Press 1-37.
- Russel LD, Amlani SR, Vogl AW, Weber JE (1987) Characterisation of filaments within Leydig cells of the rat testis. Am J Anat 178: 231-240.
- Russell LD, Ettlin RA, Sinha Hikim AP, Clegg ED (1990) Histological and Histopathological Evaluation of the Testis. Clearwater, FL: Cache River Press.

- Russell LD, Warren J, Debeljuk L, Richardson LL, Mahar PL, Waymire KG, Amy SP, Ross AJ, MacGregor GR (2001) Spermatogenesis in Bclw-deficient mice. Biol Reprod 65: 318–332.
- Saen VD, Goossens E, Bourgain C, Ferster A, Tournaye H (2011) Meiotic activity in orthotopic xenografts derived from human postpubertal testicular tissue. Hum Reprod 26(2): 282-293.
- Schlatt S, Foppiani L, Rolf C, Weinbauer GF, Nieschlag E (2002a) Germ cell transplantation into X-irradiated monkey testes. Hum Reprod 17: 55–62.
- Schlatt S, Honaramooz A, Ehmcke J, Goebell PJ, Rubben H, Dhir R, Dobrinski I, Patrizio P (2006) Limited survival of adult human testicular tissue as ectopic xenograft. Hum Reprod 21: 384–389.
- Schlatt S, Kim SS, Gosden R (2002b) Spermatogenesis and steroidogenesis in mouse, hamster and monkey testicular tissue after cryopreservation and heterotopic grafting to castrated hosts. Reproduction 124(3): 339-346.
- Schlatt S, Westernstroer B, Gassei K, Ehmcke J (2010) Donor-host involvement in immature rat testis xenografting into nude mouse hosts. Biol Reprod 82(5): 888-895.
- Schmahl J, Rizzolo K, Soriano P (2008) The PDGF signaling pathway controls multiple steroid-producing lineages. Gene Dev 22: 3255–3267.
- Shan LX, Hardy MP (1992) Developmental changes in levels of luteinizing hormone receptor and androgen receptor in rat Leydig cells. Endocrinology 131(3):1107-1114.
- Sharpe RM, McKinnell C, Kivlin C, Fisher JS (2003) Proliferation and functional maturation of Sertoli cells, and their relevance to disorders of testis function in adulthood.Reproduction 125: 769-784.
- Sharpe RM, Maddocks S, Kerr JB (1990) Cell-cell interactions in the control of spermatogenesis as studied using Leydig cell destruction and testosterone replacement. Am J Anat 188(1): 3-20.
- Shinohara T, Inoue K, Ogonuki N, Kanatsu-Shinohara M, Miki H, Nakata K, Kurome M, Nagashima H, Toyokuni S, Kogishi K, Honjo T, Ogura A (2002) Birth of offspring following transplantation of cryopreserved immature testicular pieces and *in-vitro* microinsemination. Hum Reprod 17(12): 3039-3045.

- Silberstein GB, Van Horn K, Strickland P, Roberts CT Jr, Daniel CW (1997) Altered expression of the WT1 wilms tumor suppressor gene in human breast cancer. Proc Natl Acad Sci U S A. 94(15): 8132-8137.
- Simard J, Ricketts ML, Gingras S, Soucy P, Feltus FA, Melner MH (2005) Molecular biology of the 3beta-hydroxysteroid dehydrogenase/Δ_5-Δ_4 isomerase gene family. Endocr Rev. 26(4): 525-582.
- Skakkebaek NE, Jensen G, Povlsen CO, Rygaard J (1974) Heterotransplantation of human foetal testicular and ovarian tissue to the mouse mutant Nude. Acta Obstet Gynecol Scand 53: 73–75.
- Snedaker AK, Honaramooz, A, Dobrinski, I (2004) A game of cat and mouse: Xenografting of testis tissue from domestic kittens results in complete cat spermatogenesis in a mouse host. J Androl 25(6): 926-930.
- Song Y, Cheng KM, Silversides FM (2010) Formation of testicular structure under skin after auto-transplantation of dispersed testicular cells in the chicken. Anim Reprod Sci 120: 125-128.
- Sridharan S, Simon L, Meling DD, Cyr DG, Gutstein DE, Fishman GI, Guillou F, Cooke PS (2007) Proliferation of adult sertoli cells following conditional knockout of the Gap junctional protein GJA1 (connexin 43) in mice. Biol Reprod. 76(5): 804-812.
- Stocco DM (2000) The role of the StAR protein in steroidogenesis: challenges for the future. J of Endocrinol 164 (3): 247–253.
- Stocco DM, Clark BJ (1996) Regulation of the acute production of steroids in steroidogenic cells. Endocr Rev 17(3): 221–244.
- Tanaka SS, Toyooka Y, Akasu R, Katoh-Fukui Y, Nakahara Y, Suzuki R, Yokoyama M, Noce T (2000) The mouse homolog of Drosophila Vasa is required for the development of male germ cells. Genes Dev 14(7): 841-853.
- Teerds KJ (1996) Regeneration of Leydig cells after EDS – a model for postnatal Leydig cell renewal. In The Leydig cell, pp 203–220. Eds LD Russell, AH Payne & MP Hardy. Vienna, IL: Cache River Press.
- Teerds KJ, de Boer-Brouwer M, Dorrington JH, Balvers M, Ivell R (1999) Identification of markers for precursor and Leydig cell differentiation in the adult rat testis following ethane dimethyl sulphonate administration. Biol of Reprod 60: 1437–1445.

- Teerds KJ, Rijntjes E, Veldhuizen-Tsoerkan MB, Rommerts FF, de Boer-Brouwer M (2007) The development of rat Leydig cell progenitors in vitro: how essential is luteinising hormone? J Endocrinol 194(3): 579-593.
- Tena-Sempere M, Rannikko A, Kero J, Zhang FP, Huhtaniemi IT (1997) Molecular mechanisms of reappearance of luteinizing hormone receptor expression and function in rat testis after selective Leydig cell destruction by ethylene dimethane sulfonate. Endocrinology 138: 3340–3348.
- Thuillier R, Wang Y, Culty M (2003) Prenatal exposure to estrogenic compounds alters the expression pattern of platelet-derived growth factor receptors alpha and beta in neonatal rat testis: identification of gonocytes as targets of estrogen exposure. Biol Reprod 68(3): 867-880.
- Toyooka Y, Tsunekawa N, Takahashi Y, Matsui Y, Satoh M, Noce T (2000) Expression and intracellular localization of mouse Vasa-homologue protein during germ cell development. Mech Dev 93: 139–149.
- Turner CD (1938) Intra-ocular homotransplantations of prepubertal testes in the rat. Am J Anat 63: 101-159.
- Van Vorstenbosch CJ, Colenbrander B, Wensing CJ, Ramaekers FC, Vooijs GP (1984) Cytoplasmic filaments in fetal and neonatal pig testis. Eur J Cell Biol 34: 292-299.
- Vergouwen RPFA, Huiskamp R, Bas RJ, Roepers-Gajadien HL, Davids JAG, de Rooij DG (1993) Postnatal development of testicular populations in mice. J Reprod Fertil 99: 479–485.
- Walker WH, Cheng J (2005) FSH and testosterone signaling in Sertoli cells. Reproduction 130(1): 15-28.
- Walsh EL, Cuyler WK, McCullough DR (1934) The physiologic maintenance of the male sex glands. Am J Physiol 107: 508–512.
- Wilker CE, Ing NH, Welsh TH Jr, Johnson L (1995) Syngeneic testicular grafts produce testosterone and express Sertoli cell genes characteristic of intact rats. J Androl 16 (56).
- Wilker CE, Johnson L (1995) Effects of donor age, host weight, and ear location on size of syngeneic testicular grafts. J Androl 16(59).
- Wilson JD (2001) Prospects for research for disorders of the endocrine system. J Am Med Assoc 285: 624-627.

- Wing TY, Ewing LL, Zirkin BR (1984) Effects of luteinizing hormone withdrawal on Leydig cell smooth endoplasmic reticulum and steroidogenic reactions which convert pregnenolone to testosterone. Endocrinology 115: 2290–2296.
- Wistuba J, Luetjens CM, Wesselmann R, Nieschlag E, Simoni M, Schlatt S (2006) Meiosis in autologous ectopic transplants of immature testicular tissue grafted to *Callithrix jacchus* Biol Reprod 74(4): 706-13.
- Ye L, Su ZJ, Ge RS (2011) Inhibitors of Testosterone Biosynthetic and Metabolic Activation Enzymes. *Molecules 16*(12): 9983-10001.
- Youngblood GL, Nesbitt MN, Payne AH (1989) The structural genes encoding P450scc and P450arom are closely linked on mouse chromosome 9. Endocrinology 125: 2784–2786.
- Zhang FP, Poutanen M, Wilbertz J, Huhtaniemi I (2001) Normal prenatal but arrested postnatal sexual development of luteinizing hormone receptor knockout (LuRKO) mice. Mol Endocrinol 15:172–183.