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GENERAL REVIEW

DIFFERENTIATION OF MESENCHYMAL TISSUES DURING PHALLIC MORPHOGENESIS WITH EMPHASIS ON THE OS PENIS: ROLES OF ANDROGENS AND OTHER REGULATORY AGENTS

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Summary—This article reviews various aspects of differentiation and growth of phallic mesodermal tissues with special reference to the os penis. In many species of certain mammalian orders the penile interior contains an os penis or baculum with bona fide bone. Mechanisms of phenotypic sex differentiation and the androgenic regulation of morphogenesis of genitourinary tracts of both sexes are first overviewed. Thereafter the various mesodermal tissues in fully developed penes and clitorides are discussed. The developmental fate of mesenchymal cells in the fetal genital tubercles is then considered in detail, including considerations of epithelial–mesenchymal interactions. The review concludes with a discussion of the possible roles of certain polypeptide growth factors acting in concert with androgenic steroids. Special emphasis is placed on the potential role of bone morphogenetic proteins in formation of the os penis in a restricted number of eutherian mammalian taxa.

INTRODUCTION

In eutherian mammals, the interior of the penis is mainly comprised of corpora cavernosa that are involved in the process of erection which normally is a necessary prelude to the expulsion of seminal fluid. In many species of a restricted number of mammalian orders, the penile interior also contains an os penis or baculum with bona fide bone including osteogenic cells and extracellular matrices. All cells in the erectile bodies and the os penis are of mesenchymal origin, and their differentiation and growth are strictly controlled by androgenic steroids. In eutherian females, the clitoris and certain other components of the vulva contain erectile tissues, and in some species a small os clitoridis or baubellum also develops. This article focuses on various fundamental issues related to mechanisms of differentiation and growth of phallic mesodermal tissues, and proposes some new lines of investigation. Because of its relevance to many aspects of the aforementioned topics, the first section that follows provides a brief overview of mechanisms of phenotypic sex differentiation, and also of androgen action in eutherian mammals.

ANDROGENIC REGULATION OF THE MORPHOGENESIS OF MAMMALIAN MALE AND FEMALE GENITOURINARY TRACTS

The extragonadal organs of male and female eutherian genitourinary tracts differentiate in the fetus from four types of anlagen that in a given species are initially indistinguishable morphologically between the two sexes. These primordia are: (a) The paired Wolffian ducts, which develop into the epididymides, vasa deferentia and seminal vesicles in males, but involute in females. (b) The paired Mullerian ducts that are the precursors of the oviducts, uterus and upper vagina in females, but regress in males. (c) The urogenital sinus, from which the urinary bladder and the urethra develop in both sexes, and which gives rise to the prostate and

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bulbourethral glands in the male and to the lower segment of the vagina (vestibule) and the paraurethral glands in the female. (d) The external genital primordia which are the precursors of the male penis and scrotum or the female vulva. The time-course of differentiation of these anlagen in the fetus (and in some species also during the early postnatal period) is strikingly species-dependent, and when completed results in formation of sets of immature male or female organs that are reproductively non-functional. The final growth and functional maturation of the reproductive systems of both sexes is brought about during puberty as a result of heightened secretion of gonadotrophins by the anterior pituitary and of steroid sex hormones by the ovaries or testes. The differentiation of reproductive tracts in male fetuses are strictly dependent on output of (i) androgens by the Leydig cells, and (ii) Mullerian inhibitory substance (MIS) by the Sertoli cells, of the fetal testes. Sex differentiation in the female fetus, by contrast, is not influenced by hormones secreted by the fetal ovary. That androgens and MIS secreted by the fetal testis are respectively essential for virilization of genitourinary tract primordia and for regression of the Mullerian ducts is strikingly illustrated by observations that extirpation of the fetal testes before the onset of male sex differentiation results in normal genetic (XY) males in the formation of a primitive female tract that includes oviducts, uterus, vagina and vulva, but no masculine structures, and is also supported by many other lines of evidence (for reviews see Refs [1–4]).

In most eutherian species, testosterone is the major potent androgen of fetal or adult testicular origin that ultimately stimulates the differentiation and growth of male genitourinary organs in the fetus and in adults. However, as reviewed in detail elsewhere [2–6], many though by no means all androgen-responsive cells readily convert testosterone to 5α-dihydrotestosterone (DHT) by the action of steroid 5α-reductase(s) that are largely associated with endoplasmic reticulum and nuclear membranes. (In contrast to the X-linked gene for the androgen receptor, the gene(s) for steroid 5α-reductase(s) are autosomally located.) DHT is a more potent androgen than testosterone in many androgen bioassay systems, and DHT has a relatively higher avidity for the androgen receptor. In the majority of androgen-responsive cells that transform testosterone into DHT, most of the testosterone that enters the cells ends up in nuclei in the form of DHT complexed to the androgen receptor, which apparently acts as the proximal androgenic stimulus. In all species that have been studied, the fetal urogenital sinus and external genital primordia, and also various component tissues of the penis, scrotum and male accessory glands and ducts in adult eutherians, are replete with steroid 5α-reductase and extensively convert testosterone to DHT. By contrast, Wolffian ducts before the onset and during the early stages of male phenotypic sex differentiation do not produce DHT from testosterone, though by the time of birth, the seminal vesicles and epididymides acquire this capacity. Moreover, in adults of various species, segments of nephrons of metanephric kidneys, and also certain perineal and other skeletal muscles—which are all highly androgen-responsive—do not significantly convert testosterone to DHT and exhibit negligible steroid 5α-reductase activity. Thus, in the latter organs and in fetal Wolffian ducts, testosterone apparently acts via binding to the androgen receptor as such, even though tissues of these organs are strongly stimulated by exogenous DHT. Noteworthy in the aforementioned contexts is that there is no persuasive evidence indicating the existence of separate types of androgen receptors that selectively bind DHT but not testosterone, or vice versa [5].

It has long since been recognized that specific androgen receptor proteins mediate virtually all responses of tissues to this class of steroid hormones, and that the gene encoding the androgen receptor (AR) is present on the X chromosome of eutherian mammals. Only quite recently, however, have the structures of the AR, and of its mRNA and gene, been characterized in several species. As reviewed by Liao *et al.* [5], AR proteins are comprised of three domains: (i) A C-terminal region that includes the androgen-binding site; (ii) a more central and relatively short DNA-binding domain that is rich in cysteine and contains two "zinc finger" structures and (iii) an extensive N-terminal domain whose complete amino acid sequences are quite species-variable, and which (at least in the cases of human and rat ARs) contain sequences that are oligomers or polymers of the same amino acid, including 20/21-mer polyglutamine stretches. The human AR gene of more than 90 kilobase pairs and its AR coding region is spread over 8 exons, one of which is very large and encodes the entire N-terminal region of the AR protein [5].

Various mutant forms of ARs that have been described in man and certain rodent species. Point mutations that evoke sequence changes or deletions of various regions of ARs are implicated as causes of functional defects in these receptors associated with complete or partial resistance to the actions of androgens as is evident, for example, in various forms of the testicular feminization syndrome [2, 4, 5]. In addition, genetically-determined partial deficiencies in steroid 5α-reductase are related to defects in stimulation by testosterone of normal male phenotypic sex differentiation [2, 4, 6]. ARs are considered as members of a large receptor family that includes specific receptors cognate to various other categories of steroid hormones, thyroid hormones and retinoic acid. All of these receptors, and their genes and mRNAs, exhibit common structural features, such as the hormone binding sites being associated with the C-terminal regions of the receptor proteins, and the presence of an interior DNA-binding region whose sequences are highly conserved [5, 7, 8]. Currently it is widely held that androgens and other sorts of steroid hormones act primarily at the level of regulation by the corresponding steroid–receptor complexes of initiation of transcription of clusters of nuclear genes, resulting in often positive but sometimes negative changes in the production and accumulation of various specific mRNAs or other types of RNA molecules, which eventually bring about alterations in the production in specific proteins in a given cell. Effects of various steroid hormone on cellular levels of specific mRNAs and proteins can differ greatly between various types of cells from one responsive organ to another in a particular organism. To explain this, it has been proposed that there may be a requirement for additional tissue-specific DNA-binding regulatory proteins besides appropriate hormone-occupied steroid receptors for the formation and/or stabilization of macromolecular complexes that can initiate transcription of particular genes at nuclear chromosomal sites. Details of the participation of such tissue-specific DNA-binding regulatory proteins in the initiation of specific gene transcriptions influenced by androgen receptors have not yet been worked out. However, it is evident from comparable very recent studies on estrogen and glucocorticoid receptors [7, 8] that the molecular biology of transcription initiation processes regulated by all categories of steroid hormones is immensely complex and unlikely to be unraveled definitively in the near future. Furthermore, there are increasing indications, at least with regard to androgen action mechanisms, that ARs may function not only at the level of transcription initiation but also may perhaps regulate mRNA processing or stability at post-transcriptional levels [5].

As considered below, it appears likely that morphogenesis and growth of the penis or clitoris may entail actions of certain growth factors or related polypeptides in addition to androgenic hormones. It is conceivable that androgens may regulate production of these polypeptide growth factors and/or their corresponding receptors in appropriate cells of external genital primordia, and that the growth factors may exert their actions by autocrine or paracrine mechanisms.

MESODERMAL TISSUES OF FULLY DEVELOPED PENES AND CLITORIDES

The functional morphology of eutherian penes is reviewed by Prasad [9], Sachs and Meisel [10], Benson [11] and Williams-Ashman [4]. The following outline of mesodermal structures present beneath the integuments of the penis or clitoris in sexually mature eutherians is presented before later discussion of the development of these phallic structures.

The penis

In eutherian mammals the penis is traversed from its proximal root to the distal glans by the relatively long male urethra, which permits the flaccid penis often to eliminate urine throughout postnatal life, and the erect phallus in sexually mature males to act as a conduit for delivery of seminal fluid, which occurs with much lower frequency than micturition.

In most species, the penis contains three erectile bodies comprised predominantly of fibroelastic, smooth muscle and vascular elements plus associated nerve fibers. These are: (1) a pair of corpora cavernosa that traverse the penile shaft, terminate below the glans, and end proximally as tapering crura on which are inserted the striated ischicavernosus muscles. (2) A single corpus cavernosus urethrae (corpus spongiosum) whose proximal end (bulb) that is attached to the striated bulbospongiosus muscle extends distally through the penile shaft and is expanded inside the glans. The penile segment of the male urethra runs through the central core of the corpus spongiosum and terminates

near the tip of the glans. The three corporal erectile cylinders are each bounded by separate fibrous sheaths, from which trabecluae ramify inwards so as to divide the interiors of the corpora into vascular spaces (sinusoids) that are lined with attenuated endothelium. Inside the trabeculae are fibroblasts, smooth muscle cells, extracellular matrix components including collagen and elastin fibers as well as glycosaminoglycans, plus neuronal axons and nerve terminals. The sinusoids interconnect with various penile arteries and veins. In species equipped with more "vascular" types of penes, sexual excitement triggers arterial vasodilation but evokes lesser changes in venous resistance, and also relaxation of trabecular smooth muscle cells. As a result, the sinusoids become engorged with blood, which causes the corpora to enlarge and stiffen, so that the penile shaft and/or glans increase in length and diameter. By contrast in eutherian species that have more "fibroelastic" types of phalli (e.g. many ungulates), the trabeculae in the relatively thin corpora contain extensive networks of elastin fibers but few smooth muscle cells. In flaccid state, the entire shaft of "fibroelastic" sorts of penes is concealed inside the prepuce, and is bent proximally into an S-shaped sigmoid flexure as a result of tonic contraction of a special retractor penis smooth muscle inserted onto the shaft, which relaxes during erection to evoke lengthening of the penile shaft but with little increase in its diameter, while vascular sinusoids inside the glans expand via engorgement with blood.

In many though not all species of six eutherian orders—Insesctivora, Chiroptera, Rodentia, Pinnipedia, Carnivora and Primates (excluding man)—bone and or cartilage structures develop inside the glans and the distal region of the shaft that are designated as the os penis or baculum. The morphology of the baculum is often strikingly species-typical, and has been used as an index of phyletic relationships among certain taxa [12]. Ossa penes differentiate from mesenchymal precursor cells that also give rise to corpora cavernosa. Speculations that bacula may serve significant reproductive or protective functions for the penis have been amply debated [4, 13, 14].

In certain species (e.g. ram, boar, domesticated dogs and cats), substantial accumulation of adipocytes crammed with triglycerides is evident in the corpora cavernosa of adult creatures. Conspicuous modulation of mesenchymal cells into adipocytes in penile corporal bodies is not normally evident in the penes of various rodents or humans. The extensive endothelium lining the vascular sinusoids of the erectile bodies and their blood vessels, and bone and cartilage of the os penis, are, of course, of mesenchymal origin.

The clitoris

Species variations in the morphology of the clitoris and other components of the vulva in eutherian mammals, if normalized in terms of unit body size, are not as extensive as corresponding differences in penile anatomy, even though clitorides in a minority of species have some unusual characteristics, most notably the gigantic and peniform-like clitoris and pseudoscrotum of the external genitalia of normal female spotted hyenas (*Crocuta crocuta*) [3, 4]. The clitoris or the penis develop in eutherian fetuses from the genital tubercle component of the external genital primordia, which in a given species are morphologically identical in both sexes before and onset of differentiation of extragonadal organs of the male and female reproductive tracts. There are three regions of the clitoris: the distal glans, the middle corpus or shaft, and the bifurcated proximal segment comprising the roots. Usually only the tip of the glans is visible externally, as the rest of the glans and part of the clitoridal shaft are covered by the skin of the prepuce, which is contiguous with the integument of the labia minora that hang below the clitoris on either side of the vulva introitus. The interior of the clitoris contains erectile tissue which structurally resembles that of the penile corpora cavernosa, and branches proximally into two crura. In the vast majority of species, the female urethra (which is relatively shorter but wider in diameter than in the male) opens below the clitoris in the vestibule of the lower vagina (which is derived embryologically from the fetal urogenital sinus). There are rare exceptions to the latter rule in which female urethra perforates the clitoris—so that in those species where this occurs, the female urinates through her clitoris (e.g. guinea pig, laboratory rats and mice; elephant; mole; all species of lorisiform prosimian primates; and the spotted hyena). The clitoris lacks a true corpus spongiosum as found in the male penis. (The paired vestibular bulbs, which in women are located under the bulbospongiosus muscles between the vaginal wall and each clitoridal crus contain erectile tissue which resembles that of the penile corpus spongiosum.) The vesticular bulbs as well as the erectile tissues of the clitoris

become engorged with blood during female sexual excitement. Structures in the integument and interior of the clitoris are liberally supplied with various types of motor and sensory neurons and their terminals. In contrast to studies on penile corpora cavernosa, the ultrastructure and physiology of erectile tissues in the clitoris have apparently not been investigated in depth [4].

DEVELOPMENTAL FATES OF MESENCHYMAL PROGENITOR CELLS IN FETAL GENITAL TUBERCLE CELLS *IN VIVO*

When they first arise in the fetus, the sexually indifferent external primordia of both sexes are comprised of a genital tubercle (precursor of most parts of the penis or clitoris), which is attached posteriorly to two inner genital folds that surround the opening of urogenital sinus, and are flanked on each side by the genital swellings. In normal males the genital folds fuse to enclose the penile shaft and urethra, while the genital swellings fuse medially to form the scrotum in the big majority of eutherian species that possess scrota. In females, the genital folds and swellings never fuse, and serve respectively as anlagen of the minor and major labia of the vulva [1, 2].

Descriptive morphologic studies on fetuses at various stages of gestation and early postnatal periods in the human [15] have provided much information about the dynamics of phallic development. Observations on patients with genetic defects of the action of genes coding for the androgen receptor, or steroid 5α-reductase or certain enzymes of testosterone biosynthesis (reviewed by Williams-Ashman [3, 4]) have given insight into the effects of inadequate testosterone production or androgen action mechanisms on masculine patterns of external genital differentiation. However, experimental analyses of phallic morphogenesis and growth based on expedients such as gonadectomy of fetuses or juvenile animals, or administration of drugs that impede the formation and/or functions of androgen receptors of steroid 5α-reductase—which have been productively applied to research on external genital development in certain laboratory or domestic animals—obviously cannot be applied to investigations in humans.

Murakami and Mizuno [16, 17] reported that rudiments of corpora cavernosa, and also of the proximal (p-) and distal (d-) segments of the os penis in males, are evident as clusters of mesenchymal cells in the genital tubercules (GTs) of fetal male Norway rats at 16.5–18.5 gestation days. Erectile corporal tissues containing trabeculae and sinusoids were well formed by about 1 week after birth. The entire p-segment of the os penis initially consists of membrane bone in its distal part plus hyaline cartilage situated proximally. The p-segment of the baculum is formed by fusion of membrane with the cartilage, and its further growth occurs by endochondral ossification postnatally. The d-segment of the os penis is not evident post partum until 4 weeks after birth and starts to ossify after the 10th week.

Glucksmann *et al.* [18] observed that at roughly the 16th day of gestation, in fetuses of male and female laboratory mice there is formation of mesenchymal cells that extend parallel to the ventral side of the urethra inside the GT. No further phallic differentiation occurs in males or females before parturition. At 1 day post partum in males, metachromasia was seen in the intercellular spaces of the mesenchyme. On day 2 after birth, bone began to form in the more peripheral regions, and cartilage replete with metachromasia was produced in the central district. Osteogenesis in the periphery was very rapid as membrane bone was formed without intermediary production of cartilage. More proximally in the penis, cartilage was formed and then maintained, until much later postnatally it gradually underwent endochrondral ossification. Growth of the bone began to subside at about 60 days postpartum and all cartilage was replaced by bone at 180 days after birth. Closer to the base of the penis, the dense mesenchymal mass differentiated into cavernous erectile tissue, while towards the tip of the penis the mesenchyma developed into the d-segment of the os penis; definitive cartilage was evident at day 20 and ossified subsequently so that the tip of the d-segment of the baculum was situated in the glans penis. Noteworthy in the aforementioned contexts is that in some strains of mice, a tiny os clitoridis—which Glucksman *et al.* [18] believed is analagous to the p-segment of the male's os penis—normally forms by direct ossification of female GT mesenchyme during the early postnatal phase of clitoridal development. The size of the os clitoridis in female mice is increased tremendously by treatment with exogenous testosterone or DHT. By contrast, female laboratory rats normally do not develop an os clitoridis, but the formation of bone in the clitoris can be induced by administration of high doses of androgens to neonatal rats [19, 20].

DIFFERENTIATION OF PHALLIC STRUCTURES IN GENITAL TUBERCLES TRANSPLANTED BENEATH RENAL CAPSULES OF ADULT RATS

Experimental studies on regulatory features of phallic morphogenesis *in vivo*, either in fetuses or postnatally, are both tedious and difficult to control precisely, especially with regard to artificial local application of hormones or other potential morphogens to external genital primordia. Recent investigations by Murakami [17, 21] involving transplantation of rat GTs, from fetuses at various times of gestation, under the renal capsules of heterologous syngeneic hosts revealed the following findings. In GTs transplanted from rat fetuses of 16.5–18.5 gestation days, an initial formation of dense mesenchymal cell masses that serve as progenitors of phallic cavernous tissue, cartilage and bone is not dependent on androgens of host testicular origin as indicated by studies using syngeneic adult male or castrated male, or female hosts. The subsequent overt differentiation of penile corpora cavernosa and of bone and fibrocartilage elements of the os penis required testicular androgens as shown by appropriate endocrine manipulations. However, the potential for chondrogenesis and osteogenesis was roughly equal in GTs transplanted androgen—induced from male or female fetuses. The latter conclusion was derived from observations that cartilage and bone similar to that in the male baculum plus a highly developed clitoridal corpus cavernosum were formed when GTs from female fetuses at 18.5 days of gestation were transplanted under renal capsules of adult syngeneic male rats for 14 days (normally the clitoridal corpus cavernosum in female rats is poorly developed and devoid of bone or cartilage).

That the first stage of mesenchymal cell condensation in the formation of rudiments of the penis or clitoris in eutherian GTs is independent of an androgenic stimulus, whereas androgen action is essential for later stages of penile morphogenesis, was indicated by the studies of Murakami [22] on phallic development in normal, wild-type mice compared with congenitally androgen-insensitive (*Tfm*) mice. In the mouse *Tfm* syndrome, which is due to recessive mutations of the X-linked gene for the AR that render the AR non-functional, the afflicted XY males cannot respond their own androgens produced by their permanently abdominal testes, or to exogenous testosterone or DHT. There are no defects in steroid 5α-reductase in *Tfm* males that possess no Wolffian or Mullerian duct derived structures in their reproductive tracts, which present a vulva containing a small clitoris rather than a normal male penis and scrotum.

EPITHELIAL–MESENCHYMAL INTERACTIONS

There are numerous examples of inductive interactions between epithelium and mesenchymal tissues during early phases of organogenesis, as reviewed by Reddi [23]. Murakami and Mizuno [17] separated the urethral and surface epithelia from the mesenchyme of rat GTs and transplanted the mesenchyme beneath renal capsules of adult male syngeneic rats. GT mesenchyme from fetuses of 14.5 gestation days formed only small amounts of cavernous erectile and connective tissues containing no specialized structures. Mesenchyme from 15.5 day fetuses developed into corpus cavernosum penis tissue and also hyaline cartilage of the p-segment of the baculum; occasionally membrane bone characteristic of the p-segment of the os penis was also produced, but neither the d-segment of the baculum nor its rudiment were ever formed. GT mesenchyme freed from epithelia from 15.5 day fetuses developed in the same fashion as transplants with intact epithelial elements. But when the separated epithelial and mesenchymal tissues were combined and then cultivated under renal capsules of adult male hosts, the transplants grew more profusely than transplants of the mesenchyme solo. When GT mesenchyme from fetuses at 16.5 gestation days was recombined with heterologous epithelia from the dorsal epidermis or urinary bladder from fetuses of the same age, on transplantation of the recombinant tissues under kidney capsules it was evident that the epithelia induced the formation of fibrocartilage of the d-segment of the os penis as well as of the hyaline cartilage and membrane bone of the p-segment of the baculum.

Beresford and Clayton [24] and others have averred that genesis of penes from fetal GTs resembles the morphogenesis of vertebrate limbs, inasmuch as the corresponding embryonic rudiments increasingly protrude from the trunk when they differentiate and grow in an orderly manner, and are stiffened with erectile tissue (and bone in some species). In chick limb morphogenesis, mesenchymal cells in the tip of the limb bud induce the overlying ectoderm to thicken and form the apical ectodermal ridge

(AER), which plays a key role in determining the subsequent spatial patterns of outgrowth and differentiation of mesodermal tissues [25]. In chick limb development, the AER is essential for laying down of future limb parts in a strict proximal-distal order and retinoic acid is implicated as a morphogen in these processes [25–27]. However, in eutherian GTs, no structures resembling the AER of vertebrate limb buds are produced [17]. The mechanisms by which progenitor cells of the corpora cavernosa penis and the baculum develop and become arranged spatially under androgenic stimulation are not understood. Of related interest are observations of Murakami [28] in fetal mouse GTs that were incubated for short periods with tritiated testosterone *in vitro*, preputial and periurethral mesenchymal cells from fetuses of 15.5 gestation days predominantly sequestered the steroid. And in genital tubercles from 17.5 days gestation, the anlagen of the p- and d-segments of the os penis were very active in taking up labeled testosterone. These findings suggest that mesenchymal cells serve as prime targets for androgen action during phallic morphogenesis.

REGULATION OF GENE EXPRESSION DURING VARIOUS PHASES OF PHALLIC DEVELOPMENT: CANDIDATE MORPHOGENS

Androgenic steroids are essential regulators not only of the later stages of male external genital morphogenesis in eutherian fetuses and neonates, but also of the further growth and functional maturation of the penis and scrotum during puberty. Androgenic stimulation is apparently not required for differentiation of the clitoris or other vulva structures. However, when XY female fetuses at appropriate critical stages of development receive large amounts of potent exogenous androgens they develop peniform phalli and scrota. Moreover, excessive androgenic stimulation of juvenile, pubertal or sexually mature females evokes extensive clitoridal growth including proliferation of mesodermal cells. By contrast it is noteworthy that, at least in the human, whereas postnatal administration of testosterone or DHT or potent synthetic androgens (or hyperandrogenization due to excessive endogenous production of testosterone) in juvenile males or during the early stages of puberty can stimulate penile growth, in sexually mature men the penis usually will not enlarge extensively in response to large doses of exogenous androgenic hormones [4].

There are indications that postnatal growth and maturation of the penis may be influenced by other regulatory agents besides androgens. For example, defects in the production of insulin-like growth factor 1 (IGF-1) under the influence of pituitary growth hormone have been attributed as a cause of micropenis (an exceptionally tiny but otherwise morphologically normal penis) in so-called Laron dwarfs and perhaps in other types of patients exhibiting micropenis [4].

In the authors' opinion, future research on the regulation of male or female patterns of fetal genital tubercle development should take into account the recent rush of discoveries on participation of many types of polypeptide growth factors and other regulatory molecules in differentiation of mesodermal tissues at various stages of early development. Experimental analysis of the potential relevance of these advances to phallic differentiation would be facilitated if excised genital tubercles could grow and differentiate in organ cultures supported by media of completely defined chemical composition, and notably without supplementation with fetal blood sera. Murakami and Mizuno [29] cultured GTs from male and female rat fetuses [20] gestation days in an *in vitro* synthetic system using medium plus 20% fetal bovine serum with media changes every 3 days. After 1 week in culture, no rudiments of the os penis were formed. But after 2–3 weeks *in vitro*, more than 60% of the explants developed hyaline cartilage of the p-segment of the baculum, only when testosterone at 4 ng/ml was in the medium. Under the latter culture conditions, there was virtually no ossification of any part of the os penis. (It would be preferable to use DHT rather than testosterone as the applied exogenous androgen because of possible limitations in the 5α-reductase-catalyzed conversion of testosterone to DHT under *in vitro* culture conditions.)

Requirements for chemically ill-defined fetal blood sera to support differentiation of GTs cultured *in vitro* might be effectively replaced by addition of various pure protein growth factors or other regulatory substances known to influence tissue development in other biological contexts. Protein growth factors influencing bone development and physiology are comprehensively reviewed by Hauschka [30]. Among the regulatory agents that could be effective are the

following. (1) The Fibroblast Growth Factors aFGF and bFGF (note that bFGF profoundly influences proliferation of endothelial cells [30, 31], which abound in the vascular sinusoids of phallic erectile corpora). (2) Insulin, IGF-1 and IGF-2. (3) Members of the Epidermal Growth Factor (EGF) and Platelet-derived Growth Factor (PDGF) families of polypeptides. (4) Assorted cytokines (e.g. Interleukin-1, which suppresses enhancement of endothelial proliferation stimulated by bFGF, probably by down-regulation of FGF receptors evoked by this cytokine [31]). (5) Various proteins of the Transforming Growth Factor-β (TGF-β) superfamily of growth factors. (6) Various types of recently discovered Bone Morphogenetic Proteins (BMPs). Categories (5) and (6) above merit further comment with respect to phallic mesodermal tissue proliferation and differentiation.

The TGF-β superfamily of proteins includes the widely-studied TGF-β_1 which influences differentiation of several types of cells and can also exert either inhibitory effects (especially on epidermal cells) or stimulatory actions (e.g. on osteoblasts) on cell proliferation. TGF-β_1 stimulates synthesis and inhibits degradation of extracellular matrix components. (For overviews on TGF-β_1 structure, specific receptor-mediated actions, and storage in certain mesenchymal cell extracellular matrices, see Refs [30, 32, 33]). The TGF-β superfamily includes various activins and inhibins, various segments of which have amino acid sequence homologies with segments of TGF-β_1. Activins and inhibins were first described as peptides of adult gonadal origin that respectively activate or inhibit release of FSH from the anterior pituitary gland [34]. Recently, Mitrani *et al.* [35] showed that activin B is the endogenous inducer of axial limb structures in the chick hypoblast. And studies by Thomsen *et al.* [36] indicated endogenous activin B induces dorsal axial mesoderm and anterior structures in early *Xenopus* embryos. That certain activins or inhibins might serve as regulators of the proliferation or differentiation of mesodermal cells in the early stages of phallic development from genital tubercles seems particularly worthy of investigation.

Why cartilage and bones in ossa penes are normally found only in a limited number of eutherian taxa is puzzling. The differentiation of bacula might well entail local production, and possibly extracellular storage, in mesodermal progenitor cells of various sorts of recently identified bone morphogenetic proteins (reviewed by Hauschka [30], Reddi *et al.* [37] and Wozney *et al.* [38]), and especially osteogenin or BMP-3 [39–42]. Judicious use of *in situ* hybridization with cDNA probes for appropriate mRNAs, and of monospecific/monoclonal antibodies directed against various BMPs or their corresponding specific receptors, might aid experimental analysis of possible roles of BMPs in the morphogenesis of ossa penes in GTs cultured *in vitro*.

Retinoic acid apparently functions as a natural morphogen in limb bud development in the chick and certain urodele amphibians [25–27], and stimulates expression of certain homebox genes [26]. Whether retinoic acid, or any other retinoids, may play regulatory roles in external genital morphogenesis in eutherian mammals is an open question. The authors are unaware of any reports that derangements in phallic development occur as a result of nutritional deficiencies, or excessive administration, of any types of retinoids. The obvious possibility that development of phallic bones may be regulated by the osteotropic agents parathyroid hormone, calcitonin or 1α,25-dihydroxy cholecalciferol appears not to have been explored. Research on phallic morphogenesis and regulation is on the threshold for systematic study of the synergism between androgenic steroids, polypeptide growth factors and bone morphogenetic proteins.

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