# Molecular approaches to contraceptive development

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The next generation of contraceptives will be based on the identification of novel molecules essential for reproductive processes and will rely on the refinement of older as well as newer technologies. Functional analysis of naturally occurring reproductive genetic disorders and creation of mice null for specific genes would greatly assist in the choice of genetic targets for contraceptive development. Structure-based design of drugs as exemplified by the preparation of an orally active non-peptide gonadotropin releasing hormone (GnRH) would revolutionize drug formulation and delivery for a peptide analogue. This review examines some of the molecular targets that may change contraceptive choices in the future.

## 1. Introduction

In the past fifty years, tremendous progress has been made in chemistry, biology and information technology. Techniques that have made a major impact in the last decade include combinatorial chemistry, high throughput biological screening and structure based drug design (Ohlstein *et al* 2000). A significant influence on the development of next generation contraceptives will come from peptide based orally active non-peptide analogues (Snider *et al* 1991), recombinant proteins (Penichet and Morrison 2001), DNA vaccines (Donnelly *et al* 1997), orally active vaccines in transgenic plants, antisense RNA, oligo-nucleotide technologies (Wahlestedt and Good 1999) and ribozyme-based techniques (Cech 1992) etc. Molecular approaches for contraceptive development rely on the identification of genes and target validation. Some major genomic technologies for identification of gene targets are: expressed sequence tags (est), secreted protein analysis, differential display (Liang and Pardee 1992), DNA micro array (Debouck and Goodfellow 1999) and positional cloning. Recent advances in protein analysis, termed as proteomics, which characterizes proteins of cells and tissues as apposed to mRNA transcripts, will be used increasingly for the identification of target proteins for contraceptive development (Banks *et al* 2000). Transgenic approaches are invaluable in the functional analysis of selected gene targets (Jaenisch 1998). In this review some of the development that has taken place in the identification of gene targets and an update on the structure based design of non peptide orally active mimics will be discussed.

Mammalian reproduction is a complex physiological process that involves interaction amongst factors secreted by the hypothalamo–pituitary and gonadal axes. The reproductive axis is highly regulated, integrating a diverse array of molecular signals (Knobil 1994). An understanding of its molecular mechanism and genetic basis is essential for the manipulation of reproductive ability.

The first breakthrough in the contraceptive technology based on a knowledge of the hypothalamo-pituitary – gonadal axis, was the development of the contraceptive pill by Pincus and Chang (Pincus 1965). The pill provided a method which was highly effective, easy to use and not directly linked to intercourse. The first generation of pills has undergone refinements in terms of drug load and delivery systems. The next significant breakthrough was the synthesis and evaluation of the anti progesterone,

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Abbreviations used: AFZ, Azoospermia factor; COX-2, cyclooxygenase-2; FSH, follicle stimulating hormone; GnRH, gonadotropin releasing hormone; LH, luteinizing hormone; LIF, leukaemia inhibitory factor; NSAIDs, nonsteroidal anti-inflamatory drugs; PACAP, pituitary adenyl cyclase activating peptide; PK, progesterone receptor; RA, retinoic acid.

RU 486. Mifepristone could induce abortion in early pregnancy and is licensed in combination with prostaglandin for this purpose (Lebeau and Baulieu 1994).

The rationale for the next generation contraceptive development to a large extent will be non hormonal, but again will be based on knowledge gleaned from an analysis of healthy individuals with reproductive failure and from the study of genes essential for reproductive processes from reverse genetics, e.g. mice null for specific genes (Harrison and Rosenfield 1996). There is sufficient evidence to show that genes that encode factors involved in reproduction are structurally and functionally conserved between human beings and mice.

## 2. Approaches to new contraceptive methods for females

A thorough knowledge of the female reproductive process starting from oogenesis, follicular maturation, ovulation, fertilization and implantation will have a direct bearing on contraceptives strategies. Successful follicular development and ovulation is orchestrated by three hormones: gonadotrophin releasing hormone (GnRH), luteinizing hormone (LH) and follicle stimulating hormone (FSH) and by gonadal steriods. Follicular maturation and ovulation in females and spermatogenesis in males can be tampered by GnRH agonists or antagonists. The primary structure of GnRH was elucidated two decades ago.

### 2.1 *Gonadotrophin releasing hormone*

GnRH or pGlu·His·Trp·Ser·Tyr·Gly·Leu·Arg·Pro·Gly·$NH_2$ is produced from a precursor polypeptide in hypothalamic neurons and secreted in a pulsatile manner to stimulate LH and FSH from the pituitary (Fraser and Bouchard 1994). A normal or physiological GnRH pulse frequency induces the synthesis of mRNAs for the common *a*-subunit as well as that of *b*-subunits of the LH and FSH. The activity of the GnRH pulse generator is governed by several factors including gonadal steroids and some neurotransmitters. Noradrenalin stimulates the system, while dopamine and opiates such as *b*-endorphins inhibit it. Low doses delivered in a pulsatile manner restore fertility in hypogonadal patients and has been used to treat cryptorchidism and delayed puberty (Conn and Crowley 1994). The reduction of the physiological frequency of GnRH pulses from one pulse every 60–90 min increases the circulatory concentration of FSH and reduces those of LH. This suggests a method of modulating the function of either LH or FSH by regulating the pulsatile release of GnRH (Knobil 1990).

Many potent agonists and antagonists of GnRH have been made and tested (table 1). Agonists operate in a biphasic manner initially, stimulating and ultimately inhibiting the release of GnRH. Antagonists on the other hand act by blocking the receptor. Suppressing the action of GnRH either by the use of antagonist or by antibodies against the hormone requires replacement with gonadal steroid hormone. The desensitization with GnRH analogues also has a major disadvantage in that it stimulates the pituitary and gonadal axis before becoming refractory. Even though this can be overcome by GnRH antagonists the dosage required is high – about milligrams (Amory and Bremer 2000). Further, these peptides are not orally active and require administration by injection or nasal route. Biodegradable depot preparation has also been examined as an alternate means of delivery. However, withdrawal of treatment when desired by patients or the physician becomes difficult. The solution for this is in

**Table 1.** Structure of GnRH agonist and antagonist.

| Agonist | Structure | Antagonist | Structure |
|---|---|---|---|
| Leuprolide | pGlu-His-Trp-Ser-Tyr-DLeu-Arg-Pro-Et$NH_2$ | Nal-Glu | [*N*-Ac-DNal$^1$, DpCl-Phe$^2$, DPal$^3$, Arg$^5$, DGlu$^6$(AA), DAla$^{10}$]GnRH |
| Triptorelin | pGlu-His-Trp-Ser-Tyr-DTrp-Arg-Pro-Gly-N | Detirelix | [*N*-Ac-DNal$^1$, DpCl-Phe$^2$, DTrp$^3$, DhArg(ET$_2$)$^6$, DAla$^{10}$]GnRH |
| Buserelin | pGlu-His-Trp-Ser-Tyr-Dser[0'bu]-Arg-Pro-Et$NH_2$ | Cetrorelix | [*N*-Ac-DNal$^1$, DpCl-Phe$^2$, DPal$^3$, DCit$^6$, DAla$^{10}$]GnRH |
| Hirsterlin | pGlu-His-Trp-Ser-Tyr-Dhis[Bzl]-ArgPro-Azaglyn$H_2$ | Gamirelix | [*N*-Ac-DNal$^1$, DpCl-Phe$^2$, DPal$^3$, DhArg(ET$_2$)$^6$, DhArg(ET$_2$)$^8$, DAla$^{10}$]GnRH |
| Nafarelin | pGlu-His-Trp-Ser-Tyr-DNal[2]-ArgPro-Gly $NH_2$ | Antide | [*N*-Ac-DNal$^1$, DpCl-Phe$^2$, DPal$^3$, Lys(Nic)$^5$, DLys(Nic)$^6$, Lys(iPr)$^8$ DAla$^{10}$]GnRH |
| Goserelin | pGlu-His-Trp-Ser-Tyr-Dser[0'bu]-Arg-Pro-Azaglyn$H_2$ | Antarelix | [*N*-Ac-DNal$^1$, DpCl-Phe$^2$, DPal$^3$, D(Hci)$^6$, Lys(iPr)$^8$, DAla]GnRH |
| | | Azaline B | [*N*-Ac-DNal$^1$, DpCl-Phe$^2$, DPal$^3$, DAph(at,)$^5$, DAph(at,)$^6$, Lys(iPr)$^8$, DAla$^{10}$]GnRH |

creating an entirely non-peptide molecule that mimics the three-dimensional features of GnRH. The advantage of non-peptide mimics is that they provide structural diversity (necessary to allow optimization of oral bioavailability), specificity and pharmacokinetic properties.

### 2.2 *Non-peptide GnRH mimic*

The essential features necessary for the design of a non-peptide mimic are the X-ray crystal structure or molecular model of the receptor and a map of the amino acid residues involved in hormone binding. The cloning of the GnRH receptor gene from a mouse pituitary cell line revealed that it is a member of the large G protein family related to the rhodopsine *b*-adrenergic receptor super family which is characterized by seven transmembrane domains connected by intracellular and extracellular domains (Tsutsumi *et al* 1992). Following the cloning of the murine GnRH receptor gene, many mammalian GnRH receptors including human GnRH receptor have been cloned and over expressed (Chi *et al* 1993; Zhou *et al* 1995). A molecular model for the GnRH receptor has also been generated (Miller *et al* 2000). Critical amino acid residues involved in hormone binding have been identified using the approach of site directed mutagenesis. The GnRH binding pocket has indicated the approximate size of the hormone binding region. Based on this a non-peptide molecule that could occupy this site and function as a competitive antagonist was synthesized and assayed for its activity (Kakar *et al* 1992). The lead molecules were further modified to improve binding affinity and biological activity. Because of its commercial potential much of the relevant information has been patented. Abbots and Takeda are two major pharmaceutical players in this field. The structure of the non-peptide analogues (Cho *et al* 1998) that are reported are shown in figure 1. The first non-peptide antagonist is a fused tetracyclic benzodiazepine which blocked ovulation in rats at a concentration of 0·5 mg/kg body weight. The antifungal drug ketoconazole inhibited rat pituitary GnRH with an apparent 2 μM. Addition of a number of groups to this core structure, such as di- and tri-peptides related to GnRH improved affinity to 500 nM. Abbot has recently reported a modified erythromycin A with potent GnRH antagonist properties (Miller *et al* 2000). Quinolones and derivates of indoles with GnRH antagonistic properties have been described by Merck (DeVita *et al* 2001). Intravenous administration of this drug to rhesus monkeys resulted in the suppression of LH (79% decrease in area under the curve) and testosterone (92% decrease in area under the curve), at a dose of 3 mg/kg. The interaction of non-peptide GnRH antagonists with specific residues in the GnRH receptor remains to be elucidated. The successful entry of non-peptide orally active GnRH analogue would form the foundation for a new generation of contraceptive and therapeutic agents. This will be the proto type model for preparing non-peptide mimics based contraceptives from their peptide analogues.

### 2.3 *Follicle stimulating hormone*

The family of human glycoproteins, including FSH, are hetrodimeric proteins, each composed of subunits that are non covalently linked but tightly associated. FSH and its receptor structure has also been extensively studied with the hope that knowledge gained could be used to design new hormone mimetics with appropriate agonistic or antagonistic properties devoid of side effects such as immunological resistance or hyper stimulation of gonads. To study the structure and function relationship several methods were employed using synthetic peptides, site directed mutagenesis and monoclonal antibodies for epitope maping. This has provided a reasonable model to map the receptor-binding region of FSH. The *a*-subunit of FSH features prominently in the receptor binding region and the *b*-subunit determinant loop serves as a discriminator in addition to stabilizing the binding interaction with the receptor (Dias 1996). Production of a biologically active single chain gonadotropin of both FSH and LH will certainly help in the design of suitable analogues (Sengupta and Dighe 2000).

The FSH receptor is a glycoprotein consisting of an extracellular domain (ECD) in excess of 300 amino acid and 7 *trans*-membrane segments typical of G protein–coupled receptor (GPCR). Hormone binding sites are located in the N-terminal region of FSH R-ECD (Nechamen and Dias 2000). Use of site directed mutagenesis has led to the identification of amino acids involved in the interaction of FSH and its receptors. It should be possible with the molecular modelling of the receptor and with a knowledge of the sequence of the FSH binding region to prepare non-peptide mimic for either FSH analogues or antagonists. Already a lead molecule, Suramin – poly sulphanated naphthyl urea – has been shown to compete with human FSH for its receptor (Stevis *et al* 1999). It is not unrealistic to hope that a contraceptive based on a non-peptide FSH could become reality.

Requirement of FSH in follicular development and maturation is well established and was conclusively proved by the creation of mice null for FSH as well as the FSH receptor (Kumar *et al* 1997; Dierich *et al* 1998). The phenotype of mice null for FSH receptor, FSH-R-/- is reminiscent of human hyper gonadotropic ovarian dysgenesis and infertility. Recently a single amino acid Ala 189 to Val mutation, affecting the extracellular domain of the FSH receptor gene, was found to be associated with

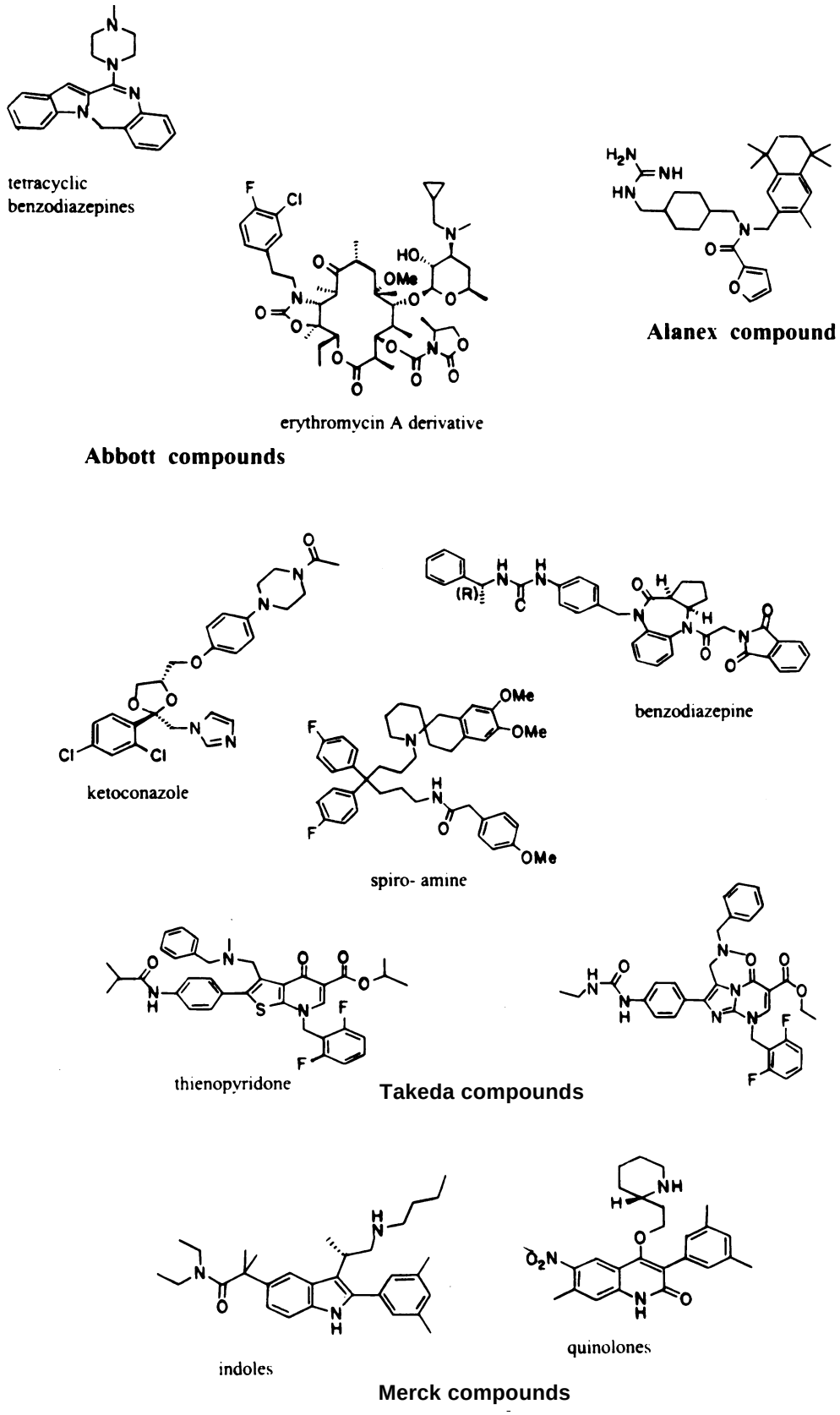


**Figure 1.** Chemical structures of the non-peptide GnRH.

premature ovarian failure in 22 women in 6 families in Finland presenting with primary amenorrhea (Aittomaki *et al* 1996).

A number of genes which are regulated by FSH, and which mediate FSH action have been identified and their function analysed. These could be evaluated for contraceptive potential. Some of these are inhibin/activin, cyclins and c-*MOS*.

### 2.4 *Inhibin analogues*

Male and female gonads produce a number of non-steriodal factors which fine-tune the hypothalmo-pituitary gonadal axis by autocrine, paracrine and endocrine mechanisms. A molecule that could suppress FSH selectively would be an attractive method to suppress spermatogenesis with out affecting testosterone levels and function. Therefore, there is a tremendous interest in developing analogues for inhibin.

The ovary undergoes continuous morphological and biochemical changes that result in the production of mature oocytes. The cyclical proliferation and differentiation of cell types is essential for ovarian cell functions. These are controlled by endocrine/paracrine and autocrine factors. Among the regulatory factors produced within the ovary are the polypeptide hormones inhibin and activin. Inhibin and activin are dimeric polypeptide hormones that belong to the transforming growth factor (TGF$b$), superfamily of growth and differentiation factors. Inhibins suppress FSH secretions from anterior pituitary gonadotrophic cells, whereas activins stimulate FSH synthesis and secretion. Inhibin are heterodimers of a unique $a$-subunit and one of two highly related $b$-subunits (inhibin A = $ab_A$ and inhibin B = $ab_B$). Activins on the other hand are formed by the hetero dimeric combination of 2$b$-subunits (activin A = $b_Ab_A$, activin AB = $b_Ab_B$ and activin B = $b_Bb_B$). Foliostatin by binding to activin regulates the action of activin. To understand the critical role of this group of gene products in ovarian physiology, the transgenic approach was exploited by creating mice null for these genes. However, the role of inhibin could not be established unequivocally, since ovarian tumours was observed in female mutant mice by the 5th week of age (Matzuk *et al* 1992). In the case of activin, it has been reported that it can promote follicular assembly and growth. However, knockout study with the activin receptor, Acti RcII did not support this hypothesis (Matzuk *et al* 1995). Females deficient in activin receptor gene had normal follicular development until the pre-ovulatory stage. However, ovulation was hampered due to inefficient preparation of granulosa cells (due to the decreased FSH) for the LH surge.

In the case of males, germ cells and somatic cells such as sertoli cells produce inhibin/activin. It was therefore thought to be involved directly in the process of spermatogenesis (de Kretser *et al* 1999). However, knockout data with receptors for activin failed to support this suggestion completely but a delayed fertility was apparent.

It is believed that in human and non human primates, the requirement of inhibin for spermatogenesis is different from that of rodents. The molecular cloning of inhibin, activin and its receptors have been accomplished, critical amino acid residues involved in the interaction of the hormone with the receptor could be identified using site directed mutagensis and other methods. It is likely that structure based approachs will lead to a rational method for the design of analogues. The molecules could then be examined for suppression or over-stimulation of follicular development in females and to inhibit or stimulate spermatogenesis.

### 2.5 *Cyclin $D_2$ and* c-MOS

These gene products were thought to be intimately associated with meiotic maturational events having a critical role in male and female gametogenesis. With this in mind, mice null for *c-MOS* were created (Hashimoto *et al* 1994). Normal spermatogenesis was seen in males and reduced fertility in females, indicating that the gene was not essential for gametogenesis.

There are three genes involved in cell progression: Cyclin $D_1$, Cyclin $D_2$ and Cyclin $D_3$. Targeted disruption of cyclin $D_2$ was attempted (Sicinski *et al* 1996) and the resulting mutant males were found to be normal but had a small testis while the females were infertile. Increased expression of these were associated with neo-plastic growth.

### 2.6 *Genes associated with ovulation*

A contraceptive which would block ovulation by inhibiting key genes without hampering the menstrual cycle would be desirable. Inhibition of ovulation as a mode of contraception by the non-hormonal method is an attractive concept. It is not unrealistic to envisage the possibility that a woman could accurately determine impeding ovulation and block crucial ovulation gene products by inhibitors. There are kits already available in the market for determining safe periods and the time of ovulation.

Three gene product have been demonstrated to be essential for ovulation. They are cyclooxygenase-2, (COX-2), CCAAT/enhancing binding protein $b$(C/EB P$b$) and progesterone receptor (Orly 2000). The genes are induced and regulated by a LH surge or molecules that trigger ovulation. Mice null for these also show loss of ovulation. It is now essential to extend these studies to human and non-human primates to see if LH induces the same genes during ovulation in them too.

### 2.7 *Cyclooxygenase-2*

COX-2 is the hormone-inducible isoform of COX, also known as prostaglandin endoperoxide H synthase-2, PGHS-2, which catalyzes the first and rate-limiting step in the conversion of arachidonic acid to the prostaglandins (PGs), thromboxane, and prostacyclin. PGHS-2 is induced in granulosa cells of the periovulatory follicles by LH surge (Sirois *et al* 1992). Mice null for this gene failed to ovulate and lacked corpora lutea (Dinchuk *et al* 1995). Due to their importance in the pharmaceutical industry for the development of potent inhibitors, the enzymes cyclo-oxygenase-1 and 2 have been characterized thoroughly. There are two classes of nonsteroidal anti-inflamatory drugs (NSAIDs). All clasical NSAIDs can inhibit both forms of the enzyme. About 1% of users of NSAIDs develop ulcers or other serious gastrointestinal complications each year due to the inhibition of prostaglandin synthesis by PGHS-1. On the other hand COX-2 inhibitors can prevent inflammatory reactions without causing drugs-associated health risks (Smith *et al* 2000; Fabiola *et al* 2001). Already several inhibitors for COX-2 are available. Experimentation and clinical trials are required to assess if these drugs can suppress ovulation.

### 2.8 *CCAAT/enhancing binding protein*

The transcription factor, C/EBP is associated with the regulation of ovarian function. Analysis of the PGHS-2 promotor revealed a recognition site for this factor (Orly 2000). Mice null for the C/EBP gene showed ovulation defects (Sterneck *et al* 1997). However, PGHS-2 activity was constitutively expressed indicating that transient expression triggers programmed events leading to ovulation. Specific inhibitors which block the interaction of the transcription factor with its specific promoter could lead to suppression of ovulation. The inhibitor could be a specific oligonucleotide or could be a structure-based molecule.

### 2.9 *Progesterone receptor*

The progesterone receptor (PR) is a member of the nuclear receptor transcription factor super family. Progesterone has a crucial role in regulating female fertility. Although usually associated with the maintenance of pregnancy, progesterone is essential for ovulation as demonstrated by prevention of ovulation by progesterone receptor blockers as well as inhibitors of progesterone biosynthesis. Use of antiprogesterone as a method of contraception and abortificient (with RU 486) is well known. PR is induced within few hours following a surge in the concentration of LH (Natraj and Richards 1993). PR is not mediated via estrogen responsive element in the PR promoter since follicular estradiol production drops before the LH surge, and also PR in granulosa cells dose not respond to estrogen. Mice null for PR are sterile and show ovulation defects (Lydon *et al* 1996). Recently Robker *et al* (2000) have shown that proteases ADAMTS-1 (a disintegrin and metalloproteinase with thrombospondin like motifs) and cathepisin L (a lysomal cystein protease) are essential and progesterone responsive genes are involved in ovulation. The challenge is now to design a specific inhibitor which could inhibit these enzymes and prevent ovulation.

### 2.10 *Genes essential for implantation*

Implantation of the embryo in the uterus depends on a complex series of interactions between the trophoblast of the embryo and the deciduas of the uterus. Endometrial development is dependent on the cyclic influence of estrogen and progesterone, but the molecular mechanism by which these hormones control implantation is not well understood (Simon *et al* 1995). Progesterone induces the several genes that may be involved in this process including leukaemia inhibitory factor (LIF), calcitonin and feritin heavy chain (Bagchi 2000). Several integrin subunits exhibit dynamic regulation in human endometrial epithelial cells during menstrual cycle and in implanting trophoblasts (Coutifaris *et al* 1998; Lessey *et al* 1995). In transgenenic mice in which the LIF gene was mutated, the embryos failed to implant due to uterine hostility. LIF analogues which could inhibit this process could again be suitable for contraceptive development. HOX genes, important regulators of tissue differentiation in the embryo, have been demonstrated to play a critical role in endometrial development and receptivity. HOX genes act by either activating or repressing downstream genes, which determine patterning of body segments. HOX genes all have a similar 180 bp sequence termed the homeobox that codes for a 60 amino acid homeodomain which binds to DNA via a helix–turn-helix motif. Two HOX genes have been shown to be involved in female fertility. Female mice with a targeted disruption of either HOXA 10 or 11 are viable but have uterine factor infertility. They produce a normal number of embryos and these embryos are viable in a wild type surrogate mother. However, neither these embryos nor the wild type embryos survive or implant in the uterus of HOXA 10–11 knockout mice. In the human HOX 10 and HOX 11 are expressed in the endometrial glands of the human uterus through the menstrual cycle. It is suggested that estrogen and progesterone regulate HOX genes, which in turn regulate other genes in a pathway that leads to proper molecular development of the human endometrium and receptivity to implantation. A mutation

in the HOXA 13 gene causes reproductive tract anomalies in females further strengthening the hypothesis that HOX genes have a role in endometrial function (Taylor 2000).

## 3. Approaches to new contraceptive methods for males

There is a need to improve contraceptive options, particularly for men. At present there are four options (i) abstinence from heterosexual intercourse, (ii) withdrawal prior to ejaculation, (iii) condoms and (iv) vasectomy. Excellent reviews have appeared on this topic and on the development of vaccine based on sperm antigens and will not be discussed here (Anderson 2000; Amory and Bremner 2000; Herr 1996; Shah 1999). New approaches would involve, targeting spermatogenesis using inhibition of FSH secretion, or FSH action using receptors blockers, inhibiting meiosis, affecting epididymal function to disrupt sperm maturation, inducing premature acrosomal activation identifying genetic loci that affect gamete development or behaviour and developing inhibitors of these functions. Control of male fertility by the inhibition of meiosis is a fascinating concept. The major advantage is that it is extremely selective in its action.

### 3.1 *Role of FSH in spermatogenesis*

The critical role of FSH in spermatogenesis, particularly in non human primates has been well documented (Moudgal *et al* 1992). Neutralization of endogenous FSH with specific antibody in the adult male bonnet monkey results in disruption of spermatogenesis leading to oligospermia and infertility. Molecular cloning of FSH receptor has been accomplished and has been over expressed and recombinant protein has also been prepared (Sharma and Catterall 1995; Khan *et al* 1997). Immunization of male monkeys with FSH receptor corresponding to residues 1–134, resulted in receptor blocking antibodies. The immunized monkeys showed a 50% reduction in transformation of spermatogonia (2c) to primary spermatocytes (4c) as demonstrated by flowcytometry. Breeding experiments indicated that the monkeys were infertile (Moudgal *et al* 1997). Thus these observation again suggest an important role for FSH spermatogenesis. Men with a FSH receptor gene mutation show variable spermatogenic defects (Tapanainen *et al* 1997). Near normal levels of spermatogenesis in FSH/FSH receptor mutated mice (Kumar *et al* 1997) suggest a possible species specificity for the requirement of FSH in spermatogenesis.

### 3.2 *Estrogen receptor*

The requirement of estrogen in ovarian function is well known and was clearly demonstrated in estrogen receptor *a* knockout mice. Female animals failed to ovulate and ovaries had large hemorrhagic cysts. This effect could be due to a hyper androgenic response to the ovary. Studies in males confirmed an essential role of estrogen in sperm maturation since these male mice had high proportions of abnormal sperms and were infertile. Further analysis revealed that estrogen is required for fluid resorption in the efferent ductules to maintain a concentrated milleu of sperm maturation factors. Mice null for the recently discovered estrogen receptor *b* on the other hand did not show any effect on male fertility even though high ER *b* level is seen in epididymis and prostate. On the other hand, female mice were infertile (Couse and Korach 1999). Tamoxifen, an estrogen inhibitor antagonist, when administered in male monkeys showed a high percentage of abnormal sperms and the animals were infertile (Rao *et al* 1998). The study of a wide range of estrogen modulators which bind to the estrogen receptor but produce tissue specific effects is in progress. A male contraceptive based on antiestrogen could be available if the effort succeeds.

### 3.3 *Genes implicated in meiosis: check point regulators*

The normal human cell has 46 chromosomes arranged on 22 pairs of autosomes and with two X chromosomes in the female or an X and Y in the male. On these chromosomes are anywhere between 30,000 to 100,000 genes. The male determining gene SRY is located on the short arm of the Y chromosome. However, several genes involved with spermatogenesis are located on the long arm of Y chromosomes. Spermatogenesis is unique because the DNA content of sperm is half that of spermatogonia. In the initial stages mitotic division give rise to spermatocites. These undergo meiotic divisions to form haploid round spermatids. The round spermatids elongate in a process called spermiogenesis and during this process, DNA becomes compacted in the sperm head. These changes are under genetic control. It is estimated that there are 2000 genes which regulate spermatogenesis, most of these being present on autosomes with approximately 30 genes being on the Y chromosomes (Hargreave 2000). In general autosomal genes that regulate spermatogensis are also involved in the regulation of other metabolic processes in other cells in the body, whereas Y-linked genes are not essential for vital functions other than reproduction. Understanding the genetics of spermatogenesis holds the promise for the development of novel non-hormonal based male contraception.

The phenomenon of meiosis is crucial for the survival of the species. The individual stages of meiosis involving chromosome pairing, recombination and the two cellular

divisions after meiotic prophase are highly conserved in single cell to multicell eukaryotes. In recent years, major advances have been made in understanding the regulation of cell cycle through studies using yeast. Many gene products involved in this process are highly conserved. The integrity of genetic information is maintained through the operation of cell-cell check points. Check points refers to control mechanisms that enforce the proper order of cell-cycle events. Check points are not confined to cells that divide mitotically but operate during meiosis in gametes. In particular, a checkpoint prevents exit from the pachytene stage of meiotic prophase when meiotic recombination and chromosome synapsis are incomplete (Roeder and Bailis 2000).

A gene targeting approach has been rewarding in understanding the genes involved in reproduction. The number of genes that are specific for reproductive tissues as well as other tissues that lead to reproductive failure have been discovered indicating that special care needs to be exercised in the identification of molecular targets for the development of contraceptives (Kumar and Matzuk 2000). Some of the gene products that hold promise are discussed below. Male infertility due to chromosome anomalies have been well documented. The molecular basis for this can now be examined by analysing for these gene products.

### 3.4 *DMC1*

DMC1 is a meiosis–specific gene first discovered in yeast that codes for a protein with homology to RecA and may be a component of recombination modules. Mice null for this gene are sterile with arrest in the first meiotic prophase. Chromosomes in mutant spermatocytes fail to synapse, despite the formation of axial elements that are the precursor to the synaptonemal complex (Pitman *et al* 1998).

### 3.5 *MutS*

MutS gene is a member of of the mammalian mismatch repair protein family. MutS and Mutl homologues have been implicated in post replicative mis-match correction and chromosome interactions during meiotic recombination. Ablation of Msh5, a homologue of Muts, results in male and female sterility in mice (de Vries *et al* 1999). Histological and cytological examination showed an extended zygotene stage, due to impaired and aberrant chromosomes synapsis, that was followed by apoptotic cell death. Recently, a human homologue of MutS, named as hMSH5 has been characterized. The expression of this gene is induced during spermatogenesis between the late primary spermatocyte and elongated spermatid phases.

### 3.6 *Morc*

Recently, a gene named Morc, of unknown function, has been associated with a genetic disorder called micro-orchidia, a term for abnormally small testes. The Morc product is expressed during the earliest stage of sperm development. This gene and its product are highly conserved functionally. Mice null for Morc to start with were normal but the spermatogenesis machinery was affected. Morc function is highly selective with female mice being normal and fertile. Establishment of the function of this gene could provide new targets for the development of male contraceptives (Watson *et al* 1998).

### 3.7 *PMS2*

PMS2 is a DNA mismatch repair enzyme. Its function is seen only male reproduction (Baker *et al* 1995). Meoisis defects leads to abnormal spermatozoa. The epididymis contains fewer and abnormal sperms in male mice null for this gene.

### 3.8 *HR6B*

The ubiquitin pathway in mammals is involved in the selective removal of a number of short lived cell cycle regulator molecules, transcription factors and cell surface receptors. Gene ablation in mice (Roest *et al* 1996), revealed the involvement of this gene in post meiotic chromatin condensation of spermatids. Absence of this gene resulted in accelerated apoptosis in the spermatogenic cells. The HR6B path way is implicated in histone degradation and subsequent protamination that is critical for post meiotic chromatin remodelling.

### 3.9 *HSP70-2*

The G2/m phase transition during mitotic and meiotic cell cycles is dependent on CDC2 kinase activity. Heat shock proteins are protein associated chaperones vital for folding, transport, assembly and disassembly of polypeptides. Of the four, hspa and hsp70-2 are testis specific and developmentally regulated. Mice null for either genes are infertile. Careful examination of the mutants indicated that HSP70-2 has critical role in three processes: meiosis, apoptosis and synaptonemal complex function in pachytene spermatocytes (Dix *et al* 1996).

### 3.10 *Heat shock transcription factor*

In mammals, testicular temperature is lower than body temperature, and the vulnerable nature of spermatogenesis to thermal insult has been known for a long time (Setchell

1994). The primary target that is affected by increase in temperature is not known. Nakai *et al* (2000) have shown male mice expressing an active form of heat shock transcription factor (HSF-1) in the testis are infertile due to a block in spermatogenesis. The germ cells entered meiotic prophase and were arrested at the pachytene stage with an increase in apoptotic germ cells. In wild type male mice, a single heat exposure caused similar changes. Male infertility due to thermal insult could be due to activation of HSF-1. It is now necessary to see if a similar transcription factor is present in humans and if it gets up regulated by thermal insult in human and higher animals (Nakai *et al* 2000). Use of oligonucleotides which inhibit or promote HSF-1 binding to DNA would be an attractive way to modulate the action of HSF-1 and spermatogenesis.

### 3.11 *CREM*

Signalling by cAMP plays a major role in spermatogenesis and is induced by sertoli cells in response to FSH and pituitary adenyl cyclase activating peptide (PACAP). PACAP is also produced in high concentration in the testis. The expression of genes induced by cAMP is regulated by two transcription factors CREB (cAMP response element binding protein) and CREM (cAMP response element modulating protein). Involvement of CREM protein in spermiogenesis was conclusively proved by creation of mice null for this gene (Nantel *et al* 1996).

### 3.12 *Azoospermia factor*

Disruption of spermatogenesis centred on post translational control of gametogenesis offers an opportunity to develop contraceptive strategies. It is known that approximately 13% of human azoospermia is due to *de novo* deletion of the azoospermia factor (AFZ) region of the Y chromosome, which encodes several RNA binding proteins. It is believed that since these deletion have no deleterious effect on general health, contraception based on the use of a molecule that could inhibit RNA–protein interaction may mimic changes similar to mutations in genes encoding essential RNA binding proteins and therefore result in infertility. While the identity of the critical missing gene is yet to be established, a strong candidate is the putative RNA binding protein DAZ (deleted in azoospermia). In humans, three microdeletions are associated with male infertility, designated as AZF*a*, AZF*b* and AZF*c* (Reijo *et al* 2000). The *daz* genes encode testis-specific RNA binding proteins with similar sequence structure. The levels of these genes are much higher in the testis than in the ovary suggesting that these genes act at an early stage of meiosis but are more prevalent in active testis than ovary, where meiosis is suspended in fetal life. In humans, DAZ proteins are located apically in the seminiferous epithelium and in the tails of spermatozoa suggesting that these may have a role in late spermatids. DAZLA, an autosomal version of DAZ, is expressed in mice and rats. Dazl immunoreactivity was found predominantly in the cytosol of primary pachytene spermatocytes. A weaker but clearly detectable signal was present in intermediate and B spermatogonia and in early spermatocytes from preleptotene to zygotene. The highest expression patterns were observed between stages IV and VIII during the spermatogenic cycle when spermatocytes prepare for the first meiotic division. Specific staining could also be observed in step 11–19 elongating spermatids in the acrosome region (Rocchietti-March *et al* 2000). The disruption of DAZLA in mice leads to complete absence of sperms beyond the spermatogonial stage (Ruggiu *et al* 1999). The results point to the importance of DAZLA in spermatogenesis.

## 4. Epididymal gene products

The tissues of the male reproductive tract including the epididymis are targets for androgens. Androgens are found in high concentrations in the epididymal lumen and the importance of 5 $\alpha$-dihydrotestosterone in the regulation of epididymal function is well known (Fan and Robaire 1998). However, attempts to inhibit the enzyme to down-regulate gene products have not been successful. Androgen withdrawl leads to apoptosis along the length of the epididymis. Agents that would inhibit androgen induced genes would have male contraceptive potential. Therefore there is an interest in examining the genes regulated by androgens.

### 4.1 *c-ros tyrosine kinase receptor*

Yet another gene product that has important selective function and is involved in epididymal function is a c-ros tyrosine kinase receptor. Male mice null for this gene are healthy and produce normal sperm but lack pre-pubertal differentiation of the epididymal initial segment. Computerized analysis of the motility of spermatozoa maturing in the epididymis revealed only minor defects. However, the majority of motile mature sperm released from the cauda epidymis showed various extents of flagellar angulation that could not be corrected by raising extracellular osmolality. Flagellar angulation could be relieved upon demembranization by Triton X-100, confirming that it was a swelling phenomenon that was affected. The flagellar

angulation may account for the reduction in sperm numbers in the oviduct of mated females and failure to fertilize *in vivo* (Yeung *et al* 1999).

### 4.2 *Retinoic acid and retinoic X receptor*

The regulation of male reproduction by vitamin A and its derivatives is well known. Retinoic acid (RA) interacts with two types of receptor family RAR and RXR. The RAR family ($a, b, g$) is activated by both all *trans* and 9 *cis* RA. RXR family is activated exclusively by the *cis* form. Mice null for these genes have been created and analysed (Lufkin *et al* 1993; Kastner *et al* 1996). Gene targetting studies showed that mutation affected only male fertility. A null mutation in RAR *g* (Lohnes *et al* 1993) showed defects in accessory glands. Both seminal vesicles and prostate gland exhibit squamous metaplasia. Kerotinization of glandular epithelia without any secretion was seen.

In addition to these, several epididymal proteins have been characterized but their functional significance is yet

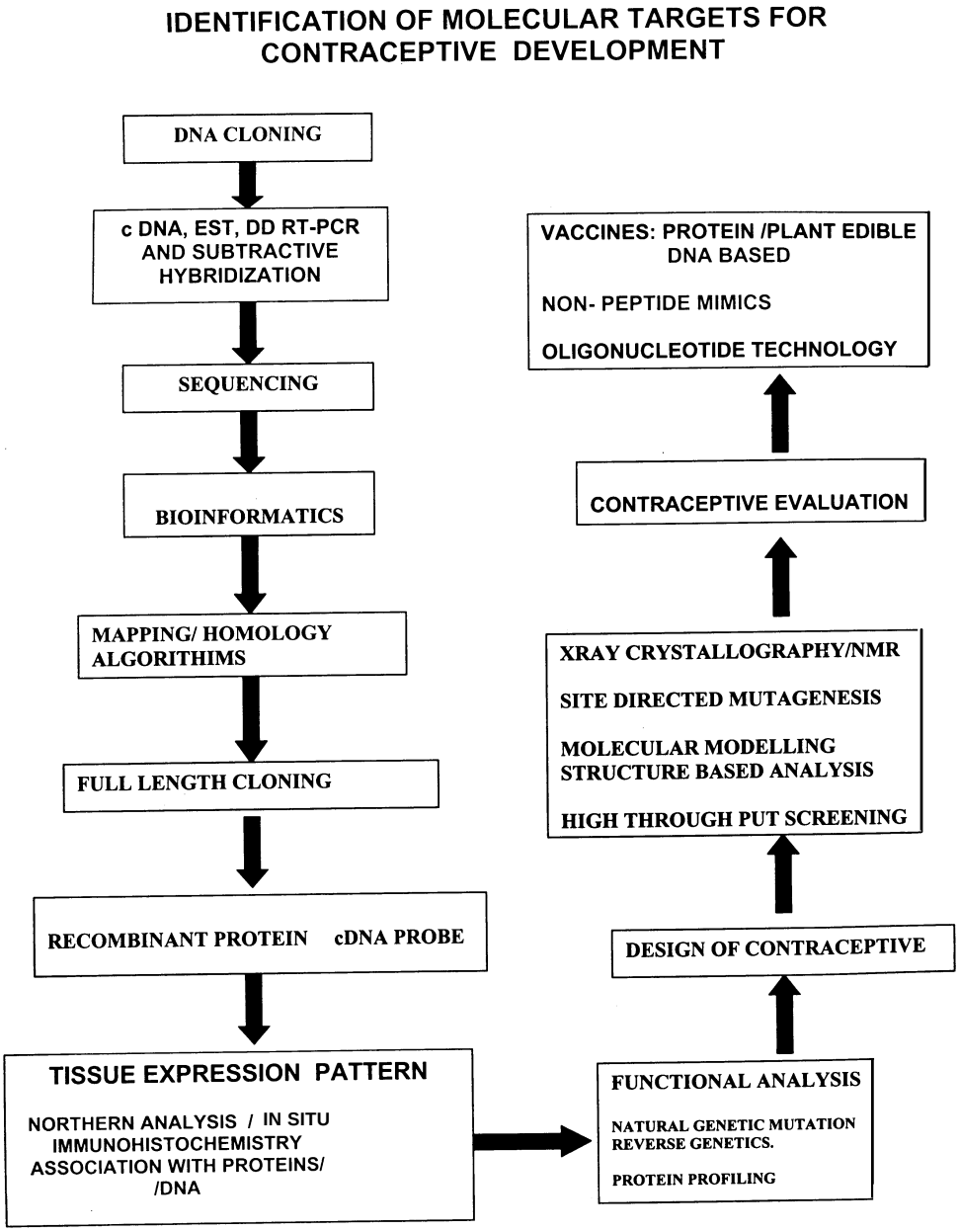


**Figure 2.** Flow chart of methodologies that will be used for identification of target gene for contraceptive evaluation.

to be established (Hegde *et al* 1991; Cohen *et al* 2000; Khole *et al* 2000; Saalmann *et al* 2001). Apart from the genes that were discussed in this article there are several genes that are involved in male fertility. These gene products were identified by the knockout technology and have been recently reviewed (Kumar and Matzuk 2000).

## 5. Conclusion and future directions

One can be optimistic that in the next few years, several target genes crucial for specific reproductive pathways would be identified. This would be possible due to rapid advances in molecular cloning, combinatorial chemistry, bioinformatics, NMR, X-ray crystallography and rational structure based method for drug development. The procedure that could be adopted is out lined in figure 2. As detailed in this article, another analytical tool that has aided in our understanding of the function of specific genes is the creation of transgenic animals. Analysis of genes responsible for genetically inherited conditions is an extremely important aspect for drug development. The next critical step in the contraceptive development is to assess the viability of the approach. An ideal contraceptive candidate should be effective with minimal side effect and be reversible. Ideally it should be delivered at the site of action. In the case of male contraceptives, there are additional requirements due to blood testis and blood-epididymal barriers.

As exemplified by the development of an orally active non-peptide mimic for GnRH described in this article, it is likely that orally active non peptide mimics could be prepared. With the availability of these the question of effective delivery will also have to be addressed. One possibility would be to link it with molecules that have specific binding sites on target tissues. In the case of the testis, FSH has specific receptors on sertoli cells and conjugating with FSH or linking with monoclonal antibodies to the FSH receptor would be a possible approach. The future of contraceptive development will depend on drug targetting and delivery. This will further strengthen the growing interdependency of biology, chemistry, physics, biopolymers and material sciences. As we enter the next millennium there will be a number of opportunities for scientists working in varied fields to come together with a common goal for ameliorating the human condition through population control.

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