SMALL NUCLEAR RING FINGER PROTEIN SNURF/RNF4 IN GONAD DEVELOPMENT AND TESTICULAR TUMORS

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To Family

CONTENTS

ABSTRACT

Steroid hormones, estrogens, androgens, progestins, glucocorticoids, and mineralocorticoids, regulate a variety of biological functions related to development, cell proliferation, differentiation, reproduction, and metabolism. The effects of the steroid hormones are mediated by steroid receptors, trans-acting transcription factors, which bind to regulatory sequences on target genes upon ligand binding, and either stimulate or repress gene expression. During recent years, a group of coregulatory proteins modulating steroid receptor responses in target gene regulation has been described. In gene inactivation experiments, some of the coregulatory proteins have been found to play essential roles in steroid receptor-mediated signaling, fertility, and fetal development. Small nuclear RING finger protein SNURF/RNF4, originally identified by its interaction with androgen receptor (AR), interacts with several transcription factors, such as TATA-binding protein (TBP) and promoter specificity protein 1 (Sp1). SNURF possesses a C-terminal RING finger domain, a motif typical of many E3 ubiquitin ligases and tumor suppressor proteins.

Despite several *in vitro* interaction and expression studies reported during recent years, the biological function of SNURF has remained largely unknown. However, the expression of SNURF to high levels in testis suggests a role in reproduction and fertility. To gain insight into the role of SNURF in reproduction and testicular cancer, the expression of SNURF mRNA and protein was investigated during murine prenatal gonad development, in rodent postnatal folliculogenesis and spermatogenesis, and in human normal testis and testicular germ cell tumors. In murine fetal gonads, the expression of SNURF mRNA was detected from E10.5 dpc onwards in both sexes. SNURF mRNA was expressed to high levels in round and elongating spermatids in postnatal human and rat testis, and in oocytes of preantral follicles in postnatal murine ovary, respectively. Transcription from the murine SNURF promoter was governed by a single promoter, which showed very strong basal activity in reporter gene assays in mammalian cells. The proximal GC box at +9 nt of the SNURF promoter was identified as the main element governing transcription from the murine SNURF gene, and the Wilms' tumor 1 (WT1) gene product was found as one of the potential activators of the murine SNURF promoter. Furthermore, the expression of SNURF mRNA was regulated by testosterone *in vivo* in rat testis and by gonadotropins and estrogen *in vivo* in rodent ovary. SNURF protein colocalized with estrogen receptor β (ERβ) protein in fetal and postnatal male germ cells. Down-regulated expression of SNURF and ERβ mRNA and protein was detected in human testicular germ cell tumors, suggesting a role for SNURF and estrogen signaling in the pathogenesis of testicular germ cell cancer.

ORIGINAL PUBLICATIONS

This thesis is based on the following original articles, referred to in the text by their Roman numerals:

I Hirvonen SJ, Santti HH, Jänne OA, and Palvimo JJ (2002) GC-rich elements flanking the transcription start site govern strong activation on the SNURF gene. *Biochem Biophys Res Commun* **291**: 897-902.

II Yan W, Hirvonen-Santti SJ, Toppari J, Palvimo JJ, and Jänne OA (2002) Expression of nuclear RING finger protein SNURF/RNF4 during rat testis development suggests a role in spermatid maturation. *Mech Dev* **118**: 247-253.

III Hirvonen-Santti SJ, Rannikko A, Santti H, Savolainen S, Nyberg M, Jänne OA, and Palvimo JJ (2003) Down-regulation of estrogen receptor β and transcriptional coregulator SNURF/RNF4 in testicular germ cell cancer. *Eur Urol, in press.*

IV Hirvonen-Santti SJ, Sriraman V, Anttonen M, Savolainen S, Palvimo JJ, Heikinheimo M, Richards JS, and Jänne OA (2003) Nuclear RING finger protein SNURF/RNF4 expression during gonad development: regulation by gonadotropins and estrogen in postnatal ovary. *Endocrinology, submitted, in revision.*

In addition, some unpublished data are presented.

ABBREVIATIONS

AF	activation function
AP-1	activating protein-1
AR	androgen receptor
CBP	CREB (cyclic AMP-responsive element -binding protein) -binding protein
CREM	cyclic AMP-responsive element modulator
DBD	DNA-binding domain
DES	diethylstilbestrol
dpc	days post coitum
DRIP	vitamin D receptor-interacting protein
E2	estradiol
EDS	ethylene dimethane sulphonate
EMSA	electrophoretic mobility shift assay
ER	estrogen receptor
FSH	follicle-stimulating hormone
GR	glucocorticoid receptor
HAT	histone acetyltransferase
hCG	human chorionic gonadotropin
HDAC	histone deacetylase
HRE	hormone response element
LBD	ligand-binding domain
LH	luteinizing hormone
MAA	methoxyacetic acid
N-CoR	nuclear receptor corepressor
NF-κB	nuclear factor κB
NLS	nuclear localization signal
Nrip1	Nuclear receptor -interacting protein 1
PGC	primordial germ cell
PIAS	protein inhibitor of activated STAT
PMSG	gonadotropin from pregnant mare serum
PPAR	peroxisome proliferator-activated receptor
PR	progesterone receptor
RNF4	ring finger protein 4
RT-PCR	reverse transcription polymerase chain reaction
SMRT	silencing mediator for retinoic acid receptor and thyroid receptor
SNURF	small nuclear RING finger protein
Sp1	promoter specificity protein 1
SRC	steroid receptor coactivator
SUMO	small ubiquitin-like modifier
T	testosterone
TBP	TATA-binding protein
TGC	testicular germ cell cancer
TRAP	thyroid receptor-associated protein
WT1	Wilms' tumor 1 gene product

REVIEW OF THE LITERATURE

Introduction

During fertilization, the fusion of sperm and egg, the specific combinations of genes encoded in the parental chromosomes are brought together to give life to an offspring in mammals. The female egg and male sperm originate from primordial germ cells (PGC) and differentiate into oocytes and gonocytes, respectively, prenatally. The germ cells gain full maturity during postnatal development in response to hormone signals and gonadal transcription factors. Disturbances during gonad development are likely to disrupt fertility and predispose to gonadal tumorigenesis, and thus better understanding of the mechanisms underlying gonadal development is mandatory for management of infertility and gonadal cancer. In this review, fetal and postnatal gonad development, testicular tumors, male infertility, steroid receptor signaling mechanisms, and coregulators are discussed.

Sex Determination and Fetal Gonad Development

Sex determination in mammals is a complex process, which is usually divided to determination of the gonad and its differentiation. During the first phase of gonad development, an indifferent bipotential primordium is established in both female and male mice from the thickening of coelomic epithelium on the ventrolateral surface of the mesonephros between embryonic day 10.5 and 11.5 (E10.5-11.5) dpc (for review, Capel 2000, Koopman *et al*. 2001, McLaren 2000, Swain and Lovell-Badge 1999, Veitia *et al*. 2001). The genital ridge is composed of the somatic cells derived from the mesonephros and proliferating primordial germ cells (PGC), which have migrated via the hindgut and mesonephros from the extraembryonic mesoderm at the base of the allantois. Expression of Wilms' tumor 1 (WT1) and steroidogenic factor-1 (SF1) begins in the bipotential ridge by E10.0 dpc. SF1 is an orphan nuclear receptor that regulates genes involved in steroid synthesis in both the gonad and the adrenal gland. Impairment of WT1 and SF1 genes resulted in complete regression of the initially formed gonadal ridges by E14.5 dpc and E12.5 dpc, respectively (Kreidberg *et al*. 1993, Luo *et al*. 1994). The gonad initially develops in a non-sex-specific manner, being morphologically identical in XX and XY embryos up until E12.0 dpc.

At E10.5-11.0 dpc, Sry, the sex determining gene on Y chromosome, begins to be expressed in the male genital ridge, and it guides the initial testis determination by

triggering differentiation of pre-Sertoli cells (Clarkson and Harley 2002, Koopman *et al*. 2001). In addition, Sry-related Sox9 (Sry-like HMG-box protein 9) is expressed in the male gonad from E11.5 dpc onwards persisting there throughout the life, while it is not seen in the ovary (Clarkson and Harley 2002). SF1 and Sox9 activate the expression of the anti-müllerian hormone gene (AMH, also called Müllerian inhibiting substance, MIS), which induces the regression of the female urogenital tract forming Müllerian ducts in males (de Santa Barbara *et al*. 1998, Shen *et al*. 1994) (Fig. 1). Transgenic experiments where XX mice carrying the Sry and Sox9 genes develop as males have established the necessity of these factors in testis determination (Koopman *et al*. 1991, Vidal *et al*. 2001). By E12.5 dpc, the Sertoli cells have encircled germ cells and formed testis cords, surrounded by peritubular myoid cells. Other somatic cells in the developing testis are Leydig cells, which produce testosterone, critical for the development of Wolffian ducts to male urogenital tract and the formation of all male secondary sexual characteristics (Fig. 1, Table 1).

At E13.5 dpc, oogonia in the murine ovary enter the meiotic prophase arresting at diplotene stage, while the majority of the germ cells within the testicular cords undergo mitotic arrest. Dax1, an orphan nuclear receptor, is expressed concomitantly with Sry in both sexes, but it is down-regulated in the testis and stays in the ovary during further prenatal gonad development (Swain *et al*. 1996). Dax1 has been thought to act antagonistically to Sry in a dosage-sensitive manner, since duplication of the Xp21 locus bearing the Dax1 gene resulted in XY sex reversal (Swain *et al.* 1998). However, murine Dax1 was essential for normal spermatogenesis in later life (Jeffs *et al*. 2001). In addition, Wnt-4 factor was involved in the suppression of Leydig cell formation in the ovary (Vainio *et al*. 1999). Wnt-4 null mutant female mice were masculinized (no Müllerian ducts and persistence of Wolffian structures), while the male phenotype was normal (Vainio *et al*. 1999).

Table 1: Timeline for murine gonad development

dpc	
E9.5-E11	PGCs migrate from yolk sac to posterior body wall, where they induce formation of genital ridges.
E11.0	Cells from the coelomic epithelium of mesonephros proliferate.
E12.5	Sertoli cells encircle the germ cells and form testicular cords.
E13.5	Testicular morphology recognizable. Female oogonia enter I meiosis, while male germ cells enter mitotic arrest. Development of external genitalia begins.

Modified from Rey R and Picard JY, *Baillieres Clin Endocrinol Metab*, 1998.

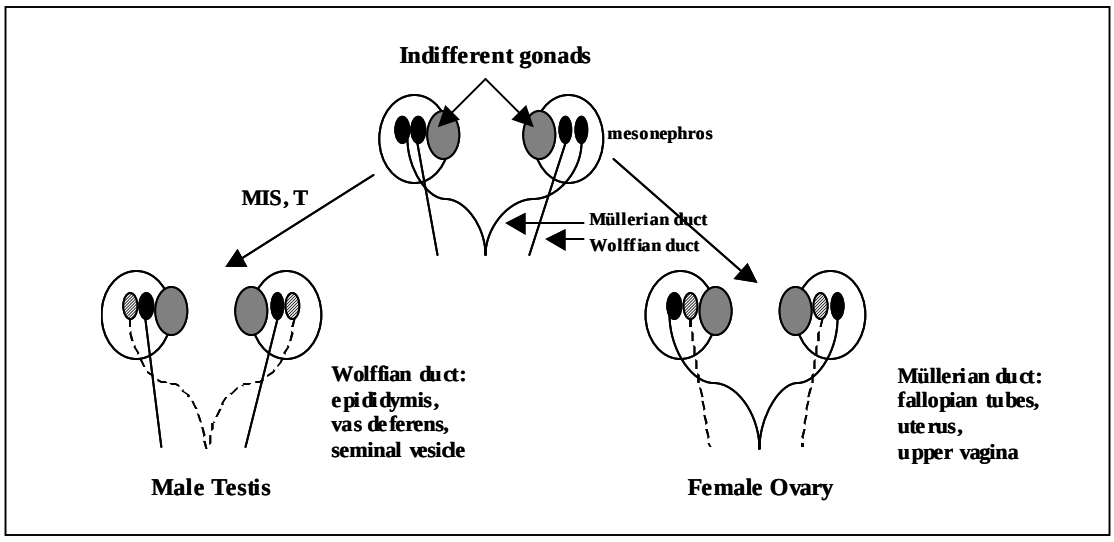

Fig. 1 Development of the male and female internal genitalia from bipotential gonadal primordial.
Pre-Sertoli cells and Leydig cells in the fetal testis produce Müllerian inhibiting substance (MIS) and testosterone (T), respectively. MIS induces the regression of Müllerian ducts in the male and T stimulates the development of the male urogenital tract from the Wolffian ducts and the formation of external genitalia. Modified from Rey and Picard, *Baillieres Clin Endocrinol Metab*, 1998.

Postnatal Gonad Development

Ovarian folliculogenesis

During folliculogenesis, the primordial follicles consisting of an oocyte and a single layer of squamous granulosa cells develop into large preovulatory antral follicles, which contain several layers of columnar granulosa cells surrounding the oocytes (for review, Epifano and Dean 2002, Matzuk *et al*. 2002, Richards 2001) (Fig. 2). Growth of the primordial follicles to primary follicles requires the Kit ligand produced by the granulosa cells and c-kit receptor expressed in the egg as well as growth and differentiation factor 9 (GDF-9), an oocyte-specific member of TGFβ growth factor family (Elvin *et al*. 1999). GDF-9 null mice exhibited failed organization of theca cells, abnormal oocyte growth, and arrested follicle maturation to the primary follicle stage (Elvin *et al*. 1999, Richards 2001).

The initiation of follicle growth occurs independently of pituitary gonadotropins, but follicle-stimulating hormone (FSH) is mandatory for the final growth of the preovulatory follicles. FSH promotes granulosa cell proliferation and stimulates expression of CYP450 aromatase in the granulosa cells (Richards 2001). The granulosa cells are not only the site

of action of FSH in the control of follicular growth, but they also produce estrogens by conversion from androgens diffused from the neighboring theca cells (Fig. 4). The granulosa cells also provide nutrients and chemical messengers critical for the oocyte maturation, and the oocyte itself promotes granulosa cell proliferation and differentiation (Matzuk *et al*. 2002). Luteinizing hormone (LH) enhances theca cell differentiation and androgen production. The oocytes have entered the first meiosis already prenatally and have arrested at diplotene of the first meiotic division. LH surge induced by the increasing plasma estrogen levels triggers the completion of the first meiotic division, terminates the follicular growth by turning on genes, such as progesterone receptor (PR) and prostaglandin synthase-2, controlling ovulation and luteinization (Richards 2001) (Fig. 4).

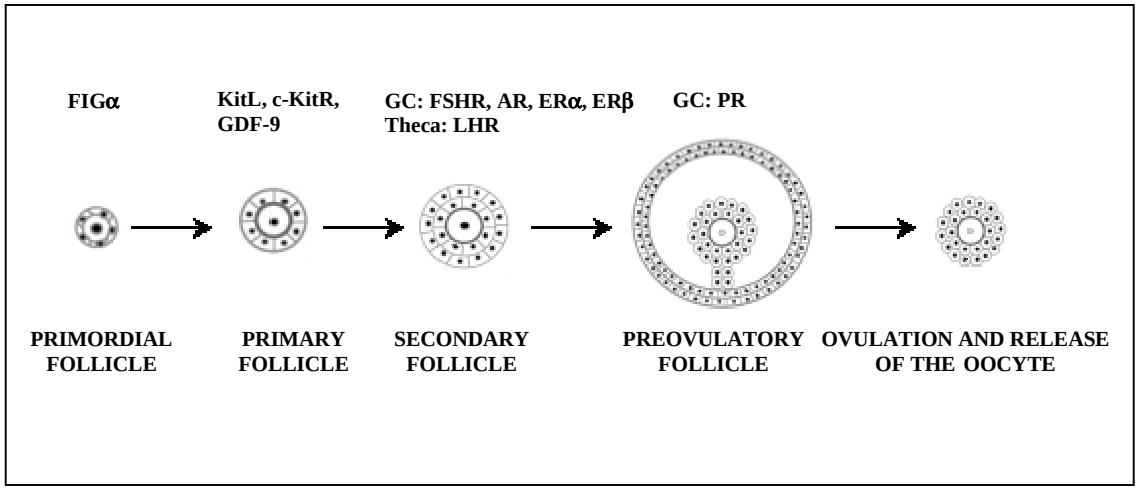

Fig. 2 Ovarian folliculogenesis. Communication between the oocyte and the surrounding granulosa cells is established perinatally in primordial follicles, where a single layer of squamous granulosa cells is gathered around the egg. Along with the progression of folliculogenesis, granulosa cells proliferate and differentiate in response to FSH to form several layers of columnar cells around the oocyte in preovulatory follicles. Oocyte-granulosa cell communication is mediated by secreted paracrine factors and gap junctions. After the LH surge, the first meiosis is completed, and the follicle wall is ruptured resulting in the release of the oocyte. The second meiotic division is completed only after fertilization. Factors critical for each follicular development stage are indicated. AR; androgen receptor, c-kitR; c-kit receptor, ER; estrogen receptor, FIGα; factor in the germline α, FSHR; follicle-stimulating hormone receptor, GC; granulosa cell, GDF; growth and differentiation factor, KitL; Kit ligand, LHR; luteinizing hormone receptor, PR; progesterone receptor, Theca; Theca cell. Modified from Matzuk *et al. Science* 2002.

Spermatogenesis

Spermatogenesis is a complex multi-step process, in which diploid spermatogonia differentiate into mature haploid spermatozoa in the epithelium of the seminiferous tubules during the postnatal testis development (for review, Grootegoed *et al*. 2000, Hecht 1998). Spermatogenesis is often divided into proliferation, meiotic, and spermatogenic phases (Russell *et al.* 1990). In the proliferative phase, most spermatogonia, which have arisen from the PGCs and gonocytes, located close to the basement membrane of the tubule, enter mitosis to give rise to a reserve of undifferentiated stem cells, and only a minority of spermatogonia become diploid preleptotene spermatocytes. During the meiotic phase, the diploid preleptotene cells pass through two meiotic divisions to produce haploid round spermatids. The long prophase of the first meiotic division lasting approximately three weeks in rodent testis, is initiated at leptotene spermatocytes and is subsequently followed by zygotene phase, in which homologous sister chromatids are paired and a synaptonemal complex is formed. In the ensuing pachytene spermatocytes, genetic material is recombined (crossing-over), and in diplotene spermatocytes it is segregated to sister chromatids. During the meiotic cell divisions, the germ cells remain connected by cytoplasmic bridges, and the syncytia move towards the tubular lumen. In differentiation or spermiogenic phase, the round spermatids undergo a series of molecular and morphological changes to generate mature spermatozoa. Changes in the composition of chromatin resulting in the formation of the spermatid head, acrosome development, and flagellum formation, take place during spermiogenesis to allow transmission of the paternal chromosomes to the oocyte at fertilization (Fig. 3).

Germ cell development takes place in close association with the somatic Sertoli cells of the seminiferous epithelium (Fig. 3). Sertoli cell-germ cell interaction provides physical support, tight junctions, growth factors, and nutrients critical for germ cell development (for review, Griswold 1995, Griswold 1998). Importantly, the Sertoli cells form blood-testis barrier, which allows them to create a microenvironment in the tubular compartment, in which meiotic divisions and postmeiotic modifications take place. The associations between the Sertoli cells and the germ cells at different phases of maturation are referred to as stages and comprise the cycle of the seminiferous tubule (Leblond and Clermont 1952). Groups of spermatogonia enter the spermatogenic process at regular intervals, and the cycle is defined as a time interval between the appearance of germ cells at the same stage at the defined point of the tubule. In mice and men, spermatogenesis lasts for 35 and 70 days, respectively.

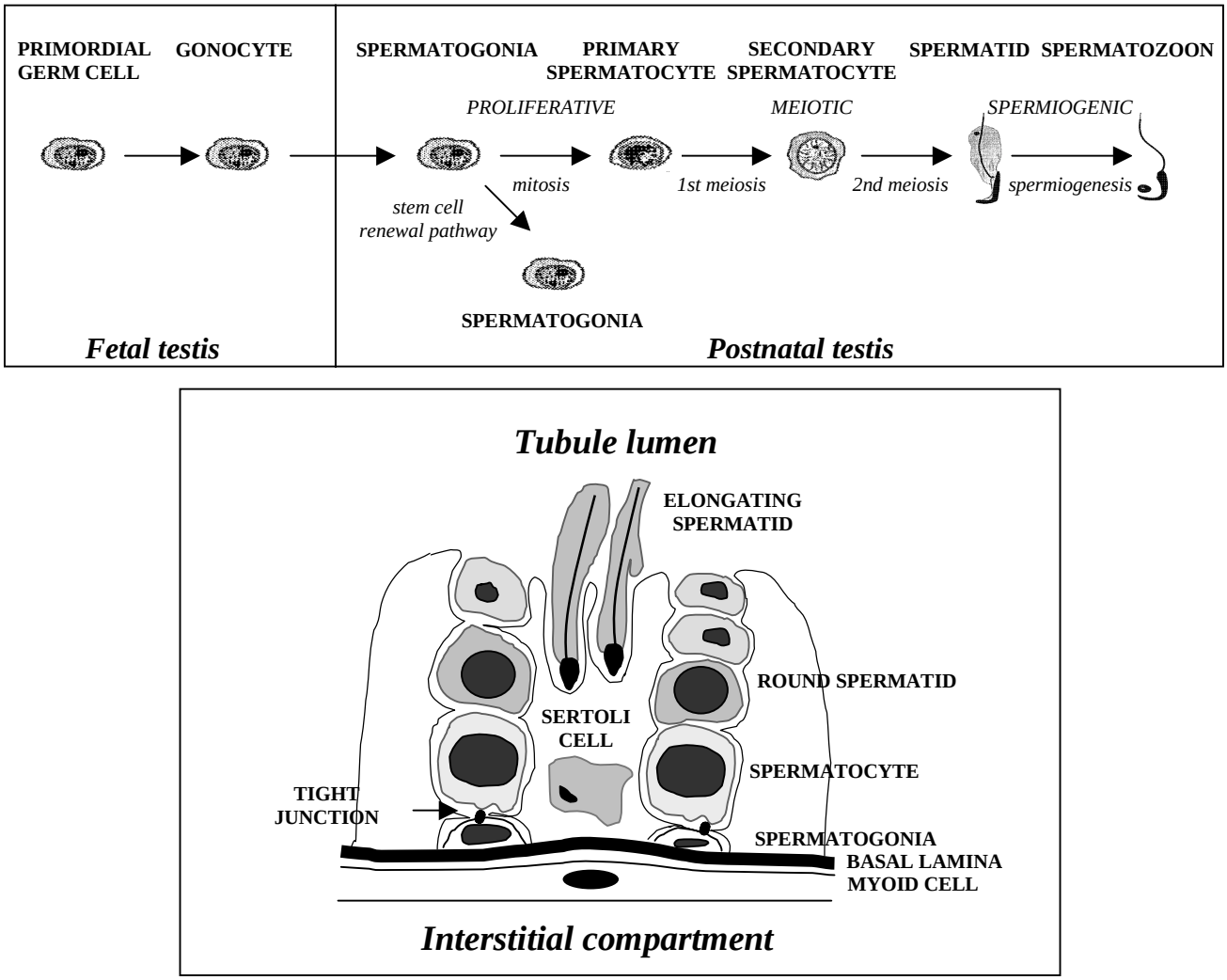

Fig. 3 Top: The Flow of Spermatogenesis. During the fetal period, primordial germ cells (PGC) differentiate into gonocytes, which give rise to spermatogonia right after birth. Spermatogonia proliferate to maintain a pool of undifferentiated germ cells or, more rarely, give rise to preleptotene primary spermatocytes. The primary spermatocytes proceed via the first and second meiotic divisions to become secondary spermatocytes and round spermatids, respectively. During spermiogenesis, chromatin is compacted, and acrosome and flagellum develop to generate mature haploid spermatozoa. Modified from Sassone-Corsi, *Cell,* 1997.

Bottom: Organization of the developing germ cells and Sertoli cells in the seminiferous tubules in testis. Spermatogonia, spermatocytes, and round spermatids are seen in successively higher levels within the seminiferous epithelium. Sertoli cells and spermatogonia contact the basement membrane. Sertoli cells reach from the base of the tubule to the lumen and encircle the developing germ cells. Tight junctions, gap junctions, and desmosomes connect Sertoli cells to each other to create the blood-testis barrier and a microenvironment for germ cell maturation. Modified from Russell *et al.*, 1990.

After the completion of germ cell development, the mature spermatozoa are released to the tubular lumen and proceed to the epididymis for capacitation, a process of biochemical changes for the development of sperm motility and capablity of fertilization (Grootegoed *et al.* 2000). Differentiation of germ cells is governed by the cyclic hormonal stimulation by the hypothalamic-pituitary axis, which secretes two anterior pituitary hormones LH and FSH (McLachlan *et al.* 1996, Zirkin 1998) (Fig.4). In the testis, LH and FSH receptors are found only in the Leydig and Sertoli cells, respectively, and their actions are mediated from these cells to developing germ cells by paracrine signaling. Leydig cells produce testosterone (T) in response to LH. T then diffuses into the seminiferous tubules and gives rise to high tubular testosterone concentration important for germ cell maturation. T and FSH induce Sertoli cells to secrete glycoproteins, proteases, and other paracrine substances essential for differentiating germ cells (Griswold 1995, Griswold 1998, Zirkin 1998). FSH is required for the neonatal Sertoli cells to divide. However, FSH-deficient mice are fertile (Kumar *et al.* 1997, Meacham *et al.* 1996, Singh and Handelsman 1996). Likewise, FSH receptor-deficient male mice are fertile but produce poor quality sperm (Krishnamurthy *et al.* 2000). Moreover, men homozygous for an inactivating mutation of the FSH receptor gene show spermatogenic failure (Tapanainen *et al.* 1997). The Sertoli cells have the capacity to support only a limited number of maturing germ cells, and apoptosis plays an important role in the regulation of sperm numbers and elimination of defective gametes. Both T and FSH suppress germ cell apoptotic death and enhance germ cell survival (Billig *et al.* 1995, Erkkilä *et al.* 1997, Tapanainen *et al.* 1993).

Estrogens are synthesized from androgens by action of CYP450 aromatase in Leydig cells, Sertoli cells, and germ cells resulting in high estrogen levels in both the interstitial tissue and tubular fluid (Carreau and Levallet 1997, Genissel *et al.* 2001, Janulis *et al.* 1998, Nitta *et al.* 1993, O'Donnell *et al.* 2001). Important actions of testosterone on developing germ cells are mediated via Sertoli cells. The male germ cells lack androgen receptor (AR) and estrogen receptor α (ERα), but estrogen receptor β (ERβ) is detected in male gametes (Carreau 2001, Enmark *et al.* 1997, Iwamura *et al.* 1994, Suarez-Quain *et al.* 1999, van Roijen *et al.* 1995). However, βERKO male mice with disrupted ERβ gene are fertile and possess normal sperm counts (Couse *et al.* 1999, Krege *et al.* 1998). In αERKO male mice, reduced fertilization capability of spermatozoa, dilatation of efferent ductules, and defects in fluid reabsorption hindering the entry of sperm to epididymis, result in infertility (Eddy *et al.* 1996, Hess *et al.* 1997, Hess *et al.* 2000, Lee *et al.* 2000). The phenotype of αβERKO male mice resembles that of αERKO mice (Couse *et al.* 1999, Dupont *et al.* 2000). Interestingly, ovaries of adult αβERKO females manifest postnatal sex reversal and

exhibit follicle transdifferentiation to structures resembling seminiferous tubules of the testis including Sertoli cell-like cells (Couse *et al.* 1999). Estradiol (E2) influences the negative feedback to the hypothalamus and the pituitary via conversion from androgens, and it controls the secretion of LH and FSH (Lindzey *et al.* 1998, O'Donnell *et al.* 2001). Thus, absence or inappropriate exposure to estrogens could disturb the balance of the hypothalamic-pituitary axis. In addition, estrogens play a role in testicular descent, the hormone-regulated migration of testes from the abdominal wall to scrotum, by regulating fetal Leydig cell gene expression (Hutson *et al.* 1997, Nef and Parada 2000, O'Donnell *et al.* 2001).

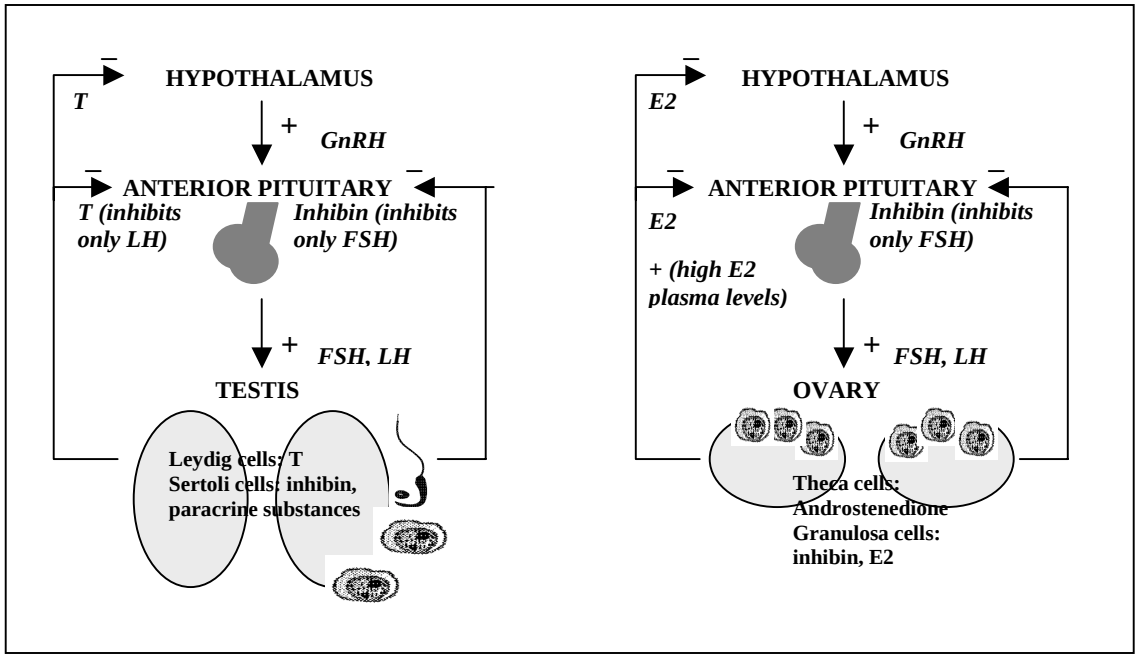

Fig. 4 Hypothalamic-pituitary-gonadal axis in the regulation of gonad function. Testis: Inhibin, a protein hormone secreted by the Sertoli cells, feeds back to the anterior pituitary and mainly inhibits secretion of FSH. Secretion of LH and gonadotropin-releasing hormone (GnRH) is inhibited mainly by testosterone (T) secreted from the Leydig cells. T, acting locally, is essential for spermatogenesis. **Ovary:** Estradiol (E2), in low plasma concentration during the early and middle follicular phase, feeds back negatively to the hypothalamus and the anterior pituitary to decrease secretion of GnRH and LH, respectively. Inhibin, secreted from the granulosa cells, acts on the pituitary to inhibit the secretion of FSH. Estrogen, in high plasma levels preceding ovulation, triggers the secretion of LH surge from the pituitary (positive feed-back). High plasma concentration of progesterone during the luteal phase and pregnancy inhibits the hypothalamic neurons that secrete GnRH. Androgens produced in response to LH in theca cells diffuse into granulosa cells, in which they are converted to estrogens by CYP450 aromatase. Modified from Vander *et al*., Human Physiology, McGraw Hill, 2001.

Marked stage-specific gene regulation underlies the morphological changes of developing germ cells during spermatogenesis (for review, Sassone-Corsi 1997, Steger 1999). Various promoters display differential activity in male gametes due to the presence of testis-specific transcription factors (Foulkes *et al.* 1992). Some general transcription factors, such as TATA-binding protein (TBP), TFIIB, and RNA polymerase II are differentially regulated in germ cells and accumulate in postmeiotic round spermatids where high transcriptional activity takes place (Schmidt and Schibler 1995). In addition, many ubiquitously expressed genes generate testis-specific isoforms by the usage of alternative promoters, splicing, or polyadenylation (Daniel and Habener 1998, Moilanen *et al.* 1998b, Schmidt *et al.* 1997). The hypothalamic-pituitary axis controls the postmeiotic gene activation by enhancing accumulation of the CREM activator protein CREMτ in the male germ cells in response to FSH (Foulkes *et al.* 1993). CREMτ is a consensus cAMP response-element (CRE) binding protein, that is highly expressed from round spermatids onwards and regulates transcription from several postmeiotic genes, such as protamines, calspermin, and transition protein (Kistler *et al.* 1994, Sassone-Corsi 1998, Sun *et al.* 1995). In addition to CREMτ activator, the CREM gene encodes transcriptional repressors CREMα, β, and γ, which are expressed to low levels in premeiotic germ cells (Foulkes *et al.* 1992). Targeted disruption of the CREM gene results in complete arrest of the germ cell differentiation at the first step of spermiogenesis (Blendy *et al.* 1996, Nantel *et al.* 1996). Recently, male germ cell-specific transcriptional coregulators have also been identified, as exemplified by the LIM-only protein ACT, that enhances CREM-mediated transcription in the germ cells (Sassoni-Corsi 2002).

Transcription ceases several days before the completion of spermatogenesis because of the condensation of the haploid genome to a volume of approximately 5% of that of somatic cell nuclei (Ward and Coffey 1991, Ward 1993). In round spermatids, the histones and non-histone proteins are replaced by transition proteins, and in elongating spermatids, the transition proteins are removed and replaced by protamines, the principal basic nuclear proteins in spermatozoa (Dadoune 1995, Oliva and Dixon 1991, Wouters-Tyrou *et al.* 1998). Translational regulation plays a significant role in maturing spermatids: a number of proteins appear in the late stages of spermiogenesis and must be translated from mRNA in postmeiotic germ cells (for review, Steger 1999). Translational regulation is exemplified by direct repression of mRNA by RNA-binding proteins, such as 70-kDa poly-A binding protein (PABP), and activation by deadenylation of long poly-A tails (Gu *et al.* 1995, Venables and Eperon 1999).

Testicular Tumors and Male Infertility

Testicular germ cell cancer (TGC)

Testicular germ cell cancers (TGC) comprise a heterogenous group of neoplasms originating from cells belonging to the male germ cell lineage (Dearnaley *et al.* 2001). Seminoma recapitulates some aspects of spermatogenesis, and it is the most common type of TGC accounting for about half of the cases. Nonseminomas, including embryonal carcinoma, yolk sac tumor, teratoma, and teratocarcinoma, represent progeny of pluripotent embryonic cells (for review, Dearnaley *et al.* 2001) (Table 2). Although TGCs comprise only approximately 1% of all human malignancies, they are the most frequent cancers in young men between ages 20-40 (Chaganti and Houldsworth 2000). In Finland, approximately 70-90 testicular cancers are diagnosed annually (Black *et al.* 1997, Sundström *et al.* 2001).

Table 2: Classification of testicular cancer types.

Germ cell tumors		
Seminomas		50-60 %
	Classic	(82-85 %)
	Anaplastic	(5-10 %)
	Spermatocytic	(2-12 %)
Nonseminomas		
	Embryonal carcinoma	20-25 %
	Teratocarcinoma	25-30 %
	Teratoma	5-10 %
	Yolk sac tumor	<1%
Stromal tumors		
	Leydig cell tumor	1-3 %
	Sertoli cell tumor	<1%
	Mixed tumor	

Modified from Nurmi *et al*., Urologia, *Duodecim*, 2002.

Several risk factors for TGC development have been identified, including cryptorchidism, testicular dysgenesis, Klinefelter's syndrome, prior history to germ cell cancer, and a positive family history (Bosl and Motzer 1997, Chaganti and Houldsworth 2000, Forman *et al.* 1992). The onset of the disease at early adulthood suggests that the initiating event occurs prenatally, and endogenous or environmental estrogens affecting the embryonic testis have been suggested to participate in the pathogenesis of TGC (Henderson *et al.* 1979, Sharpe 2003, Weir *et al.* 2000). Gene amplification is a common mechanism of

increased gene expression in human cancers. The most common cytogenetic aberration in TGC is the presence of an isochromosome (duplication) of the short arm of chromosome 12 in up to 80% of all TGC cases (Chaganti and Houldsworth 2000). In accordance with the overpresentation of 12p, mRNA encoded by the cyclin D2 gene, located at 12p13, was over-expressed in 69% of the TGCs by a mean factor of 7.9 as judged by semi-quantitative RT-PCR assay (Schmidt *et al.* 2001). Cyclin D2 along with the CDK4 protein regulates phosphorylation of the RB tumor suppressor protein and controls the G1-S cell cycle checkpoint (Schmidt *et al.* 2001). In addition, specific gains and losses from several other chromosomal regions, e.g. overpresentation of 17q, have been reported in TGC (Skotheim *et al.* 2002). Furthermore, the first mouse model of classical testicular seminoma has been identified in transgenic mice over-expressing glial cell-derived neurotrophic factor (GDNF), a member of TGFβ superfamily expressed also in Sertoli cells (Meng *et al.* 2001).

The treatment of testicular tumors consists of radical orchiectomy (removal of the testis), and chemotherapy and/or radiotherapy depending on the tumor stage (Dearnaley *et al.* 2001, Nurmi *et al.* 2002). To date, cisplatin-containing chemotherapy has enabled cure rates >90-95% in lower-stage testicular malignancies (Dearnaley *et al.* 2001, Nurmi *et al.* 2002). However, liver, bone, or central nervous system metastases, high alpha-fetoprotein (>1000 ng/ml) or human chorionic gonadotropin (hCG>10 000 IU/l) marker levels, or the presence of primary mediastinal tumor indicate poorer prognosis with 70% 5-year survival rate among the Finnish testicular cancer patients (Aareleid *et al.* 1998, Mäenpää *et al.* 1996, Nurmi *et al.* 2002).

Male infertility

Approximately 10% of couples trying to conceive suffer from subfertility, with approximately one third of the cases deriving from male factor infertility. Potential causes of male infertility comprise varicocele, undescended testes, antibodies against spermatozoa, microdeletions in the Y-choromosomal AZF region, testicular and genital tract infections, chromosomal disorders such as Klinefelter's syndrome (47XXY), systemic disease, external factors (such as drugs), radiotherapy, chemotherapy, and insufficient or excess reproductive hormone production. However, most often the reason for the poor quality of semen remains undetermined (for review, Cahill and Wardle 2002, Ford 2001, Hirsh 2003, Khorram *et al.* 2001). Patients suffering from hypogonadotropic hypogonadism as a major cause of infertility benefit from medication (Khorram *et al.*

2001). Furthermore, introduction of intracytoplasmic sperm injection (ICSI) has dramatically improved the treatment of male factor infertility (Braude and Rowell 2003, Cahill and Wardle 2002, Ford 2001, Khorram *et al.* 2001).

Nuclear Receptors

Nuclear receptor superfamily

The actions of lipophilic hormones, including steroids, retinoids, vitamin D, and thyroid hormone, are mediated through the conserved family of nuclear receptors which function as ligand-regulated, DNA-binding transcription factors (for review, Aranda and Pascual 2001, Beato *et al.* 1996, Mangelsdorf *et al.* 1995, McKenna *et al.* 1999). Fourty-eight human genes encoding members of the nuclear receptor superfamily sharing extensive homology have been identified to date (Mangelsdorf *et al.* 1995, McKenna and O'Malley 2002b). Identification of genes with structural features similar to those found in nuclear receptors has lead to the discovery of orphan receptor members, such as PPAR, ERR, Nurr1, LXR, and PXR, of the family without prior knowledge of their putative ligands (Giguère 1999). Recently, ligands have been found for many orphan receptors: LXR binds oxysterols, FXR bile acids, PXR pregnanes, and PPAR several fatty acids and prostaglandins (Giguère 1999). This chapter focuses mainly on the steroid receptors, especially androgen receptor (AR) and estrogen receptors (ER).

Androgen receptor (AR)

Physiological roles of androgens

Androgen receptor (AR) mediates the responses of androgens that affect a male body in multiple ways. Androgens regulate the development of sex organs, the growth of facial, body, and pubic hair, the enlargement of vocal cords, the growth of the prostate, the production of sperm, and the development of muscle strength, and masculine behavior (Mooradian *et al.* 1987, Quigley *et al.* 1995). AR has two physiological ligands, testosterone and 5α-dihydrotestosterone (DHT), which is converted from testosterone by 5α-reductase in target cells (Mooradian *et al.* 1987). In certain cells, testosterone is converted to 17β-estradiol by CYP450 aromatase. After binding the androgen ligand, AR regulates the bodily functions by interacting with hormone response elements (HRE) located in the regulatory regions of the target genes. This leads to transcriptional activation

or repression of the regulated genes (Beato *et al.* 1996). Among genes activated by AR are those encoding rat probasin, mouse sex-limited protein (slp), human glandular kallikrein (KLK2), and prostate-specific antigen (PSA) (Adler *et al.* 1992, Murtha *et al.* 1992, Rennie *et al.* 1993, Riegman *et al.* 1991).

Functional domains of androgen receptor

Androgen receptor consists of an amino-terminal region (region A/B), a DNA-binding domain (DBD, region C), a hinge region (region D), and a ligand-binding domain (LBD, region E)(for review, Gelmann 2002). The DBD and LBD of AR share 77-80% and 50-55% homology, respectively, with GR, PR, and MR. The amino-terminal modulatory A/B domain of AR is the most variable in both size and sequence among the steroid receptors. The A/B domain harbors a ligand-independent transactivation function (AF-1), communicates with other parts of the receptor and interacts with several coregulator proteins, such as SRC-1 and CBP (Alen *et al.* 1999, Ikonen *et al.* 1997). The DBD of the steroid receptors is the most conserved region, and it confers ability to recognize specific target sequences and activate genes. The DBD of AR is composed of two zinc finger modules comprising some 70 amino acid residues and a carboxyl-terminal extension that spans approximately 25 residues. The second zinc finger participates in receptor dimerization and, along with the carboxyl-terminal region, is involved in HRE recognition (Freedman 1992, Gelmann 2002, Schoenmakers *et al.* 1999).

The D domain, which is less well conserved among the nuclear receptors, serves as a hinge between the DBD and the LBD, allowing rotation of the DBD. The hinge region contains the major part of the nuclear localization signal, which mediates the transfer of AR from the cell cytoplasm to its site of action in the nucleus (Jenster *et al.* 1993, Zhou *et al.* 1994). The LBD is the site of specific, high-affinity binding of the androgen ligand. Recently, the three-dimensional structure of the AR LBD has been determined (Matias *et al.* 2000, Sack *et al.* 2001). Like other steroid receptors, AR LBD is composed of 12 α-helices that form a ligand-binding pocket. After agonist binding, helix 12 is positioned over the pocket to enclose the ligand (Sack *et al.* 2001). The LBD of AR possesses the second activation function (AF-2) and interacts with coregulators (Ikonen *et al.* 1997). Deletion of AR LBD results in a constitutively active receptor, indicating strongly that AF-1 harbors the main transactivation activity of AR (Jenster *et al.* 1993, Gelmann 2002).

Androgen receptor gene and disease

The human gene for AR encompasses approximately 80 kb and is mapped to a conserved region on chromosome Xq11-12 (Lubahn *et al.* 1988, Quigley *et al.* 1995). AR protein is approximately 110 kDa in size, and it is polymorphic due to the polymeric glutamine and proline repeats in the first exon of the AR gene. The glutamine (CAG) repeat length polymorphism contributes to the pathogenesis of Kennedy's disease (also called spinal and bulbar muscular atrophy (SBMA)), that is an adult-onset, slowly progressing motoneuron disease (LaSpada *et al.* 1991, Yong *et al.* 2000). The number of CAG repeats increases with the severity of the Kennedy's disease, and affected males show variable degrees of gynecomastia, testicular atrophy, and subfertility (LaSpada *et al.* 1991). In contrast, short CAG repeat length of AR was associated with more aggressive prostate cancer occurrence, earlier age of disease onset, and likelihood of prostate cancer recurrence (Bratt *et al.* 1999, Giovannucci *et al.* 1997, Hardy *et al.* 1996, Nam *et al.* 2000). The AR gene was amplified in 30% of hormone-refractory prostate cancers, and mutations in the LBD of AR were shown to broaden ligand specificity of the receptor in recurrent prostate cancer (Gregory *et al.* 2001). Loss of AR function in genetic (XY) males leads to complete androgen insensitivity syndrome with an external phenotype of a sterile woman, undescended testes, and lack of ovaries and uterus (Yong *et al.* 2000). In testicular feminized mice (*Tfm*) with a spontaneous single point mutation in the AR gene resulting in truncation and inactivation of AR, testicular migration and spermatogenesis are impaired (Charest *et al.* 1991, Lyon and Hawkes 1970, Lyon *et al.* 1975). Interestingly, AR knockout male mice (ARKO), generated by using the *cre-lox* conditional knockout strategy, showed female-like external genitalia, severely reduced testicular size, lowered testosterone concentration, Leydig cell hyperplasia, and arrested spermatogenesis at pachytene spermatocyte stage (Yeh *et al.* 2002).

Estrogen receptors (ER)

Estrogens have traditionally been connected to female reproduction, but during recent years they have been reported to play a role in the male reproduction, bone metabolism, and physiology of the cardiovascular and central nervous systems (Enmark and Gustafsson 1999). Estrogen signals are mediated by two estrogen receptors, estrogen receptor α (ERα) and estrogen receptor β (ERβ), of which only ERβ is expressed in the male germ cells (Fig. 5) (Enmark *et al.* 1997, Kuiper *et al.* 1996). The human ERβ gene is localized to a different chromosome than the ERα gene, to 14q22-24 (Enmark *et al.* 1997). Recently,

an ERβ variant ERβcx/ERβ2, a dominant negative mutant against the ERα without an apparent ligand-binding ability, was identified (Gaskell *et al.* 2003, Ogawa *et al.* 1998, Saunders *et al.* 2002). In the human testis, abundant amounts of ERβ protein were detected in primary spermatocytes and early round spermatids (Enmark *et al.* 1997, Pelletier and El-Alfy 2000).

Male βERKO mice were fertile and showed hyperplastic foci in the prostate of young adult mice (Weihua *et al.* 2001). Female βERKO mice were subfertile due to the early atresia of preantral follicles and ovulatory failure and exhibited defects in differentiation of ductal side branches and alveoli in mammary glands after puberty (Dupont *et al.* 2000, Förster *et al.* 2002, Krege *et al.* 1998, Weihua *et al.* 2001). Furthermore, ERβ was required for the migration of cortical neurons during late embryonic development of the brain and differentiation of pluripotent hematopoietic progenitor cells (Shim *et al.* 2003, Wang *et al.* 2003).

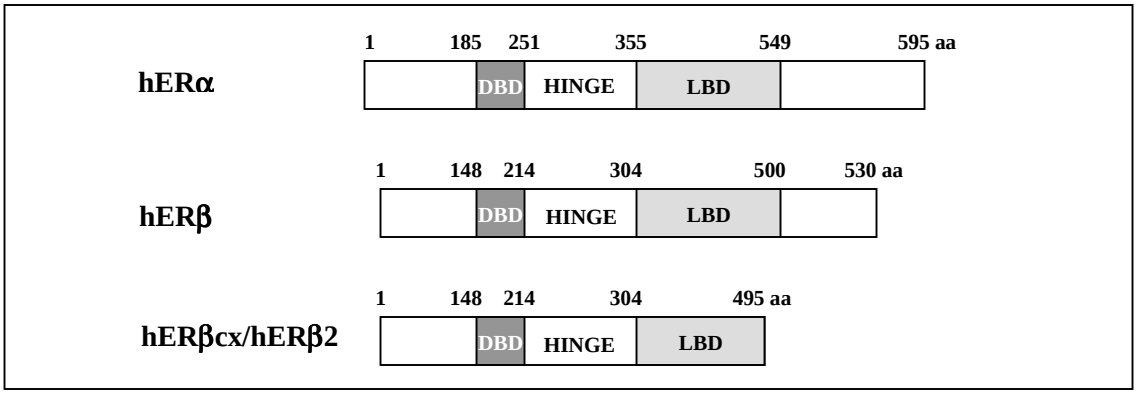

Fig. 5 Comparison of the human estrogen receptor α, estrogen receptor β, and estrogen receptor βcx/β2 structures. Human ERβ shows 97% and 54% homology to the DBD and LBD of human ERα, respectively. ERβcx/ERβ2 is identical with ERβ for the first 469 amino acids, but differs from it in the replacement of the last 61 amino acids by a unique 26-amino acid sequence. Modified from Enmark and Gustafsson, *J Int Med*, 1999.

Transcriptional control by nuclear receptors

Activating signals and 'transcriptional cross-talk'

In contrast to peptide hormones, which communicate with their intracellular targets through surface receptors and second messenger signaling pathways, lipophilic steroid hormones bind to intracellular steroid receptors. Ligand binding induces a conformational change in the steroid receptor, that dissociates from receptor-associated inhibitory heat shock proteins and facilitates receptor dimerization, nuclear transport, and interaction with target DNA motifs, resulting in transcriptional regulation of the target gene (for review, Aranda and Pascual 2001) (Fig. 6).

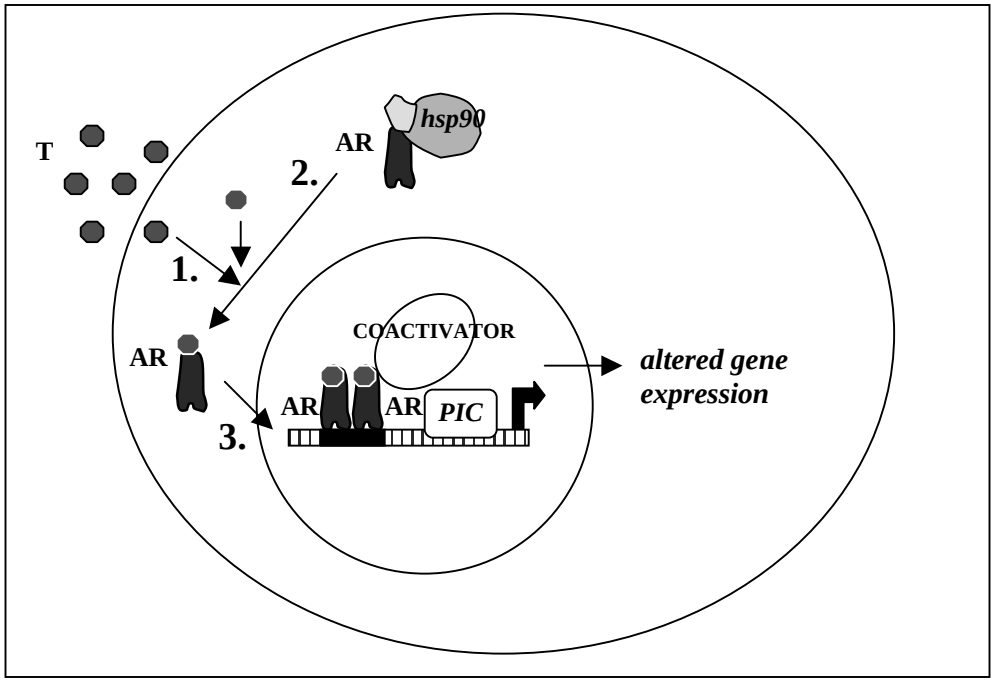

Fig. 6 Outline of androgen receptor (AR) action in cell. 1. The lipohilic testosterone ligand (T) diffuses into the cell. In some cells, testosterone is converted to 5α-dihydrotestosterone (DHT) by 5α-reductase. **2**. After ligand binding, AR undergoes a conformational change and dissociates from receptor-associated inhibitory heat shock proteins, including hsp90. **3.** Ligand binding facilitates nuclear translocation of AR, receptor dimerization, and interaction with hormone response elements (HRE) and coactivators, resulting in enhanced or repressed transcription of the target gene. PIC; preinitiation complex.

A pair of palindromic 6-bp consensus AGAACA motifs, separated by three spacer nucleotides, is recognized by androgen, progesterone, glucocorticoid, and mineralocorticoid receptors, while the motif AGGTCA is preferentially recognized by estrogen receptors (Glass 1994). Steroid receptors usually bind to HRE as homodimers, while several orphan and non-steroidal nuclear receptors, such as thyroid and vitamin D receptors, bind to their HREs as heterodimers with retinoic acid receptor X (RXR) (Glass 1994). Interaction of ligand-bound steroid receptor with its HRE often results in transcriptional activation of the target gene. However, also negative HREs that bind liganded receptors resulting in hormone-mediated transrepression of the gene have been identified, such as for GR in the pro-opiomelanocortin and prolactin gene promoters (Drouin *et al.* 1989, Sakai *et al.* 1988). A subset of nuclear receptors, such as thyroid and retinoic acid receptors, repress basal transcription when bound to HRE in the absence of the ligand. The repressor activity is due to the binding of corepressors to the unliganded receptors. Binding of the hormone ligand to the receptor releases corepressors and leads to transactivation (Casanova *et al.* 1994, Tong *et al.* 1996).

Steroid receptors interact with sequence-specific transcription factors through binding to nearby or overlapping binding sites. This is exemplified by the communication between GR and heterodimeric AP-1 transcription factors on a composite site containing both GRE and AP-1 elements at the proliferin promoter. The interaction results in GR-mediated transrepression or transactivation by AP-1 depending upon whether a Fos-Jun or Jun-Jun complex occupies the AP-1 site (Miner and Yamamoto 1992). Steroid receptors also affect transcriptional activity through positive or negative interference with other transcription factors without binding to the specific hormone response elements, referred to as 'transcriptional cross-talk' (for review, Beato *et al.* 1996, Göttlicher *et al.* 1998). For example, the collagenase type I gene is repressed by mutual antagonism between GR and AP-1 independent of DNA binding (Schüle *et al.* 1990, Jonat *et al.* 1990). In addition, AR was able to elicit both transactivation and transrepression of AP-1 factors without interacting directly with DNA-binding elements (Kallio *et al.* 1995). Moreover, NF-κB transcription factors participate in cross-talk with steroid receptors, as exemplified by mutual repression between AR and NF-κB factors by competition for a common cellular factor, the CBP coactivator (Aarnisalo *et al.* 1998, Dumont *et al.* 1998, Palvimo *et al.* 1996).

Nuclear receptors are also activated or repressed by posttranslational covalent modifications, such as phosphorylation, acetylation, ubiquitination, and sumoylation.

Steroid receptors are phosphorylated even in the absence of ligand, but ligand binding often increases the phosphorylation status of the receptors leading to enhancement of steroid receptor-mediated transcription (Kato *et al*. 1995, Kuiper *et al.* 1993, Nazareth and Weigel 1996, Orti *et al.* 1992, Shao and Lazar 1999, Weigel 1996). AR is phosphorylated on several sites, such as S81, S94, and S650, of which the phosphorylation of S650 seems to be necessary for full AR activity (Brinkmann *et al.* 1999, Zhou *et al.* 1995). Phosphorylation of AR is necessary for ubiquitination and degradation of AR by Mdm2 E3 ligase (Lee and Chang 2003). Mutation of the AR lysine residues to abrogate acetylation repressed transactivation of AR-regulated gene promoters, inhibited coactivation by several cofactors, increased corepressor N-CoR binding to the receptor, and inhibited MEKK1-induced apoptosis in prostate cancer cells (Fu *et al.* 2000, Fu *et al.* 2002). The AR is also covalently modified by small ubiquitin-like modifier 1 (SUMO-1) resulting in attenuation of AR-elicited transactivation (Poukka *et al.* 2000c).

Targeting of chromatin remodeling complexes

Two classes of chromatin remodeling factors play critical roles in transcriptional activation by nuclear receptors: ATP-driven nucleosome remodeling complexes and factors possessing histone acetyltransferase activity (for review, Narlikar *et al.* 2002, Workman and Kingston 1998, Wu 1997). Chromatin remodeling complexes, such as SWI/SNF/BRG1 and imitation of SWI (ISWI), alter the nucleosome structure in an ATP-dependent manner to facilitate promoter access, transcription factor binding, and transcription initiation (Narlikar *et al.* 2002, Owen-Hughes *et al.* 1996, Workman and Kingston 1998, Wu 1997). For example, progesterone receptor targeted ISWI-containing complexes to the mouse mammary tumor virus (MMTV) promoter and glucocorticoid receptor tethered the SWI/SNF complex to the chromatinized templates containing glucocorticoid response elements (DiCroce *et al.* 1999, Ostlund Farrants *et al.* 1997). Histone acetyltransferases covalently modify histones by decreasing their positive charge by acetylation, which is thought to result in weakened interaction with DNA and more accessible chromatin for transcription factor binding (Struhl 1998, Workman and Kingston 1998, Wu 1997). Histone acetylation usually increases transcriptional activity while hypoacetylation often correlates with transcriptional inactivity (Pazin and Kadonaga 1997, Workman and Kingston 1998). Factors with intrinsic histone acetyltransferase activity include members of the general transcription machinery, such as $TAF_{II}250$, and coactivators of nuclear receptor action, including p/CAF (p300/CBP-associated factor),

SRC family members, CREB -binding protein (CBP), and p300 (Glass and Rosenfeld 2000).

Coregulators

Introduction

Steroid receptors interact directly with components of the basal transcription machinery *in vitro* (Lee *et al.* 2000, McEwan and Gustafsson 1997). In addition, coregulators, defined broadly as cellular factors recruited by nuclear receptors that complement receptor function mediating the cellular response to endocrine signals, have been identified during recent years. Most of the coregulators were initially identified by their stimulative or repressive effects on nuclear receptor action in transient transfection assays, and the proteins are called as coactivators and corepressors, respectively (for review, Beato *et al.* 1996, Freedman 1999, Glass and Rosenfeld 2000, Hermanson *et al.* 2002, McKenna *et al.* 1999, McKenna and O'Malley 2002a,b, Xu *et al.* 1999). Chromatin immunoprecipitation studies have shown that nuclear receptors and coregulators assemble on target promoters in a sequential and rapidly cycling dynamic fashion (Shang *et al.* 2000).

Coactivators modulate nuclear receptor function in a variety of ways. Coactivators recruit additional cofactors to DNA-bound nuclear receptors, as exemplified by the recruitment of CBP by SRC family proteins (Demarest *et al.* 2002, Leo and Chen 2000, Yao *et al.* 1996). Several coactivators, such as SRC-1, SRC-3, CBP/p300, and p/CAF, acetylate nucleosomal histones at promoter regions to derepress chromatin structure (Chan and La Thangue 2001, Chen *et al.* 1997, Spencer *et al.* 1997). In addition, coactivator-associated arginine methyltransferase (CARM) and protein arginine methyltransferase (PRMT-1) function as histone methylases further relieving chromatin compaction (Chen *et al.* 1999, Koh *et al.* 2001). Some coactivators, such as the human TRAP/DRIP (TR-associated protein/VDR-interacting protein) complex, may recruit RNA polymerase II to nuclear receptor target promoters (Malik and Roeder 2000).

Coregulators interact with various domains of nuclear receptors. SRC family, Nrip1, AIB3/RAP250/ASC-2, and PBP/TRAP220 proteins interact with the LBD of nuclear receptors via α-helical motifs related to Leu-X-X-Leu-Leu (LXXLL motif or NR box) (for review, Cavailles *et al.* 1995, Feng *et al.* 1998, Freedman 1999, Glass and Rosenfeld 2000, Heery *et al.* 1997, Lee *et al.* 1998, Leo and Chen 2000, McKenna *et al.* 1999).

Table 3: Examples of Coregulators		
Coregulator	**Enzymatic activity**	**Reference**
AIB3/ASC-2/RAP250	-	Lee *et al*. 1999, Caira *et al*. 2000
ANPK	Serine/threonine kinase	Moilanen *et al*. 1998a
ARIP4	ATPase	Rouleau *et al.* 2002
CARM	methyltransferase	Koh *et al.* 2001
CBP	histone acetyltransferase	Bannister & Kouzarides 1996
E6-AP	E3 ubiquitin ligase	Nawaz *et al*. 1999, Scheffner *et al*. 1994
GT198	-	Ko *et al*. 2002
N-CoR	-	Alland *et al*. 1997
Nrip1	-	Cavailles *et al*. 1995
p300	histone acetyltransferase	Eckner *et al*. 1994
PBP/TRAP220	-	Zhu *et al*. 1997
PIAS1/GBP	E3 SUMO-1 ligase	Liu *et al*. 1998, Kotaja *et al*. 2002a
PIAS3	E3 SUMO-1 ligase	Chung *et al.* 1997, Kotaja *et al.* 2002a
PIASxα/ARIP3	E3 SUMO-1 ligase	Moilanen *et al*. 1999, Kotaja *et al*. 2002a
PIASxβ/Miz1	E3 SUMO-1 ligase	Wu *et al.* 1997, Kotaja *et al.* 2002a
PIASy	E3 SUMO-1 ligase	Liu *et al*. 1998, Kotaja *et al*. 2002a
PRMT-1	methyltransferase	Chen *et al.*1999
SMRT	-	Nagy *et al.*1997
SNURF/RNF4	E3 ubiquitin ligase ?	Moilanen *et al.*1998b, Häkli *et al.* unpubl.
SRC-1/NcoA-1	histone acetyltransferase	Spencer *et al.*1997
SRC-2/TIF2/GRIP1/NcoA-2	-	Hong *et al.*1997
SRC-3/AIB1/ACTR/RAC3/TRAM-1	histone acetyltransferase	Anzick *et al.* 1997

SNURF/RNF4, ANPK, ARIP3/PIASxα, and ARIP4 were identified by their interaction with the AR DBD and hinge region (Moilanen *et al.* 1998a, Moilanen *et al.* 1998b, Moilanen *et al.* 1999, Rouleau *et al.* 2002). In addition, GT198, a tissue-specific coactivator expressed to high levels in testis, interacts with the DBD of nuclear receptors (Ko *et al.* 2002). The best characterized corepressors, nuclear receptor corepressor (N-CoR) and silencing mediator of retinoid and thyroid hormone receptors (SMRT), repress transcription by binding to the DBD of the DNA-bound unliganded thyroid and retinoid receptors (Chen and Evans 1995, Horwitz *et al.* 1996, Hörlein *et al.* 1995) and also interact with unliganded and antagonist bound LBD of nuclear receptors. Steroid receptors, such as estrogen and progesterone receptors, bind N-CoR and SMRT in the presence of antagonists 4-hydroxytamoxifen and RU486, respectively, and corepressor recruitment is essential for the full antagonist activity (Lavinsky *et al.* 1998). N-CoR and SMRT, that are related structurally and functionally recruit cellular histone deacetylase (HDAC) complexes enhancing chromatin compaction (Alland *et al.* 1997, Kao *et al.* 2000, Nagy *et al.* 1997). The corepressors are recruited to nuclear receptors by CoRNR signature motif, resembling

the LXXLL motif of coactivators (Hu and Lazar 1999, Nagy *et al.* 1999). In the following chapters, some of the most extensively studied coregulators are described in more detail.

CBP and p300

CREB-binding protein and p300 are functionally conserved histone acetyltransferases, and coactivate nuclear receptor action (Bannister and Kouzarides 1996, Chakravarti *et al.* 1996, Chan and La Thangue 2001, Eckner *et al.* 1994, Lundblad *et al.* 1995, Ogryzko *et al.* 1996). CBP and p300 acetylate not only histones but also transcription factors, such as p53, AR, ERα, TFIIE, and TFIIF, and function as coactivators for several signaling pathways involving CREB, AP-1, p53, and STATs (Arias *et al.* 1994, Bhattacharya *et al.* 1996, Fu *et al*. 2000, Gu and Roeder 1997, Horvai *et al.* 1997, Imhof *et al.* 1997, Jancknecht and Hunter 1996, Zhang *et al.* 1996). Only limiting amounts of CBP and p300 reside in cells, and several signal transducers compete for their interaction, as exemplified by the inhibition of AP-1 activity through CBP-recruiting nuclear receptors (Kamei *et al.* 1996). CBP and p300 function as bridging proteins by contacting nuclear receptors and basal transcription factors, form a scaffold for the assembly of multiprotein complexes, and may increase the local concentration of additional histone acetyltransferases, such as SRC-1 and p/CAF (Chan and La Thangue 2001, Jenster *et al.* 1997, Kee *et al.* 1996, Spencer *et al.* 1997, Yang *et al.* 1996). In addition, CBP and p300 participate in the *in vivo* polyubiquitination, but not monoubiquitination, of p53 (Grossman *et al.* 2003).

Gene-disrupted $p300^{-/-}$, $CBP^{-/-}$, and $p300^{+/-}CBP^{+/-}$ mouse embryos died around embryonic day 10 due to the defects in neural tube closure and heart development (Yao *et al.* 1998). In addition, $p300^{-/-}$ murine embryonic fibroblasts showed retarded cell proliferation and were defective in retinoic acid-dependent transcription (Yao *et al.* 1998). $CBP^{+/-}$ mouse embryos presented abnormal skeletal patterning with similarities to patients with Rubinstein-Taubi syndrome, a disorder of mental retardation, craniofacial and skeletal defects, and increased incidence of neoplasia caused by mutations in one CBP gene locus in humans (Petrij *et al.* 1995). In addition, studies on $CBP^{+/-}$ mice suggest that CBP functions as a tumor suppressor in the hematopoietic system (Kung *et al.* 1999). Translocation between acetyltransferases MOZ and CBP resulting in MOZ-CBP fusion protein has been found in patients suffering from acute myeloid leukemia (Borrow *et al.* 1996). Moreover, biallelic mutations of p300 were found in patients with colorectal and gastric carcinomas (Gayther *et al.* 2000, Giles *et al.* 1998, Muraoka *et al.* 1996).

p160 coactivators

Three related genes, encoding approximately 160-kDa proteins, SRC1/NcoA-1, TIF2/GRIP1/NcoA-2, and p/CIP/AIB1/ACTR/SRC-3/TRAM-1/RAC3, constitute the p160 or SRC family of nuclear receptor coactivators (Anzick *et al.* 1997, Chen *et al.* 1997, Hong *et al.* 1997, Leo and Chen 2000, Li *et al.* 1997, Onate *et al.* 1995, Takeshita *et al.* 1997, Torchia *et al.* 1997). The p160 factors possess highly conserved amino-terminal basic helix-loop-helix (bHLH) and PAS domains, and the carboxyl-terminus of SRC-1 and SRC-3 harbor histone acetyltransferase (HAT) activity (Chen *et al.* 1997, Spencer *et al.* 1997). The carboxyl-terminus of the p160 factors acts as a platform for assembly of arginine methyltransferases CARM1 and PRMT1, that enhance nuclear receptor activity by methylating histones (Chen *et al.* 1999, Koh *et al.* 2001).

Mice with the disrupted SRC-1 gene were viable and fertile, but responded only partially to thyroid and steroid hormones in the development and growth of target organs, such as testis, uterus, prostate, and mammary gland (Qi *et al.* 1999, Weiss *et al.* 1999, Xu *et al.* 1998). The relatively subtle defects seen in the SRC-1 knockout mice may be explained by the compensatory over-expression of transcriptional intermediary factor 2 (TIF2) (Xu *et al.* 1998, Wang *et al.* 2000). TIF2/GRIP1 null mutant mice showed impaired fertility in both sexes (Gehin *et al.* 2002). Male hypofertility was caused by teratozoospermia (i.e., abnormalities in morphology of the spermatozoa) and age-dependent testicular degeneration, while female subfertility derived from defects in placental development (Gehin *et al.* 2002). TIF2 knockout mice possessed normal thyroid function, but double heterozygous disruption of TIF2 and SRC-1 resulted in thyroid hyposensitivity (Takeuchi *et al.* 2002, Weiss *et al.* 2002). In addition, gene deletion experiments have revealed that SRC-1 and TIF2 modulate energy metabolism in the white and brown adipose tissue (Picard *et al.* 2003).

Furthermore, the inv(8)(p11q13) resulted in a fusion between TIF2 and MOZ genes in human acute myeloid leukemia patients, and interaction of the MOZ-TIF2 fusion protein with CBP was essential for transformation (Carapeti *et al.* 1998, Deguchi *et al.* 2003). Inactivation of the gene encoding SRC-3, which is highly amplified and over-expressed in primary breast cancers, resulted in dwarfism, delayed puberty, abnormal female reproductive function, and mammary gland growth retardation (Xu *et al.* 2000, Yuan *et al.* 2002). These data underscore the biological significance of the SRC family proteins in the developmental and hormonal regulation.

Nrip1

Nuclear receptor -interacting protein 1 (Nrip1, formerly RIP140), which was originally identified as an estrogen-dependent cofactor of mouse ERα, is widely expressed in tissues and cells (Cavailles *et al.* 1995, Lee *et al.* 1998). Nrip1 inhibits the activity of several nuclear receptors, and its expression was regulated by estradiol in breast cancer cells (Cavailles *et al.* 1995, Subramaniam *et al.* 1999, Thenot *et al.* 1999, Treuter *et al.* 1998). In the ovary, the highest expression level of Nrip1 was detected in granulosa cells, whereas lower levels were detected in the thecal and interstitial compartments (White *et al.* 2000). Furthermore, Nrip1 expression was temporally regulated in the corpora lutea at different stages of pregnancy (Leonardsson *et al.* 2002). Null mutation of Nrip1 led to female infertility due to a complete ovulatory failure (White *et al.* 2000). In addition, approximately 50% of the oocytes were entrapped in luteinizing follicles in superovulated heterozygous mice, underscoring the need of the absolute level of Nrip1 for ovulation (White *et al.* 2000). However, embryo and ovarian transplantation experiments demonstrated that by by-passing ovulation, Nrip1$^{-/-}$ mice were capable of establishing and maintaining pregnancy (Leonardsson *et al.* 2002).

PPARγ –binding protein (PBP)/TRAP220

The TRAP/DRIP complex that interacts with thyroid and vitamin D receptors is recruited to nuclear receptors by an LXXLL motif of PPARγ-binding protein (PBP)/ DRIP205/TRAP220 (Fondell *et al.* 1996, Fondell *et al.* 1999, Rachez *et al.* 2000). Several proteins of the TRAP/DRIP complex are components of mediator, a protein assembly associating with RNA polymerase II, and the TRAP/DRIP complex probably acts as a bridging protein recruiting the polymerase to nuclear receptor target promoters (Rachez *et al.* 1998, Rachez *et al.* 1999).

Disruption of the PBP/TRAP220 coactivator gene resulted in embryonic lethality at E11.5 dpc due to defects in placental vascular network, heart failure, and impaired neurological development with extensive apoptosis (Ito *et al.* 2000, Zhu *et al.* 2000). In addition, deletion of the PBP gene lead to paucity of retinal pigment, defective lens formation, excessive systemic angiogenesis, a deficiency in the number of megakaryocytes, and an arrest in erythrocyte differentiation (Crawford *et al.* 2002). Moreover, heterozygous mice showed growth retardation, dysfunction of the pituitary-thyroid axis, and transcriptional impairment in the testis and in the brain (Ito *et al.* 2000). Murine embryonic fibroblasts

derived from the TRAP220$^{-/-}$ embryos showed significant decrease in thyroid receptor function, and TRAP220 was also required for PPARγ2-mediated adipogenesis (Ge *et al.* 2002, Ito *et al.* 2000). Furthermore, PBP/TRAP220 was amplified and overexpressed in breast carcinomas (Zhu *et al.* 1997, Zhu *et al.* 1999).

AIB3/RAP250/ASC-2

Amplified in breast cancer-3 (AIB3)/RAP250/ASC-2 is a coactivator of several nuclear receptors, including thyroid hormone receptors, retinoic acid receptor α, PPARα, PPARγ, ERα, and GR (Lee *et al.* 1999, Caira *et al.* 2000). The human AIB3 gene is located to a highly amplified chromosomal region 20q11-12, and AIB3 was amplified in 10% of breast cancers, 30% of colon cancers, and 13% of lung cancers studied (Lee *et al.* 1999). Since AIB3 interacts with several transcription factors, such as nuclear receptors, serum response factor (SRF), AP-1, and NF-κB, it could contribute to malignant transformation by affecting multiple signal transduction pathways governing cell proliferation and differentiation (Lee *et al.* 2000).

Murine embryos with disrupted AIB3 gene died during E9.75-13.5 dpc due to the cardiac hypoplasia and defects in placental vascular network and nervous system development (Antonson *et al.* 2003, Kuang *et al.* 2002). The placental defects included failure of labyrinthine development, dilation of maternal blood sinuses, massive erythrophagocytosis by trophoblasts, and alteration of trophoblast populations (Antonson *et al.* 2003, Kuang *et al.* 2002). In microinjection studies, anti-ASC-2 antibody abrogated the ligand-dependent transactivation by retinoic acid receptor. Moreover, the repression was fully reversible by coinjection of ASC-2 expression plasmid, thus confirming the essential role of AIB3/ASC-2 in nuclear receptor-mediated signaling in mammalian cells (Lee *et al.* 1999). In addition, AIB3 was required for full PPARγ action in murine fibroblasts (Antonson *et al.* 2003, Kuang *et al.* 2002).

PIAS protein family

PIAS1/GBP, PIAS3, PIASy, PIASxβ/Miz1, and PIASxα/ARIP3, members of the protein inhibitor of activated STAT (signal transducer and activator of transcription) family, interact with steroid receptors (Chung *et al.* 1997, Kotaja *et al.* 2000, Kotaja *et al.* 2002a, Liu *et al.* 1998, Moilanen *et al.* 1999, Wu *et al.* 1997). STAT transcription factors are activated by several cytokines, that bind to their cell surface receptors resulting in tyrosine

phosphorylation of STATs. After phosphorylation, STATs dimerize and translocate into the nucleus to induce transcription from their target genes (Levy and Darnell 2002). PIAS1 and PIAS3 inhibit DNA binding of STAT1 and STAT3 factors, respectively, whereas PIASy represses STAT1 and AR activity without affecting their DNA binding (Chung *et al.* 1997, Liu *et al.* 1998).

PIAS proteins comprise a conserved group of putative zinc finger proteins with 60-80% homology in their amino acid sequences. They contain two LXXLL motifs and a N-terminal SAF box/SAP domain (SAF-A, Acinus, PIAS) (Moilanen *et al.* 1999, Tan *et al.* 2002). PIAS1, PIASy, and PIASxα function as E3 ligases for small ubiquitin-like modifier-1 (SUMO-1) in sumoylation, a covalent posttranslational modification, of p53, TIF2/GRIP1, Sp3, LEF1, c-Jun, and AR (Kahyo *et al.* 2001, Kotaja *et al.* 2002b, Nishida and Yasuda 2002, Ross *et al.* 2002, Sachdev *et al.* 2001, Sapetschnig *et al.* 2002, Schmidt and Müller 2002). In addition, STAT1 is a substrate for SUMO-1 modification but inhibition of STAT1 by PIAS1 did not require sumoylation of STAT1 itself (Rogers *et al.* 2003, Ungureanu *et al.* 2003). Sumoylation proceeds by a three-step enzyme pathway resembling ubiquitylation (for review, Hochstrasser 2001, Jackson 2001, Kim *et al.* 2002, Müller *et al.* 2001). SUMO-1 is likely to target proteins to subcellular and subnuclear compartments, influence the activity of transcription factors, facilitate protein-protein interactions, and inhibit ubiquitylation.

PIAS3 is widely expressed in various tissues, whereas the PIAS1, PIASy, PIASxα/ARIP3, and PIASxβ/Miz1 are predominantly expressed in testis (Chung *et al.* 1997, Liu *et al.* 1998, Santti *et al.* 2003, Tan *et al.* 2000, Tan *et al.* 2002, Yan *et al.* 2003). Inactivation of Su(var)2-10, a Drosophila PIAS orthologue, disrupted mitotic chromosome condensation and organization of interphase chromatin (Hari *et al.* 2001). In addition, yeast Siz1 (PIAS homologue) interacted with the condensin complex, suggesting for a role in chromatin condensation (Strunnikov *et al.* 2001). Furthermore, PIAS1 was down-regulated in RAS-transformed fibroblasts, repression of PIASy was associated with stage-progression in chronic myeloid leukemia, and PIAS3 was down-regulated in anaplastic lymphoma (Ohmine *et al.* 2001, Zhang *et al.* 2002, Zuber *et al.* 2000).

Table 4: Homozygous null mutations of coregulators	
Targeted gene	**Phenotype**
CBP and p300	Embryonic lethality at E10.5 dpc, defects in neural tube closure and heart development
SRC-1	Partial resistence to thyroid and steroid hormones
TIF2/GRIP1	Impaired fertility in both sexes, teratozoospermia, age-dependent testicular degeneration, defects in placental development
SRC-3/AIB1/ACTR/ RAC3/TRAM-1/p/CIP	Female subfertility, dwarfism, delayed puberty, mammary gland growth retardation
Nrip1	Female infertility, complete ovulatory failure
TRAP220/PBP	Embryonic lethality at E11.5 dpc, defects in placental vascular network, cardiac failure and impaired neurological development, arrest in erythrocyte differentiation, excessive systemic angiogenesis
ASC-2/RAP250/AIB3	Embryonic lethality at E9.75-E13.5 dpc, cardiac hypoplasia, defects in placental vascular network and nervous system development

Small nuclear RING finger protein SNURF/RNF4

Structure of SNURF

Small nuclear RING finger protein (SNURF)/Ring finger protein 4 (RNF4), a steroid receptor coregulator, was originally identified in a yeast two-hybrid screen using AR DBD as a bait (Moilanen *et al.* 1998b). SNURF is phylogenetically unique to mammals: its homologues are not present in Saccharomyces cerevisiae or Caenorhabditis elegans. Rat SNURF is composed of 194 amino acids, of which approximately 30% are charged (Chiariotti *et al.* 1998, Moilanen *et al.* 1998b) (Fig. 7). SNURF possesses an N-terminal bipartite nuclear localization signal and a C-terminal C_3HC_4 type of RING (really interesting new gene) finger (Fig. 7) (Borden and Freemont 1996, Borden 2000, Joazeiro and Weissman 2000, Saurin *et al.* 1996). RING finger motifs are found in several proteins involved in developmental regulation (polycomb group proteins Bmi-1, RING1), apoptosis (inhibitors of apoptosis c-IAP1, c-IAP2), oncogenesis (proto-oncogene proteins PML, BRCA1), neurodegenerative disease (familial juvenile Parkinson disease product Parkin), viral pathogenicity (Herpes simplex virus protein IE110), regulation of cell cycle (APC11), and protein degradation (E3 ubiquitin ligases APC11, BRCA1) (Everett and Maul 1994, Futreal *et al.* 1994, Gmachl *et al.* 2000, Haupt *et al.* 1991, Kakizuka *et al.* 1991, Kanno *et al.* 1995, Rothe *et al.* 1995, Satijn *et al.* 1997, Shimura *et al.* 2000, van der Lugt *et al.* 1994).

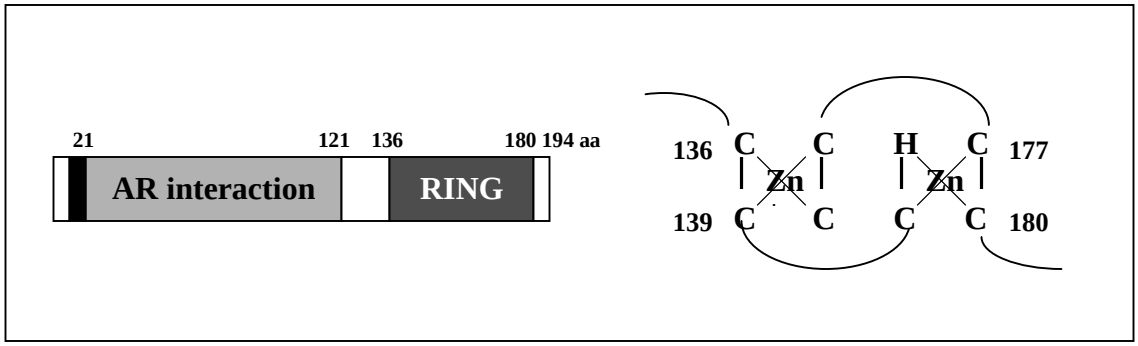

Fig. 7 Rat SNURF is composed of 194 amino acids. SNURF possesses an N-terminal bipartite nuclear localization signal (designated as a black box) and a C_3HC_4 -type of RING finger in its carboxyl-terminus. Modified from Moilanen *et al*., *Mol Cell Biol*, 1998.

Interaction partners of SNURF

SNURF interacts *in vitro* with several transcription factors, such as Sp1 (promoter specificity protein 1), SBPB (activator of stromelysin gene expression), PATZ (POZ-AT hook-zinc finger protein), Gscl (Goosecoid-like), TRPS1, and TATA-binding protein (TBP) (Fedele *et al.* 2000, Galili *et al.* 2000, Kaiser *et al.* 2003, Lyngso *et al.* 2000, Moilanen *et al.* 1998b, Pero *et al.* 2002, Poukka *et al.* 2000a). SNURF binds to the DBD and hinge regions of steroid receptors and enhances their transactivation functions in mammalian cells (Moilanen *et al.* 1998b, Saville *et al.* 2001). For example, SNURF and TATA-binding protein (TBP) synergistically enhanced ERα-mediated transactivation in ZR-75 human breast cancer cells (Saville *et al.* 2001). Moreover, SNURF facilitated nuclear import of AR and retarded its export from nuclei of mammalian cells after testosterone withdrawal (Poukka *et al.* 2000b). In addition, SNURF stimulated transcription from minimal reporter gene constructs bearing consensus elements for transcription factors Sp1 and AP-1, and enhanced binding of Sp1 and SPBP to their cognate DNA targets *in vitro* (Lyngso *et al.* 2000, Moilanen *et al.* 1998b, Poukka *et al.* 2000a). SNURF also stimulated transcription from the rat luteinizing hormone-β promoter (LHβ) by interacting with Sp1 and steroidogenic factor-1 (SF1) (Curtin *et al*., unpublished results). Binding of SNURF to AR, Gscl, TRPS1, and SF1 was mediated via the regions outside the RING finger, whereas for the interaction with TBP, Sp1, PATZ, and SPBP, an intact RING finger region was needed (Kaiser *et al.* 2003, Lyngso *et al.* 2000, Pero *et al.* 2002, Poukka *et al.* 2000a). SNURF bound to DNA without an apparent nucleotide sequence specificity, and it interacted with both supercoiled and four-way junction DNA and recognized nucleosomes (Häkli *et al.* 2001). Deletion of amino acids 1-20 and

mutations R8-11A/K9A and R8A of SNURF protein abolished DNA binding activity and severely decreased transactivation on the Sp1 minimal promoter (Häkli *et al.* 2001).

Table 5: Interaction partners of SNURF

Factor	Interaction domain of SNURF	Reference
AR	aa 20-121	Moilanen *et al.* 1998b
ERα	aa 31-65, RING finger	Saville *et al.* 2001
TBP	RING finger	Moilanen *et al.* 1998b
Sp1	RING finger	Poukka *et al.* 2000a
Gscl	aa 39-125	Galili *et al.* 2000
PATZ	RING finger	Pero *et al.* 2002
HMGI(Y)	full length protein	Pero *et al.* 2000
SBPB	RING finger	Lyngso *et al.* 2000
TRPS1	aa 6-65	Kaiser *et al.* 2003
SF1	aa 31-65	Curtin *et al*., unpublished

Expression of SNURF

SNURF is expressed as a single mRNA species of 3.0 kb in size in all human tissues analyzed. In addition to the ubiquitously expressed 3.0-kb transcript, another 1.0-1.6-kb testis-specific transcript is detected in rodents (Moilanen *et al.* 1998b). SNURF is expressed in murine embryonic tissues from E7.5 dpc on throughout the development, with high levels of expression in the developing gonads and nervous system (Galili *et al.* 2000). The gene coding for human SNURF/RNF4 maps at chromosomal region 4p16.3, which is associated with several genetic and neoplastic diseases (Chiariotti *et al.* 1998). The 47-kb spanning human RNF4 gene was located immeadiately proximal to the anonymous position D4S183, between the huntingtin (HD) and fibroblast growth factor receptor 3 (FGFR3) genes (Chiariotti *et al.* 1998).

Deletion at 4p16.3 between the markers D4S43 and D4S127, the region bearing the RNF4 gene, was detected in breast carcinomas, and the loss of heterozygosity at 4p16.3 was common in bladder carcinomas (Elder *et al.* 1994, Shivapurkar *et al.* 1999). Interestingly, expression of SNURF was down-regulated in RAS-transformed cells (Zuber *et al.* 2000). Mutational activation of the RAS genes is important in the multi-step pathogenesis of several malignancies, such as pancreas, colon, lung, and myeloid cancers. Expression of SNURF was repressed in testicular germ cell tumors of yolk sac, embryonal carcinoma, and seminoma types (Pero *et al.* 2001). In addition, ectopically overexpressed SNURF

inhibited the growth of cultured human embryonal kidney (293T) and teratocarcinoma (NTERA-2) cells (Pero *et al.* 2001).

RING finger proteins in ubiquitylation

Ubiquitin is a 76-amino acid protein. Its successive covalent attachments to target proteins result in their degradation, endocytosis, or conformational alteration (Hershko and Ciechanover 1998, Varhavsky 1997, Weissman 2001). Protein modification by the ubiquitin pathway proceeds in a three-step fashion (for review, Hershko and Ciechanover 1998, Varhavsky 1997, Weissman 2001) (Fig. 8). First, ubiquitin is activated by a ubiquitin-activating enzyme, E1. Secondly, the activated ubiquitin moiety is transferred to a cysteine residue of a ubiquitin-conjugating enzyme, E2, from which a ubiquitin ligase, E3, transfers it to the target protein. Several RING finger proteins and HECT (homology to the E6-AP carboxyl-terminus) domain ligases function as E3 ubiquitin ligases. E3 ligase facilitates the covalent attachment of ubiquitin to the substrate via the formation of an isopeptide bond between the C-terminal lysine of ubiquitin and ε-NH_2 group of an internal lysine residue of the substrate. In subsequent reactions, several ubiquitin molecules are added to the substrate, and the polyubiquitin-tagged protein is degraded by the 26S proteasome.

Many factors of the ubiquitylation pathway show remarkable evolutionary conservation, from yeast to mammalian species. The ubiquitin system participates in the regulation of several signal transduction processes, such as cell differentiation, cell cycle progression, endocytosis, immune response, programmed cell death, receptor down-regulation, and chromatin modification including DNA repair and gene silencing (Hershko and Ciechanover 1998, Weissman 2001). SNURF is auto-ubiquitylated in a RING domain-dependent fashion and may mediate ubiquitylation, suggesting its potential function as an E3 ubiquitin ligase (Häkli *et al.*, unpublished results). The Angelman syndrome-associated protein E6-AP functions as an E3 ubiquitin ligase, targets p53 protein specifically to degradation, and coactivates nuclear receptors (Nawaz *et al.* 1999, Scheffner *et al.* 1994). Inactivation of the E6-AP gene in mice resulted in reduced fertility in both genders with defects in sperm production and in ovulation, and tissue-selective steroid hormone resistance (Smith *et al.* 2002).

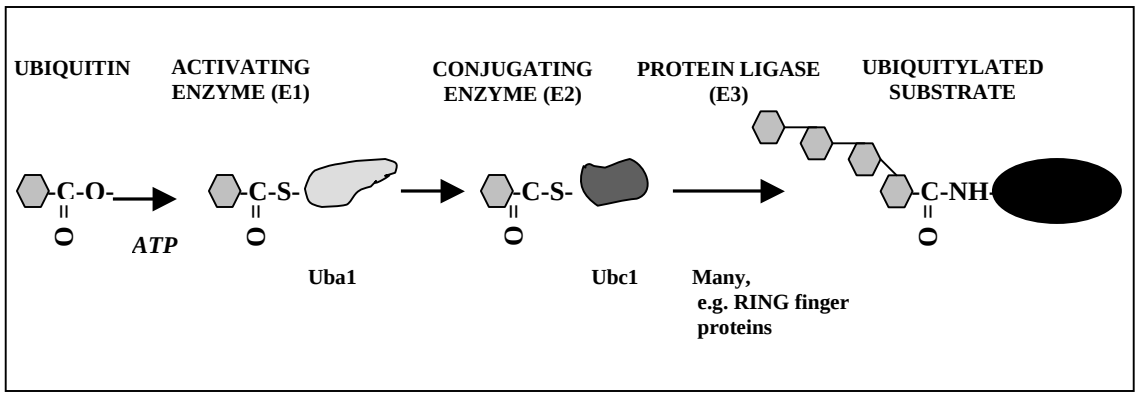

Fig. 8 Ubiquitylation pathway. First, ubiquitin is activated by the ubiquitin-activating enzyme E1 in a reaction that requires ATP hydrolysis to form a high-energy thiol ester linkage between the carboxyl-terminus of ubiquitin and the cysteine of the active site of E1. Secondly, the activated ubiquitin moiety is transferred via transthiolation reaction to the ubiquitin-conjugating enzyme, E2, from which the ubiquitin ligase, E3, facilitates the transfer to the target protein. In subsequent reactions, several ubiquitin molecules are added to the substrate and the polyubiquitin-tagged protein is degraded by the 26S proteasome. Ubiquitin can be detached from the polyubiquitin chain or substrate by members of two deubiquitinating enzyme families: ubiquitin-terminal hydrolases (UCHs) and ubiquitin-specific processing proteases (UBPs) (Wilkinson 1997). Modified from Hochstrasser, *Science*, 2000.

AIMS OF THE STUDY

The physiological role of small nuclear RING finger protein SNURF (RNF4), a transcriptional coregulator, is largely unknown despite several *in vitro* interaction and expression studies reported. However, the expression of SNURF mRNA and protein to high levels in testis suggests a role for SNURF in reproduction. This thesis work aimed at studying the regulation of the SNURF gene expression during fetal gonad development, postnatal ovarian folliculogenesis and spermatogenesis, and in testicular germ cell tumors. The specific goals were as follows:

- To study the expression of SNURF mRNA and protein during gonad determination and prenatal differentiation, as well as in postnatal ovarian and testicular development.

- To characterize the main regulatory elements governing transcription from the murine SNURF gene.

- To study the hormonal regulation of SNURF gene expression *in vivo* in rodent gonads.

- To analyze the expression of SNURF and steroid receptors in human testicular germ cell cancer (TGC).

METHODS

For detailed description of the materials and methods, the reader is referred to the original publications indicated in Table 6. In addition, some discussion on the methodology is included in the chapter 'Results and Discussion'.

Table 6: Methods used in the study	**Described in publication no.**
Cell culture and transfections	I, IV
Electrophoretic mobility shift assay (EMSA)	I
Immunohistochemistry	II, III, IV
In situ hybridization	II, IV
Northern blotting	II
Primer extension	I
Quantitative RT-PCR	III, IV
Recombinant DNA techniques and plasmid construction	I, II, IV

Tissue samples

All animal experiments were approved by the appropriate institutional review boards of the University of Turku (II), University of Helsinki (IV), and Baylor College of Medicine (Houston, TX, USA) (IV). For the description of the hormone-treated animal models the reader is referred to Table 8. The gonad samples from the hormone-treated and wild-type mice and rats (n=2-3 in each group) were fixed in 4% paraformaldehyde and embedded in paraffin for preparation of 5-μm tissue sections for *in situ* hybridization and immunohistochemistry. The human and rat testicular specimens were snap-frozen in liquid nitrogen and stored at –70^{o}C until RNA extraction. Study plan of the testicular germ cell cancer project (III) was approved by the ethics committee of the Helsinki University Central Hospital. Paired specimens of normal testis tissue and adjacent tumor tissue were obtained from 12 patients with TGC (seminomas n=8, non-seminomas n=4), who undervent surgery at the Department of Urology, Helsinki University Central Hospital, Helsinki, Finland. Informed consent was received from each patient.

Plasmids

The SNURF promoter fragments –617/+111, -202/+111, -38/+111, -8/+111, +32/+111, and –38/+36 nt (numbering refers to the transcription initiation site) were cloned into the luciferase encoding pGL3-basic vector (Promega, Madison, WI, USA). In reporter gene assays and cotransfections, 300-350 ng of the promoter constructs, 100 ng of pEVR-Sp1, pRc/CMV-Sp3, or pCB6-WT18A, and 50 ng of pCMVβ (Clontech, Palo Alto, CA, USA) were used per $3x10^4$ cells per well.

Cell culture and transfections

COS-1 (American Type Culture Collection, ATCC), HeLa (ATCC), and mSC-1 cells (a gift from Prof. Ilpo Huhtaniemi, University of Turku, Finland) were maintained in Dulbecco's minimal essential medium containing penicillin (25 000 U/l), streptomycin (25 000 U/l), and 10% (vol/vol) fetal bovine serum. Twenty-four hours before transfection, the cells were seeded on 12-well plates ($3x10^4$ cells per well) with indicated expression plasmids and 50 ng of β-galactosidase encoding pCMVβ using FuGENE 6 reagent (Roche Molecular Biochemicals, Indianapolis, IN, USA). The cells were harvested 48 hours after transfection. Luciferase activities were measured with reagents from Promega using a Luminoskan RT reader (Labsystems, Helsinki, Finland) and β-galactosidase activities were determined according to Rosenthal (1987). The luciferase values were normalized by the β-galactosidase values.

Electrophoretic mobility shift assay (EMSA)

EMSA experiments were carried out with 1 μg of HeLa cell nuclear extract (4C Biotech, Belgium), 1.6 μg of poly(dI-dC)(dI-dC) (Amersham Pharmacia Biotech, Aylesbury, UK), and ^{32}P-labeled promoter fragments –8/+111 nt and –38/+111 nt in 20 mM HEPES (pH 7.9), 10% glycerol (vol/vol), 50 mM KCl, 0.5 mM EDTA, 1 mM DTT, 1 mM $MgCl_2$, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), and 1 μM leupeptin. Reaction products were separated on 4% polyacrylamide gels run in 22.5 mM Tris-borate, 0.5 mM EDTA for 2-3 h at 200V at 22^{o}C. In oligomer competition assays, nuclear extracts were preincubated with 100-fold molar excess of non-radioactive double-stranded oligonucleotides encompassing indicated promoter regions or consensus binding sites for NF-κB, CREB, and Sp1.

Immunohistochemistry

Paraffin-embedded sections of gonads were used for immunohistochemical staining with polyclonal anti-SNURF antibody raised against rat SNURF in rabbit (1:1000-1:2000 dilution in Tris-buffered saline (TBS) containing 1% bovine serum albumin (BSA), Moilanen 1998b), and the positive cells were visualized using Vectastain Elite ABC kit (Vector laboratories, Burlingame, CA, USA) according to the manufacturer's instructions. As a control, 500 ng of GST-SNURF were first incubated with the antibody (1:2000 dilution) for 16 h at 4°C, and the resulting preadsorbed anti-SNURF antibody was used to monitor the specificity of staining. ERβ antigen was detected using anti-ERβ 503 antibody that was raised against human ERβ in chicken (a gift from Dr. Jan-Åke Gustafsson, Novum, Huddinge, Sweden, diluted to 1:1000 in phosphate-buffered saline (PBS) with 3% BSA and 0.05% Tween 20) and peroxidase-conjugated rabbit anti-chicken IgG (1:1000 dilution in PBS and 2% rat normal serum) (Sigma-Aldrich, St. Louis, MO, USA). Samples were incubated with the primary antibodies at 4°C overnight. Peroxidase activity was visualized with diaminobenzidine as the substrate (Vector laboratories, Burlingame, CA, USA), and the sections were stained with Mayer's hematoxylin, dehydrated, and mounted.

In situ hybridization

The tissue sections of gonads were deparaffinized, permeabilized, dehydrated, and hybridized to antisense and sense SNURF RNA probes (corresponding to nt 156–536 of murine SNURF cDNA, or to nt 234-967 and nt 968-1644 of rat SNURF cDNA) synthesized in the presence of [α-^{35}S]UTP (Amersham Pharmacia Biotech, Aylesbury, UK). The tissue sections were incubated with $5x10^4$ cpm of ^{35}S-labeled probes in a total volume of 100 µl and incubated overnight at 55°C. Hybridization signals were detected after a 10-14-day exposure to NTB-2 emulsion (Eastman Kodak, Rochester, NY, USA) at 4°C.

Northern blotting

Ten micrograms of total rat testicular RNA were size-fractionated in 1% denaturing agarose gel and transferred onto Hybond-N+ nylon membrane (Amersham Pharmacia Biotech, Aylesbury, UK). After hybridization with ^{32}P-labeled SNURF probes (corresponding to nt 234-967 and nt 968-1644 of rat SNURF cDNA) at 68°C overnight, the blots were stripped and subsequently hybridized with 28S rRNA cDNA probe labeled with

[α-^{32}P]dCTP (Amersham Pharmacia Biotech, Aylesbury, UK) by the random priming method (Prime-a-gene kit, Promega, Madison, WI, USA).

Real-time quantitative RT-PCR

Real-time quantitative RT-PCR was performed using the Lightcycler system, FastStart DNA Master SYBR Green I mix (Roche Molecular systems, Indianapolis, IN, USA), and indicated primers according to the manufacturer's instructions. For detailed protocol of the RT-PCR reactions the reader is referred to Fig. 11.

Granulosa cell cultures

Granulosa cells were harvested by needle puncture from the ovaries of E2-primed (1.5 mg s.c. daily for 3 days) immature rats (aged 26 days). The cells were cultured on serum-coated 12-well culture plates at a density of 0.5x10^6 cells per 1.5 ml in Dulbecco's minimal essential medium:F12 containing 100 IU/ml penicillin and streptomycin. On the following day, the cells were washed and cultured in fresh serum-free medium containing FSH (50 ng/ml, NIH FSH-16), testosterone (10 ng/ml), forskolin (10 μM), and/or PMA (20 nM) and harvested after selected time intervals for RNA extraction.

Semi-quantitative RT-PCR

Total RNA were extracted from whole ovaries of PMSG/hCG-primed immature rats, hypophysectomized (H) rats, and E2/FSH/hCG-primed H rats (from Harlan Sprague Dawley, Inc., Chicago, IL, USA), as well as from granulosa cells using Trizol reagent (Life Technologies, Inc., Grand Island, NY, USA). Semi-quantitative RT-PCR was performed using primers specific for SNURF and the ribosomal protein L19. Total RNA (500 ng) was reverse transcribed using oligo-d(T) primer and AMV-RT at 42^{o}C for 75 min and 95^{o}C for 5 min. DNA products were amplified in the presence of [α-^{32}P]dCTP and Taq polymerase for 22 cycles at 94^{o}C for 1 min, 60^{o}C for 2 min, and 72^{o}C for 2 min. The amplified DNA was resolved on a 5% polyacrylamide gel, which was dried, and exposed to X-ray film, and the signal intensities were quantified by using Storm 860 PhosphorImager software (Molecular Dynamics, Sunnyvale, CA, USA).

RESULTS AND DISCUSSION

Expression of SNURF during murine gonad determination and prenatal differentiation (IV)

The murine gonad development begins at E10.5 dpc by migration of primordial germ cells to the urogenital ridges. The gonads first develop in a non-sex-specific manner, but by E11.0 dpc, the Sertoli cells express masculinizing transcription factors, and at E13.5 dpc, the testicular cords have been established (Capel 2000). Previously, Galili *et al.* (2000) reported that SNURF mRNA was detected at high levels in murine embryos from E7.5 dpc onwards. To gain insight into the role of SNURF in gonad determination and differentiation, we analyzed the expression of SNURF mRNA in developing murine gonads at E10.5-E17.5 dpc by *in situ* hybridization. The expression of SNURF mRNA was detected at moderate levels in fetal gonads of both sexes from E10.5 dpc onwards. SNURF mRNA accumulated to high levels in the fetal testis by E15.5-E17.5 dpc, with the most intense signal being localized to testicular cords, indicating that SNURF mRNA accumulation was developmentally regulated. Immunohistochemical staining using polyclonal anti-SNURF antibody (Moilanen *et al.* 1998b) confirmed the presence of SNURF protein in gonocytes, Leydig cells, and some Sertoli cells and in oogonia and supporting cells in the fetal testes and ovaries, respectively, at E17.5 dpc. SNURF interacts with SF1, an orphan nuclear receptor essential for gonadogenesis expressed in pre-Sertoli cells from E10.5 dpc onwards (Curtin *et al*., unpublished results, Luo *et al.* 1994). Moreover, SNURF is able to indirectly facilitate transcription elicited by GATA4, that is expressed to high levels in Leydig cells and Sertoli cells during fetal testis development (Kaiser *et al.* 2003, Ketola *et al.* 1999). Interestingly, GATA4 enhances transcription from the Müllerian inhibiting substance gene by interacting directly with SF1 (Tremblay and Viger 1999). Thus, SNURF might regulate gonad development by modulating transcriptional responses elicited by SF1 and GATA4.

Expression of SNURF in spermatogenesis (II, III)

A variety of widely expressed transcription factors exhibit alternatively spliced testis-specific transcript species. They may be differently regulated in gonads due to tissue-specific alternative promoters, or by signals from cell-specific transcription factors (Daniel and Habener 1998, Schmidt and Schibler 1995). The SNURF gene encodes a 1.0-1.6-kb alternative transcript in adult rodent testis, in addition to the ubiquitous 3.0-kb transcript

(Moilanen *et al.* 1998b, Lyngso *et al.* 2000). We isolated the rat testis-specific SNURF mRNA sequence from the same cDNA library, from which the ubiquitous SNURF transcript was originally identified (Moilanen *et al.* 1998b). Both rat transcripts encode a full-length SNURF protein of 194 amino acids. The shorter rat SNURF mRNA is generated by the use of an alternative poly(A)$^+$ sequence. Similarly, both murine SNURF transcripts encode the full-length SNURF protein and only differ in their 3'untranslated regions (Pero *et al.* 2003).

To analyze whether expression of the SNURF gene was developmentally regulated during postnatal rat testis development, we performed *in situ* hybridization experiments using a series of rat testicular samples representing different developmental phases. The 3.0-kb SNURF mRNA was expressed from newborn to mature testis, whereas the 1.6-kb mRNA appeared only after postnatal day 30, as detected using transcript-specific RNA probes in *in situ* hybridization experiments. The 1.6-kb transcript was abundantly expressed in postmeiotic haploid round and elongating spermatids (step 4-11 spermatids), while the 3.0-kb mRNA localized mainly to spermatogonia and somatic Leydig and Sertoli cells. In agreement with our data, Pero *et al*. (2003) detected the 3.0-kb SNURF transcript in murine spermatogonia and Sertoli cells, whereas the shorter transcript was seen in spermatogenic cell populations. We confirmed the cell type-specific expression of the SNURF transcripts by using methoxyacetic acid (MAA) treatment, which depletes spermatocytes at stages I-VI within 24 h of oral administration (Bartlett *et al*. 1988, Table 8). In MAA rat model, the stages are devoid of spermatocytes on day 4 after the treatment, and on days 18 and 30 the stages lack round and elongating spermatids, respectively (Bartlett *et al*. 1988). The 3.0-kb SNURF mRNA remained constant in testis on post-treatment days 4, 18, and 30. Interestingly, the level of the 1.6-kb SNURF transcript was markedly decreased on day 18, possibly due to the depletion of most of the SNURF-expressing spermatids at stages IV-VI. MAA treatment provided additional evidence that the 3.0-kb and 1.6-kb SNURF mRNAs accumulated mainly in somatic cells and spermatogonia, and spermatids, respectively.

SNURF protein was detected in moderate amounts in postnatal rat testis from day 20 onwards (Fig. 9). It localized mainly to Sertoli cell nuclei before postnatal day 30, but from day 30 onwards nuclei of both Sertoli cells and spermatids were coated with polyclonal anti-SNURF antibody in agreement with the *in situ* hybridization data. Furthermore, the expression of SNURF protein was high at stages VII-VIII, IX-XII, and II-VI, and low at

stages XIII-I. In summary, SNURF protein was expressed in developmentally regulated fashion in the rat testis.

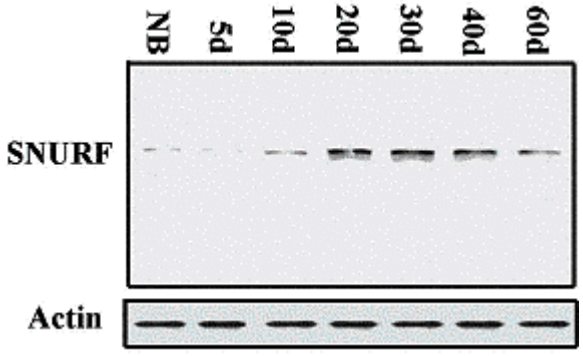

Fig. 9 Western blot analysis of SNURF protein expression during postnatal development of the rat testis. One hundred µg of total testicular protein/lane were resolved on SDS-PAGE, transferred onto nitrocellulose membrane, and immunoblotted using polyclonal anti-SNURF antibody (1:10 000 dilution). The immunocomplexes were visualized with horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (1:10 000 dilution, Zymed laboratories, South San Francisco, CA, USA) and ECL detection system (Amersham Pharmacia Biotech, Aylesbury, UK). The membrane was stripped according to the manufacturer's instructions and reprobed with mouse anti-actin antibody (ICN Biochemicals, Aurora, OH, USA) to monitor sample loading and transfer. d; days after birth, NB; newborn.

SNURF was originally identified as an AR-interacting protein in a yeast two-hybrid screen, and it was found to colocalize with AR in the epithelial cells of the prostate (Moilanen *et al.* 1998b). Since AR is not expressed in the male germ cells, expression pattern of SNURF suggests that its function is not restricted to AR-mediated transcription in testis. In addition to steroid receptors, SNURF interacts with other transcription factors expressed in male germ cells, such as Sp1, and may thus act as a transcriptional coregulator of several signal transduction pathways in the germ cells (Hapgood *et al.* 2001). SNURF is autoubiquitylated, may function as an E3 ubiquitin ligase, and it binds to single-stranded and double-stranded DNA and histones H3 and H4 (Häkli *et al.* 2001, Häkli *et al*., unpublished results). It is tempting to speculate that SNURF participates in protein degradation and chromatin compaction during spermiogenesis.

Immunohistochemical staining using anti-SNURF antibody demonstrated the expression of SNURF protein in Leydig cells, Sertoli cells, as well as in round and elongating spermatids in adult human testis. In addition, SNURF protein was detected in spermatogonia and

spermatocytes at low levels in the human testis. However, on the contrary to the rodent Leydig cells showing slight immunoreactivity, human Leydig cell nuclei stained intensively with the anti-SNURF antibody (III). In testes, SNURF localized mainly to cell nuclei, although predominantly cytoplasmic expression was reported in developing murine neural structures, such as dorsal root ganglia and the neural tube, and in human primary fibroblasts (Galili *et al.* 2000, Kaiser *et al.* 2003). Since SNURF is phosphorylated by casein kinase II *in vitro* and is ubiquitylated *in vitro* (Moilanen *et al.* 1998b, Häkli *et al*., unpublished). Subcellular localization of SNURF may be regulated by protein-protein interactions elicited by covalent modifications in specific cell types. Interestingly, SNURF enhanced nuclear transport of AR and association of AR to nuclear matrix in COS-1 cells by an unknown mechanism (Poukka *et al.* 2000b). Other nuclear receptor coregulators expressed to high levels in male germ cells include PIASxα, PIAS1, and AIB3/RAP250 (Yan *et al.* 2003, Zhang *et al.* 2003). In addition, GRIP1/TIF2 was expressed in Sertoli cell-specific fashion in murine testis (Gehin *et al.* 2002).

Expression of SNURF in murine postnatal ovary (IV)

Several factors important for spermatogenesis, such as c-Kit receptor and Kit ligand and steroid receptors, play also important roles during folliculogenesis (Richards 2001). The abundant amounts of SNURF mRNA and protein in fetal gonads and in spermatogenesis prompted us to investigate the expression of SNURF mRNA during ovarian development. A series of ovarian specimens from newborn to mature mice were subjected to *in situ* hybridization using SNURF RNA probe. We detected SNURF mRNA throughout folliculogenesis appearing at high levels in the oocytes of preantral follicles from postnatal day 14 onwards. SNURF mRNA was detected at moderate levels in granulosa cells of preantral, antral, and Graafian follicles and in the luteal gland. Moreover, immunohistochemical staining using anti-SNURF antibody demonstrated SNURF protein in oocytes, in granulosa and luteal cells, as well as in theca and interstitial cells. Unfortunately, studies of SNURF expression in human ovary and ovarian tumors was out of reach in this work. SNURF protein colocalizes with AR, ERβ, and PR in the granulosa cells, ERα in the oocytes and in the corpus luteum, suggesting that SNURF facilitates steroid receptor-mediated signaling during follicle maturation and luteinization. Some other coregulator proteins have already been implicated in the regulation of female fertility, as exemplified by Nrip1, the null mutants of which are infertile due to ovulatory failure (White *et al.* 2000). Moreover, deletion of the gene encoding SRC-3 resulted in female subfertility (Xu *et al.* 2000). In addition, female mice lacking $TAF_{II}105$, a TATA-

binding protein associated factor, are infertile because of a defect in folliculogenesis (Freiman *et al.* 2001).

Table 7. Expression of SNURF mRNA during gonad development

Prenatal murine	**Male**			**Female**		
development	Germ cells	(pre)SC	LC	Germ cells	GC	CL
E10.5 dpc	+	+	-	+	+	-
E12.5 dpc	+	+	-	+	+	-
E13.5 dpc	+	+	+	+	+	-
E15.5 dpc	++	+	+	+	+	-
E17.5 dpc	++	+	+	+	+	-
Newborn mice	+	+	+	+	+	-
Postnatal rat development						
NB	+	+	-	nd	nd	nd
5d	+	+	-	nd	nd	nd
10d	+	+	-	nd	nd	nd
20d	+	+	+	+	+	nd
30d	++	+	+	nd	nd	nd
40d	++	+	+	nd	nd	nd
60d	++	+	+	nd	nd	nd
Postnatal murine development						
7d	nd	nd	nd	+	+	-
14d	nd	nd	nd	++	+	-
25d	nd	nd	nd	++	+	-
59d	nd	nd	nd	++	+	+
11 wk	nd	nd	nd	++	+	+
Adult human						
	++	+	++	nd	nd	nd

nd; not determined, SC; Sertoli cell, LC; Leydig cell, GC; granulosa cell, CL; corpus luteum.

Posttranslational modifications, such as phosphorylation, acetylation, sumoylation, and ubiquitylation, modify steroid receptor-mediated signaling by affecting the transcriptional activity and/or subcellular localization of the receptors. In the periovulatory granulosa cells, ubiquitin-related modifier SUMO-1 and PR interacted *in vivo*, and inhibition of SUMO-1 expression by luteinizing hormone receptor stimulation was linked to periovulatory induction of PR levels (Shao *et al.* 2003). Recently, ret finger protein-like 4 (Rfpl4), exhibiting a RING finger-like structure with E3 ubiquitin ligase activity, was shown to regulate proteosome-mediated degradation of maturation-promoting factor (MPF), a heterodimer of p34Cdc2 and cyclin B1, during the progression of meiotic cell

cycle in murine oocytes (Suzumori *et al.* 2003). As SNURF possesses a RING finger domain present in many E3 ubiquitin ligases, the abundant expression of SNURF in the maturing oocytes might implicate in a role in regulation of oocyte meiosis. Although proteasomal degradation has been well established in the steroid receptor-dependent transactivation (Lonard *et al.* 2000), SNURF does not appear to mediate ubiquitylation of AR (Tian *et al*., unpublished results).

Transcriptional regulation of the murine SNURF gene (I)

Transcriptional regulation is mediated via promoter regions usually located upstream of transcription initiation sites of the target genes. The diversity of developmental and tissue-specific signals is conveyed by binding of a variety of cellular transcription factors to the specific target sequences on the promoter regions, as exemplified by binding of AR to AREs on the PEM homeodomain gene promoter (Rao *et al.* 2003). Along with the identification of the two alternative SNURF transcripts in rodent testis (Moilanen *et al.* 1998b), we became interested in the regulatory determinants governing transcription from the murine SNURF gene. To identify the regulatory regions responsible for the generation of murine SNURF transcripts, 0.7 kb of the SNURF promoter was isolated. Alternative transcripts may be generated using alternative transcription start sites or different tissue-specific promoters of the gene, as exemplified by the testis-specific promoter of TBP (Schmidt *et al.* 1997). To determine the transcription start sites of the SNURF gene, we performed primer extension assays using two different antisense oligomers and mouse total testicular RNA as a template. A single primer extension product was detected using both primers, suggesting that a single promoter governs the generation of both SNURF transcripts. In addition, Pero *et al.* (2003) published primer extension data that agree with our observations, in that the murine RNF4 transcripts also originate from the same promoter.

There are several GC-rich elements within the SNURF promoter characteristic of housekeeping genes, but a consensus TATA box or a CCAAT box were not located in the vicinity of the transcription start site. To determine the elements critical for basal promoter activity, different fragments of the promoter were cloned in front of the luciferase gene and transfected into mammalian cells. The longest construct (–617/+111 nt) showed very strong basal activity in HeLa, COS-1, and mSC-1 cells. The activity of the region –38/+36 nt exhibited 29-43% of the maximal promoter activity in mammalian cells, indicating that the fragment harbored the elements critical for the basal activity of the SNURF promoter.

Detailed sequence analysis revealed a GC box at +9 nt and a NF-κB consensus site at -8 nt within the core promoter (Fig.10). The GC box was conserved in the human RNF4 gene, suggesting functional importance of the element (Chiariotti *et al.* 1998). Although SNURF is expressed at high levels in spermatids, consensus elements for the testis-specific transcription factor CREM were not found within the SNURF promoter region (data not shown).

Binding of nuclear proteins to the SNURF promoter was examined by electrophoretic mobility shift assay using ^{32}P-labeled probes corresponding to –8/+111 nt and –38/+111 nt. Using nuclear extract, we detected one major and few minor nuclear protein-DNA complexes, which were abolished by the addition of a 100-fold molar excess of unlabeled probes or double-stranded –8/+34 or –8/+24 oligomers. Interestingly, a non-radioactive Sp1 consensus oligomer reduced the formation of the major protein-DNA complex indicating that the Sp family members might bind to the promoter. NF-κB and CREB consensus oligomers did not challenge the probe markedly. Deletion of the proximal GC box from the probe –8/+111 abolished nuclear protein binding to the SNURF promoter and reduced promoter activity to 21% and 13% of the corresponding wild-type construct activity in COS-1 and HeLa cells, respectively.

In search for factors regulating SNURF gene activity, we transfected plasmids encoding Sp1, Sp3, and WT1 gene products, factors binding to GC-rich elements, with the promoter constructs into mammalian cells (Hapgood *et al.* 2001). Ectopically expressed Sp1 marginally stimulated the SNURF promoter in HeLa cells, an effect not seen in the presence of a construct bearing the mutated proximal GC box. WT1, essential for urogenital development, activated the –8/+111 fragment >4-fold in HeLa cells. Interestingly, the WT1 gene product is expressed concomitantly with SNURF at E10.5 dpc in genital ridges of both sexes (Kreidberg *et al.* 1993). Colocalized expression of SNURF and WT1 during early gonad development supports the possibility that SNURF gene activation is dependent on WT1 signaling in fetal gonads *in vivo*. SNURF binds to Sp1, which interacts with components of the TFIID complex and several transcription factors, such as NF-κB and GATA-1 (Chiang and Roeder 1995, Emili *et al.* 1994, Gill *et al.* 1994). The critical GC element on the SNURF promoter may thus mediate activation by tissue-specific and developmental signals in addition to constitutive induction (Hirano *et al.* 1998, Merika *et al.* 1995). Interestingly, Sp1 and Sp3, present in the male germ cells, are among the factors responsible for the testis-specific expression of PIASx coregulators, and

they may thus also stimulate the SNURF promoter in the spermatogenic cells (Santti *et al.* 2003).

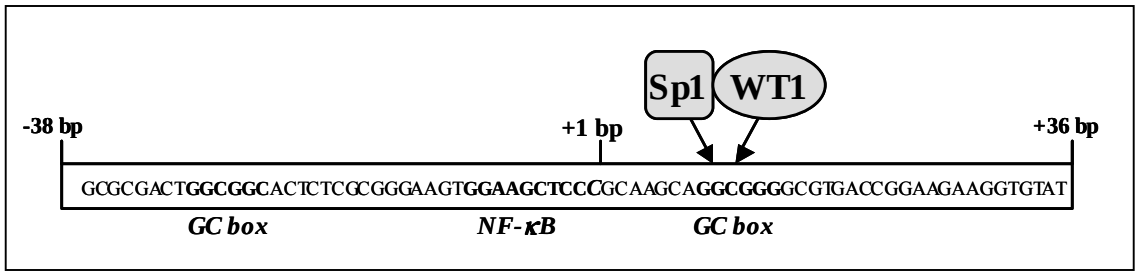

Fig. 10 Schematic presentation of the murine SNURF core promoter. The TATAless proximal promoter contains GC boxes starting at +9 nt and –25 nt, and a putative NF-κB site at –8 nt. The GC element at +9 nt was critical for the proximal SNURF promoter activity, and Sp1 and WT1 gene products were found as potential activators of the murine SNURF gene in mammalian cells.

Hormonal regulation of SNURF gene expression *in vivo* in rodent gonads (II, IV)

SNURF interacts with steroid receptors augmenting their function and is expressed to high levels in steroid hormone target tissues, the testis and the ovary. Thus, we hypothesized that SNURF mRNA might be regulated by steroids *in vivo*. Despite the presence of several steroid receptor response element half-sites in the murine SNURF promoter (at nt +58, -50, -238, -407, and –431 for AR, and +18 and –123 for ER, respectively), ectopically expressed AR or ER in the presence of testosterone or estradiol, respectively, did not affect the activity of SNURF promoter fragments in mammalian cells (data not shown). However, administration of ethylene dimethane sulfonate (EDS), which depletes testicular Leydig cells within 24 h, resulted in undetectable SNURF mRNA levels between post-treatment days 2 and 15 in rat testis concomitantly with the declining testicular testosterone (T) levels (Teerds 1996, Table 8). Twenty days after the EDS administration, by the time that the Leydig cells had recovered their function, SNURF transcript levels accumulated in concert with the raising T levels. Rats, that received EDS and T implants simultaneously exhibited higher circulating T levels and 3-5-fold increased testicular SNURF mRNA levels, respectively, compared to control rats. Steroid hormone response elements are often located up to 10 kb upstream of the transcription start site, and the cis-element responsible for androgen-regulation of testicular SNURF mRNA could be located further upstream of the SNURF promoter analyzed in the transient transfections. The effects of testosterone may also be mediated indirectly. Steroid receptors interact with other transcription factors,

such as NF-κB, and influence their transcriptional activity sometimes even without binding to DNA elements, referred to as 'transcriptional cross-talk' (Göttlicher *et al.* 1998). Androgen action to germ cells is mediated through Sertoli cells and Leydig cells that express AR, and accumulation of SNURF in germ cells is likely to be regulated indirectly by paracrine signaling from the neighboring cells. However, ERβ is expressed in male germ cells and the effects of androgens could alternatively be conveyd to the germ cells by conversion to estrogens through the action of CYP450 aromatase present in the spermatogenic cells (Enmark *et al.* 1997). Interestingly, testosterone has been shown to stimulate the expression of aromatase mRNA in the purified rat germ cells (Bourguiba *et al.* 2003).

In the ovary, estradiol is produced in the granulosa cells by aromatization of the androgen precursors diffusing from the neighboring theca cells (Richards 2001). Because testosterone increased SNURF mRNA level in rat testis either by an indirect mechanism or via conversion to estrogens, we sought to study whether estradiol regulates the accumulation of SNURF mRNA *in vivo* in the murine ovary. In the mouse model, PMSG, a hormone with both FSH and LH-like activity in the rodents, stimulates the growth of preovulatory follicles that synthesize increased amounts of estrogen (Sztein *et al.* 2000, Table 8). We exposed 3-week-old immature female mice to 2.5 IU of PMSG, that marginally attenuated the SNURF mRNA within 48 h after administration, whereas a subsequent injection of 2.5 IU of hCG, mimicking the preovulatory LH surge, slightly augmented ovarian SNURF mRNA content. However, as determined by semi-quantitave RT-PCR, SNURF mRNA level was not dramatically changed in whole ovaries of immature intact rats treated with PMSG and hCG.

The ovaries from mice treated daily with 125 μg of diethylstilbestrol for 3 days increased SNURF mRNA levels in oocytes compared to control mice treated with vehicle, an effect that was blocked by the pure antiestrogen ICI 182,780, suggesting that expression of the SNURF gene was controlled by ER-dependent signaling in the oocytes (Jain *et al.* 1999). The levels of SNURF mRNA remained unchanged in the ovaries of female mice treated with ICI 182,780 alone compared to control mice, implying that estrogen-dependent signaling is not the only mechanism that regulates SNURF gene expression in oocytes. Moreover, ERα, that is expressed in the oocyte, did not stimulate SNURF gene activity in the presence of estradiol in mammalian cells of non-ovarian origin (I).

Table 8: Hormone treatments in animal models		
model	**hormone treatment**	**Consequence**
MAA (Bartlett *et al.* 1988)	650 mg/kg p.o. single dose	Depletion of spermatocytes at stages I-VI after 24 h, lack of round and elongating spermatids 18 and 30 days after treatment, respectively.
EDS (Teerds 1996)	75 mg/kg i.p. single dose	Depletion of Leydig cells within 24 h. Testosterone undetectable between days 2 and 15.
PMSG + hCG (Sztein *et al.* 2000)	2.5 IU PMSG i.p. + after 48 h 2.5 IU hCG i.p.	Preovulatory follicles 48 h after PMSG and 5 h after hCG, post-ovulatory follicles 20 h after hCG, luteinizing follicles 5 d after hCG
DES (Heikinheimo 1997)	125 µg s.c. daily for 3 days	Stimulation of granulosa cell proliferation
ICI 182,780 (Jain *et al.* 1999)	50 µg s.c. daily for 3 days	Inhibition of estrogen-mediated granulosa cell proliferation
Hypophysectomized rats (Zhou *et al.* 1995)	1.5 mg E2 s.c. daily for 3 days + 1.0 µg FSH i.p. twice daily + 10 IU hCG i.p.	Preovulatory follicles 48 h after FSH, postovulatory follicles 16 h after hCG, luteinizing follicles 24 h after hCG

To confirm the hormone-dependent regulation of SNURF mRNA observed in the whole murine ovary *in vivo*, we studied regulation of SNURF mRNA in hypophysectomized (H) rats (Zhou *et al.* 1995). In the PMSG model, it is difficult to dissociate the effects of the steroid from those of the gonadotropin. In the H rat model, E2 is required to stimulate granulosa cell proliferation and formation of the preantral follicles, which are highly responsive to FSH (Zhou *et al.* 1995). After hypophysectomy, SNURF protein levels declined in granulosa cells *in vivo* as determined by immunohistochemical staining, whereas SNURF was constitutively expressed in rat oocytes. Treatment of H rats with E2 for 3 days restored SNURF protein expression in granulosa cells to some extent, but did not increase SNURF protein levels in the oocytes. This provided further evidence that basal activity of the SNURF gene in the oocytes was regulated in an estrogen-independent fashion. On the other hand, estradiol-mediated stimulation of SNURF gene expression might not be clearly detectable at the protein level by immunohistochemical analysis. Granulosa cells were isolated from ovaries of the H rats before and after hormonal stimulation and were used as a source of total RNA for semi-quantitative RT-PCR. SNURF mRNA levels were increased in E2-primed granulosa cells by 3-fold, as determined by semi-quantitative RT-PCR, and were increased further by 12 h after an additional treatment with FSH and hCG. To the best of our knowledge, the specific genes regulated by estradiol in the oocyte have remained speculative, but estrogen-dependent

genes in the granulosa cells include cyclin D2, cyclin E, ERβ, Foxo1 (FKHR), Foxo3 (FKHRL1), and IGF-R1β (Castrillon *et al.* 2003, Richards 2001, Richards *et al.* 2002, Robker and Richards 1998, Sharma *et al.* 1999). Several estrogen-regulated genes, such as ERβ and cyclin D2, are overexpressed in ovarian granulosa cell tumors, that are often hormonally active secreting estrogens (Chu *et al.* 2000, Sicinski *et al.* 1996). SNURF, regulated by steroids and repressed in RAS-transformed cells and testicular tumors, might also contribute to ovarian tumorigenesis (Pero *et al.* 2001, Zuber *et al.* 2000). Taken together, SNURF gene expression is regulated in part by estrogen in rodent oocytes and granulosa cells *in vivo*.

Table 9. Genes induced by testosterone or estradiol in rodent gonads/gonad-derived cells

Androgen-regulated genes	*reference*
SNURF	Yan *et al.* 2002
PIASx	Yan *et al.* 2003
PEM	Rao *et al.* 2003
Aromatase	Bourguiba *et al.* 2003
5α-reductase1	Pratis *et al.* 2003
Estrogen-regulated genes	*reference*
SNURF	Hirvonen-Santti *et al*., submitted.
ERβ	Sharma *et al.* 1999
Cyclin D2	Robker and Richards 1998
Foxo1 (FKHR)	Richards *et al.* 2002
Foxo3 (FKHRL1)	Richards *et al.* 2002
IGF-R1β	Richards *et al.* 2002
Cyclin E	Robker and Richards 1998

To study intracellular signaling mechanisms regulating SNURF gene activation in granulosa cells, E2-primed granulosa cells were treated with the LH agonists forskolin and PMA, that are known to induce several genes, such as progesterone receptor, COX-2, AP1 factors, and Egr-1, in preovulatory cells (Ochsner *et al.* 2003, Russell *et al.* 2003, Sharma *et al.* 2001, Sriraman *et al.* 2003). Moreover, the changes in SNURF mRNA were analyzed during granulosa cell differentiation in response to FSH and testosterone exposure. Forskolin and PMA transiently stimulated SNURF gene expression at 4 h after the treatment and induced SNURF in differentiated granulosa cells (i.e., in cells expressing markers, such as aromatase and LH receptor, of preovulatory phenotype) at 48 h after the FSH/T treatment. Furthermore, SNURF mRNA level was down-regulated in luteal cells when compared to preovulatory granulosa cells. These data suggest that SNURF,

interacting with PR essential for ovulation (Moilanen *et al.* 1998b), may play a role in ovulation.

Expression of SNURF and estrogen receptor β in human testicular germ cells and testicular tumors (III, IV)

The onset of the testicular germ cell malignancies at adolescence implicates in a fetal origin of the disease, and endogenous or environmental estrogens have been suggested to contribute to tumorigenesis (Sharpe 2003). Fetal exposure to excess of estrogens could suppress testosterone production and AR expression in the fetal testis as well as inhibit fetal Leydig cell gene expression, e.g. insulin-like factor-3 (InsL3) production (Nef and Parada 2000, Sharpe 2003). The role of androgens, essential for the normal testicular development and spermatogenesis, is largely unknown in testicular cancer development. Since the testicular expression pattern of SNURF implicated a role in prenatal gonocyte differentiation and spermatid maturation, we determined SNURF mRNA levels in 12 paired normal testis-testicular germ cell cancer samples by real-time quantitative RT-PCR (Fig. 11). In addition to SNURF, we analyzed levels of mRNAs encoding ERβ, AR, PIAS1, and PIASx in the TGC specimen pairs. We found statistically significant repression of ERβ mRNA by 59% in seminomas ($p=0.017$, Wilcoxon signed ranks test) and down-regulation of SNURF/RNF4 and AR mRNA by 67% and 75%, respectively, in seminomas and teratocarcinomas ($p=0.034$ for both). In addition, the mRNA coding for ERβcx/β2 isoform was down-regulated in TGC samples (data not shown), whereas PIASx and PIAS1 mRNA levels were unaltered in TGC. We also examined the levels of cyclin D2 in our TGC material, and in agreement with previously reported observations, we found overexpression of cyclin D2 mRNA with an average increase of 6.8-fold. However, in our small patient group, overexpression of cyclin D2 remained statistically non-significant ($p=0.136$). No statistically significant association was observed between ERβ and SNURF mRNA in seminomas, or between AR and SNURF mRNA levels in all tumors, respectively (data not shown).

ERβ and SNURF protein levels were also repressed in local pT1 seminomas as judged by immunohistochemical analysis. Our results confirm and complement the observations of Pero *et al*. (2001), who reported reduced expression of SNURF in TGC of yolk sac, embryonal carcinoma, and seminoma types. In normal human testis, ERβ protein localized to primary spermatocytes and round spermatids as previously described (Saunders *et al.* 2002). Recently, Pais *et al*. (2003) reported diminished expression of ERβ in seminomas,

embryonal cell carcinomas, and mixed germ cell tumors. Repressed ERβ mRNA and protein levels were reported in prostate cancer (Latil 2001). However, ERβ knockout male mice reproduced normally and showed prostatic hyperplasia, but did not exhibit testicular tumors (Weihua *et al.* 2001). The loss of AR mRNA in tumor samples probably reflects their cellular composition, i.e., the absence of AR-expressing Sertoli and Leydig cells. Accordingly, *Tfm* male mice bearing the mutated AR gene and AR knockout male mice showed Leydig cell hyperplasia, but no germ cell-derived cancer was reported (Lyon *et al.* 1975, Yeh *et al.* 2002). To our best knowledge, expression of other classical nuclear receptor coregulators has not been studied in testicular germ cell cancer. However, several studies indicate altered coregulator mRNA expression in hormone-dependent breast, ovarian, and prostate cancers (Anzick *et al.* 1997, Lee *et al.* 1999, Zhu *et al.* 1999), as exemplified by overexpression of SRC-1 and TIF2/GRIP1 proteins in hormone-refractory prostate cancer (Gregory *et al.* 2001). Examples of genes with altered expression in testicular germ cells tumors are presented in Table 10.

Table 10. Examples of genes with altered expression in TGC

Gene	*Expression in TGC vs. normal*	*Reference*
SNURF	down-regulated	Hirvonen-Santti *et al.* 2003
ERβ	down-regulated	Hirvonen-Santti *et al.* 2003
Cyclin D2	up-regulated	Schmidt *et al.* 2001
CDK4	up-regulated	Schmidt *et al.* 2001
Cyclin E	down-regulated	Schmidt *et al.* 2001
CDK2	down-regulated	Schmidt *et al.* 2001
GRB7	up-regulated	Skotheim *et al.* 2002
c-KIT	down-regulated	Rajpert-de Meyts *et al.* 1994
Ret finger protein	down-regulated	Tezel *et al.* 2002

Immunoreactivity of ERβ and SNURF proteins were detected in the gonocytes in murine fetal testis from E15.5 dpc and E10.5 dpc onwards, respectively, in agreement with the expression of ERβ in Sertoli cells and gonocytes at midgestation in fetal human testis (Gaskell *et al.* 2003). Therefore, already prenatal disturbances in the regulation of estrogen signaling and SNURF expression may play a role in the early stages of TGC development.

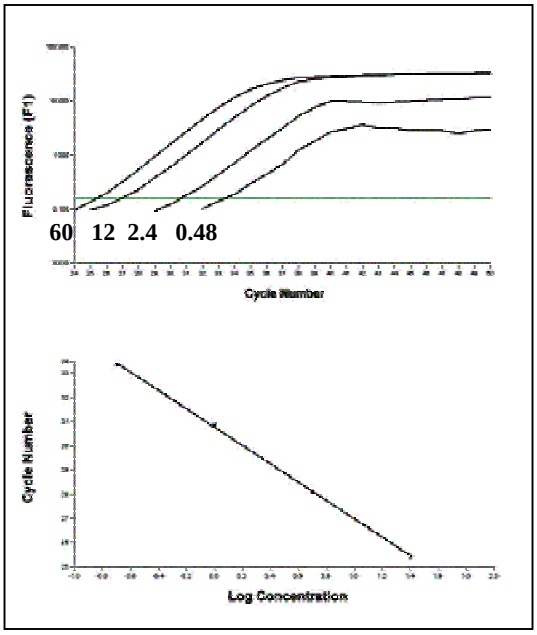

Fig. 11 Quantification of human SNURF gene expression using real-time RT-PCR with Lightcycler system (Roche Molecular Systems, Indianapolis, IN, USA). Lightcycler measures fluorescent signals, that are produced proportional to the concentrations of the PCR products. The higher the cDNA concentration of the template, the earlier a significant accumulation of the PCR product occurs, and thereby the earlier the fluorescent signal is measured. After the PCR run, logarithmic values of fluorescence are plotted against cycle number. **Top:** A logarithmic plot of fluorescence above noise level during amplification. Serially diluted (1:5) standard contained approximately 60 ng, 12 ng, 2.4 ng, and 0.48 ng of normal human testis cDNA. Total RNA from testis samples was extracted using Trizol reagent (GIBCO BRL, Life Technologies) and phenol-chloroform extraction according to the manufacturer's recommendations. Three µg of total testis RNA were annealed to 50 ng of oligo-d$(T)_{12-18}$ primer (Amersham Pharmacia Biotech, Aylesbury, UK) at 70^{o}C for 10 min, and cDNA synthesis was carried out with 5-10 U of avian myeloblastosis virus reverse transcriptase (AMV-RT, Promega, Madison, WI, USA) at 50^{o}C for 1 h and subsequently at 42^{o}C for 1 h. Thermocycling was carried out in a 20-µl reaction volume containing 0.5 µM primers, 3.5 mM $MgCl_2$, 2 µl of FastStart DNA Master SYBR Green I mix (Roche Molecular Systems, Indianapolis, IN, USA) and 2 µl of template cDNA. To avoid amplification of genomic DNA, the forward and reverse primers were designed to represent different exons. Human chromosome 1 contains an intronless SNURF pseudogene, but a number of mismatches in the pseudogene enabled the design of primers specific for the bona fide SNURF gene. No amplification of the product was seen in the absence of reverse transcriptase. **Bottom:** The crossing points (cycle number), plotted against the logarithm of cDNA copy number to construct the standard curve, with the help of which the concentrations of unknown patient samples were determined.

Moreover, the majority of seminomas (7/8) in this study were early stage pT1 tumors further indicating that dysregulation of SNURF and ERβ could be an early event in testicular tumorigenesis. It is believed that the germ cells pass through a carcinoma *in situ* stage before differentiating into a seminoma or a non-seminoma, and because SNURF is repressed in the major TGC types, it could be down-regulated already in the early stages of TGC (Fig. 12, Masters and Köberle 2003).

In summary, our results suggest that ERβ and SNURF might contribute to the development of TGC. Further investigation with larger testicular germ cell cancer sample series are needed to elucidate the roles of SNURF and ERβ during the development and progression of TGC.

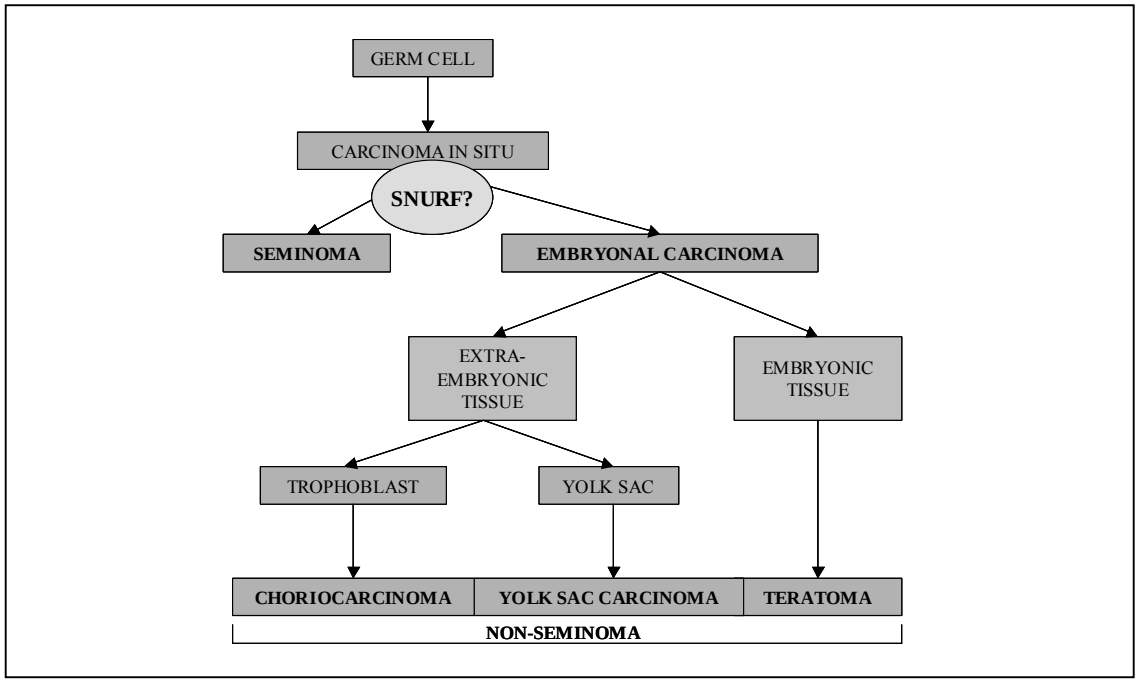

Fig. 12 Hypothetical model of SNURF gene expression in testicular germ cell cancer development. SNURF mRNA, expressed to high levels in fetal testicular gonocytes, was down-regulated in both seminomas and non-seminomas, suggesting that repression of the SNURF gene is an early event in testicular tumorigenesis. Model modified from Masters and Köberle, *Nature Reviews Cancer*, 2003.

CONCLUDING REMARKS

Gene expression data provide clues in definition of biological gene functions. To gain insight into the role of SNURF in reproduction, this thesis work characterized the temporal and spatial expression pattern of SNURF during rodent prenatal and postnatal gonad development as well as in human testicular germ cell tumors. Importantly, the results showed evidence for the regulation of SNURF gene expression in response to steroids and pituitary gonadotropins *in vivo* in the rodent gonads. This implies that SNURF may play a role in gonadal physiology, such as germ cell maturation and ovulation. The main results of the study are summarized below.

- SNURF mRNA is expressed to high levels in fetal and in postnatal germ cells as well as to lower levels in somatic cells in rodent gonads of both sexes.

- Transcription from the murine SNURF gene was governed by a single promoter, which showed very strong basal activity in reporter gene assays in mammalian cells. A proximal GC box at +9 nt relative to the transcription start site was found to be critical for transcription from the murine SNURF gene. Sp1 and WT1 transcription factors were identified as potential activators of the SNURF gene.

- SNURF mRNA levels are regulated by testosterone *in vivo* in the rat testis and by gonadotropins and estrogen *in vivo* in the rodent ovary.

- SNURF protein colocalizes with ERβ in fetal and postnatal male germ cells, and the expression of SNURF and ERβ mRNA and protein is down-regulated in human testicular germ cell tumors.

The study of the SNURF gene expression during gonad development and in testicular tumors offered an exciting area for basic research, the results of which are relevant to our understanding of steroid receptor-mediated signaling, reproduction, infertility, and testicular tumor development and progression. However, further studies using genetically modified mouse models are required for confirmation of the biological significance of the SNURF coregulator in reproduction and development. Furthermore, research with large tumor sample series is required for assessing the role of SNURF in hormone-dependent tumorigenesis.

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