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Epidermal Growth Factor Receptor (EGFR) Immunolocalization in the Male Rat Reproductive Tract During Pre-and Postnatal Periods

Celal KALOĞLU, Hüseyin Eray BULUT, Bilge ONARLIOĞLU
Department of Histology - Embryology, Faculty of Medicine, Cumhuriyet University, Sivas - TURKEY

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Abstract : A number of recent studies suggest the effects of several growth factors as well as genetic factors on the development and differentiation of the testes and excretory ducts. One of the most important growth factors affecting the development and differentiation of the above-mentioned organs may be epidermal growth factor (EGF). Therefore, the aim of the present study was to investigate the effects of EGF on the development of the testes and excretory ducts of male rats by determining the immunolocalization of EGF receptors in these organs during foetal and postnatal periods.

To do this, testicular and excretory duct tissue samples from prenatals of 13, 16 and 18 days, neonates, 10-day-olds and adults were obtained and processed for light microscopy for immunohistochemical examination. APAAP immunohistochemical staining was applied to 5-7mm thick paraffin sections.

While there was a strong immunoreactivity for EGFR in coelomic epithelium and a relatively weaker immunostaining in the mesonephric duct on prenatal day 13, differentiated testes and Wolffian ducts demonstrated strong EGFR immunoreactivity on prenatal day 16. Interstitial Leydig cells of the testes and Wolffian ducts showed strong immunostaining on prenatal day 18. This strong EGFR immunoreactivity was maintained during the postnatal period and remained positive in the adult epididymis, which differentiated from the proximal part of the Wolffian duct. The EGFR immunoreactivity in the gonocytes, observed on prenatal day 16, increased on prenatal day 18 and during the neonatal period, whereas it disappeared on postnatal day 10 and in adult germinal cell lines of the seminiferous tubules.

It may be concluded that while EGF mediates testicular and excretory duct development and differentiation during the pre- and postnatal periods, it may also regulate the functions of testicular Leydig cells and epididymal epithelial cells in adults.

Key Words : EGFR, immunohistochemistry, testis, epididymis, development, rat

Erkek Sıçan Üreme Yollarında Pre-ve-Post-natal Dönemlerde Epidermal Büyüme Faktörü Reseptörü (EGFR) İmmünolokalizasyonu

Özet : Son zamanlarda, testis ve boşaltım kanallarının gelişimi ve farklılaşmasında genetik faktörlerin yanısıra büyüme faktörlerinin de etkili olabileceği ileri sürülmektedir. Bu faktörlerden birisi de epidermal büyüme faktörüdür. Bu çalışmada, epidermal büyüme faktörü reseptörlerinin (EGFR) doğum öncesi ve sonrası gelişim sürecinde sıçan testis ve boşaltım kanallarındaki lokalizasyonunun belirlenerek, EGF'nin bu yapıların gelişimi ve farklılaşmasındaki rolünün araştırılması amaçlanmıştır.

Bu amaçla, prenatal 13, 16, 18 günlük embriyolar ile yeni doğmuş, doğum sonrası 10 günlük ve erişkin testisinden alınan doku örnekleri ışık mikroskobu işlemlerini takiben parafinde bloklandılar. Bu bloklardan alınan 5-7mm'lik parafin doku kesitlerinde EGFR lokalizasyonu APAAP immünohistokimyasal boyama yöntemi ile saptandı.

Doğum öncesi 13. günde gonadal kabarıntıyı sınırlayan sölom epitelinde kuvvetli, mezonefrik kanalda ise zayıf bir EGFR immünoreaktivitesi saptanırken, 16. günde farklılaşan testis ve Wolff kanalı güçlü bir EGFR lokalizasyonu sergilemiştir. Doğum öncesi 18. günde testisin interstisyal Leydig hücreleri ve Wolff kanalındaki güçlü EGFR immünoreaktivitesi doğum sonrası dönemde de devam etmiş ve erişkinde Wolff kanalının proksimal parçasından farklılaşan epididimisi döşeyen epitelyal hücrelerde de kuvvetli pozitif olarak izlenmiştir. Diğer taraftan, gonositlerde ilk olarak doğum öncesi 16. günde gözlenen EGFR immünoreaktivitesi, doğum öncesi 18. günde ve yeni doğanlarda artmış, doğum sonrası 10 günlük ve erişkin testisinde ise kaybolmuştur.

Sonuç olarak, doğum öncesi ve sonrası dönemlerde testis ve boşaltım kanallarının gelişimini düzenleyen EGF'nin, erişkinde de testiste özellikle Leydig hücreleri, epididimisde ise epitelyal hücreler üzerinde etkili olduğu, böylece bu yapıların fonksiyon görmesinde otokrin/parakrin bir rol aldığı ileri sürülebilir.

Anahtar Sözcükler : EGFR, immünohistokimya, testis, epididimis, gelişim, sıçan

Introduction

Testicular tissue and excretory ducts are shaped in the gonadal ridge that starts to develop in the medial region of the mesonephros with the proliferation of the coelomic epithelium and condensation of mesoderm.

It is well known that gonads develop after very complicated developmental and differentiation stages in the prenatal period, and continue to develop during the postnatal period. It has been suggested that growth factors and cytokines, having autocrine and paracrine effects, might influence the gonadal development and differentiation as well as genetic factors. Those growth factors and cytokines – transforming growth factor - α (TGF-α) (1), insulin-like growth factor (IGF) (2) and epidermal growth factor (EGF) (1,3) – have been shown to play important roles in testicular and excretory duct development and differentiation (4,5).

Along with these possible roles of EGF on testicular development and differentiation, it could affect the spermatogenesis via influencing the germinal cell lines and Leydig cells during the postnatal period (6,7).

The present study was designed to determine the possible role of EGF on testicular and excretory duct development and differentiation during the pre- and postnatal periods.

Materials and Methods

Animals

Fifteen female and 20 male rats bred and fed in standard laboratory conditions were used in the present study. Animals were obtained from the Experimental Animal Laboratory of the Faculty of Medicine, Cumhuriyet University, Sivas.

Embryos and Tissues

Female and male rats were kept together in cages overnight for copulation. The following morning, vaginal smears were obtained and if there were spermia in the smears those females were spared, and this day was classified as the first day of pregnancy. Embryos obtained on days 13, 16 and 18, and testicular/excretory duct tissues obtained from neonates, 10-day-olds and adults were processed for light microscopy.

Light Microscopy

Embryos and tissues were fixed for either 6-8 hours in Bouin's solution or 24-48 hours in 10% neutral formaline. They were dehydrated through increasing concentrations of the ethanol series, and they were blocked in paraffin. Tissue sections 5-7 µm thick were cut and immunostained for EGFR localization.

Immunohistochemistry

In the present study, monoclonal anti-EGFR primary antibody (Sigma, Clone No: 29.1), AP conjugated goat anti-rat IgG secondary antibody (Sigma), monoclonal mouse APAAP complex (Sigma) and fast red TR/Naphtol (Sigma) enzyme-substrate complex were used for immunocytochemical staining. All the staining procedures were done in humidified chambers, and PBS buffer was used between all staining steps.

All 5-7µm thick paraffin sections were briefly cleared, then rehydrated in decreasing concentrations of ethanol and put into 0.2% tripsin for proteolysis. In order to avoid undesired background staining, sections were put into 20% goat serum in PBS for 30 minutes. Monoclonal anti-EGFR primary antibody (dilution: 1/300) was applied to the sections for 2 hours at 37ºC in a humidified staining chamber. Sections were then incubated in anti-rat IgG secondary antibody (dilution: 1/1000) for 1.5 hours, and they were put into the APAAP complex for an hour. Following this step, sections were incubated in the fast red/TR naphtol mixture until the specific regions were stained red, and then the sections were either briefly put into haematoxylene in order to visualize the nuclei, or were not subjected to counterstaining. Sections were mounted with a glycerol-PBS mixture (1:1 glycerol:PBS). The control staining of some sections was performed without the primary antibody, and no EGFR immunostaining was observed in these sections, showing the specificity of the immunohistochemical staining procedure.

Results

The present study investigated EGFR localization in developing rat testes and epididymis throughout the foetal period and during the postnatal period. A semi-quantitative scoring system was applied and those findings are documented in Table 1.

Prenatal 13th Day

Thirteen-day-old embryo sections demonstrated strong EGFR immunoreactivity in the coelomic epithelium of the gonadal ridge located on both sides of the dorsal aorta, whereas a relatively weak EGFR staining was observed in the luminal site of the mesonephric duct (Figs. 1a, 1b).

Table 1. Shows the semiquantitative observations of the EGFR immunoreactivities in several compartments of the male reproductive tract throughout the foetal period and during the neonatal and postnatal periods.

Age	Wolff		Gonadal ridge		Epididym		Testes			
	Epth	Str	Coelom	Msnephros	Epth	Str	T.Albug.	Leydig	Germ cell	Sertoli
Prenatal 13	+	-	+	-						
Prenatal 16	+++	-	+++	-			strong expression in developing testicular tissue			
Prenatal 18	+++	-					-	+++	++	
Neonatal					+++	-	-	+++	++	
Postnatal 10					+++	-	-	+++	-	
Adult					+++	-	-	++	-	-

Symbols: No staining = -
Weak = +
Medium = ++
Strong = +++

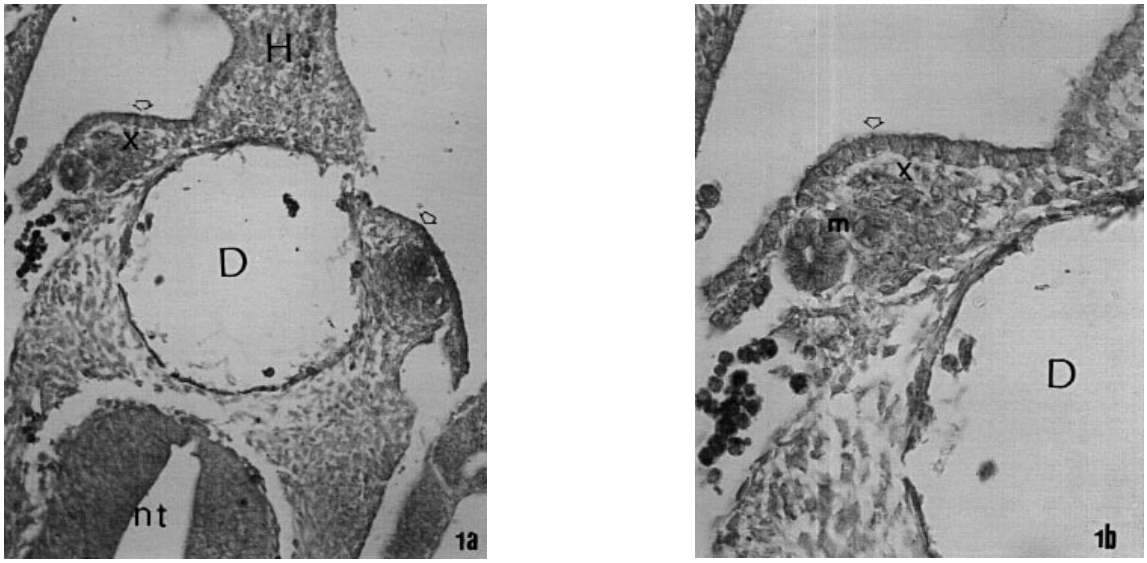


Figure 1a, b. Shows a strong EGFR immunoreactivity in the apical region of the coelomic epithelium (⇨) and relatively weak immunostaining in the luminal surface of the mesonephric duct (m) on prenatal day 13. Gonadal ridge (x), dorsal aorta (D), neural tube (nt) and hindgut (H). a: X200, b: X400.

Prenatal 16th Day

On day 16 of development, primitive testes, developing from the gonadal ridge, and Wolffian ducts neighbouring the liver were observed. A strong EGFR immunostaining was seen in developing testicular tissue and in the apical region of the Wolffian duct epithelium (Figs. 2, 3a, 3b).

Prenatal 18th Day

Testicular tissue surrounded by tunica albuginea was seen on day 18 of development, along with developing seminiferous tubuli that had no apparent lumina and separated by interstitial tissue (Figs. 4a, 4b). The genital excretory tract developed from the Wolffian duct was also observed in the neighbouring region of the testes (Fig. 5).

When the EGFR immunoreactivity was considered, a medium staining was seen in the germinal cell line of the seminiferous tubuli, whereas a strong immunostaining was evident in Leydig cells of the interstitial tissue. In addition, a strong EGFR immunoreactivity was present in the epithelial cells of the genital excretory tract.

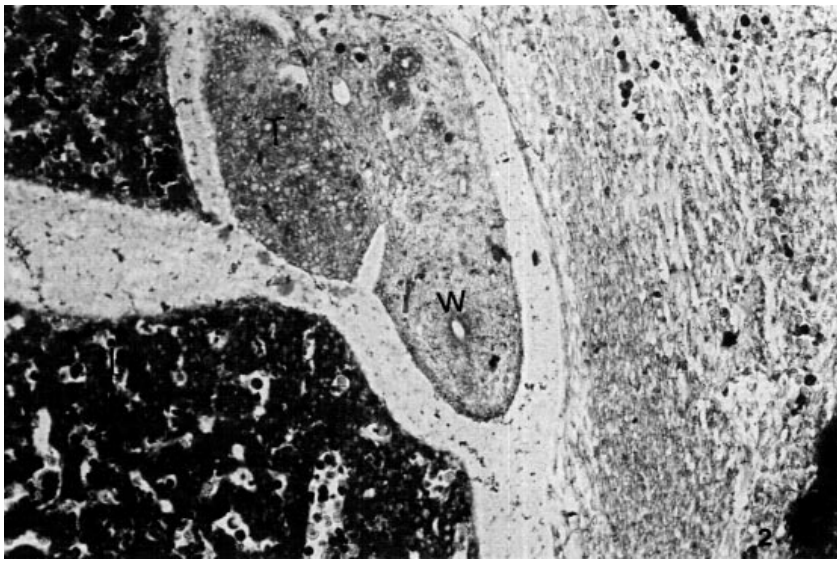


Figure 2. There is strong EGFR inmmunostaining both in testes (T) and Wolffian duct (W) on prenatal day 16. Liver (L). X200.

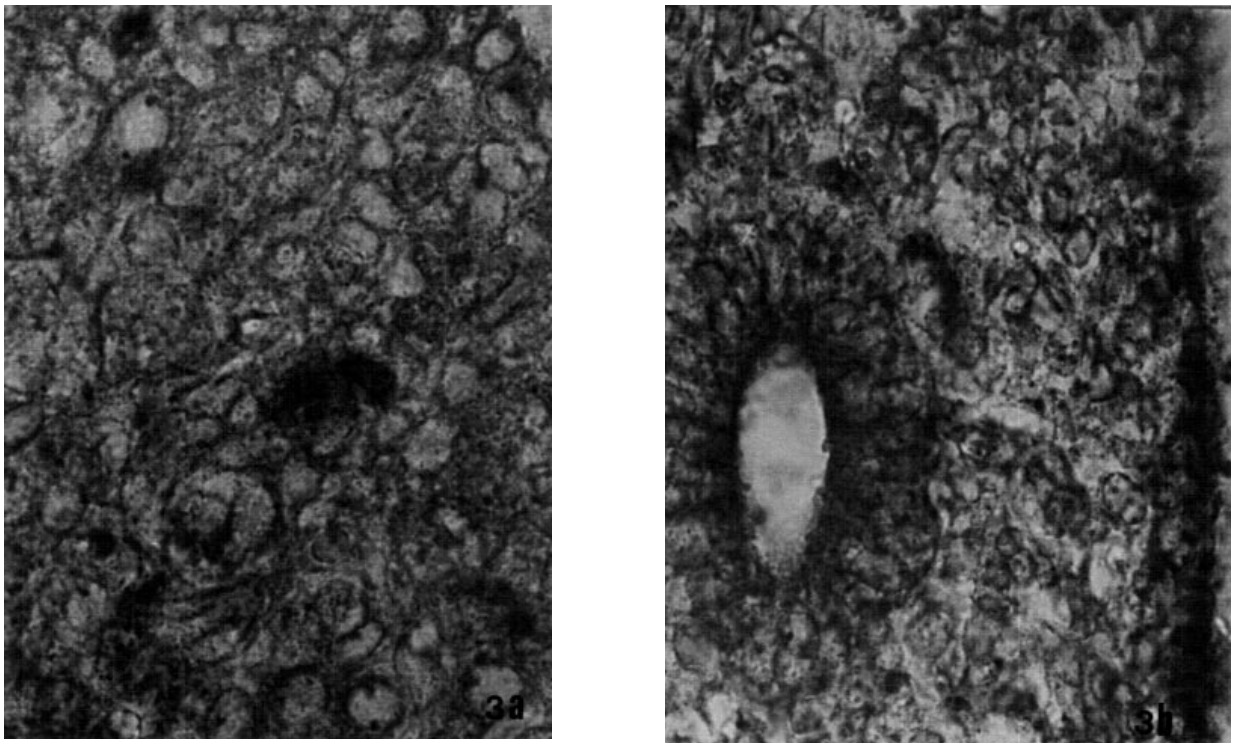


Figure 3a, b. EGFR distribution was detected in developing testes on prenatal day 16 (a) X1000. A strong EGFR immunoreactivity is also seen in the apical region of the Wolffian duct epithelium on the same day of development (b) X400.

Neonates

Testicular tissue of neonatal rats showed features similar to those seen in 18-day-old embryos, except for the increased number of Leydig cells in the interstitial tissue. While Leydig cells demonstrated a strong immunoreactivity, a relatively weak EGFR staining was present in the seminiferous tubular cells (Figs. 6a, 6b). On the other hand, EGFR immunostaining in the epididymal epithelial cells of neonates was stronger than those seen on day 18 of development (Fig. 6a).

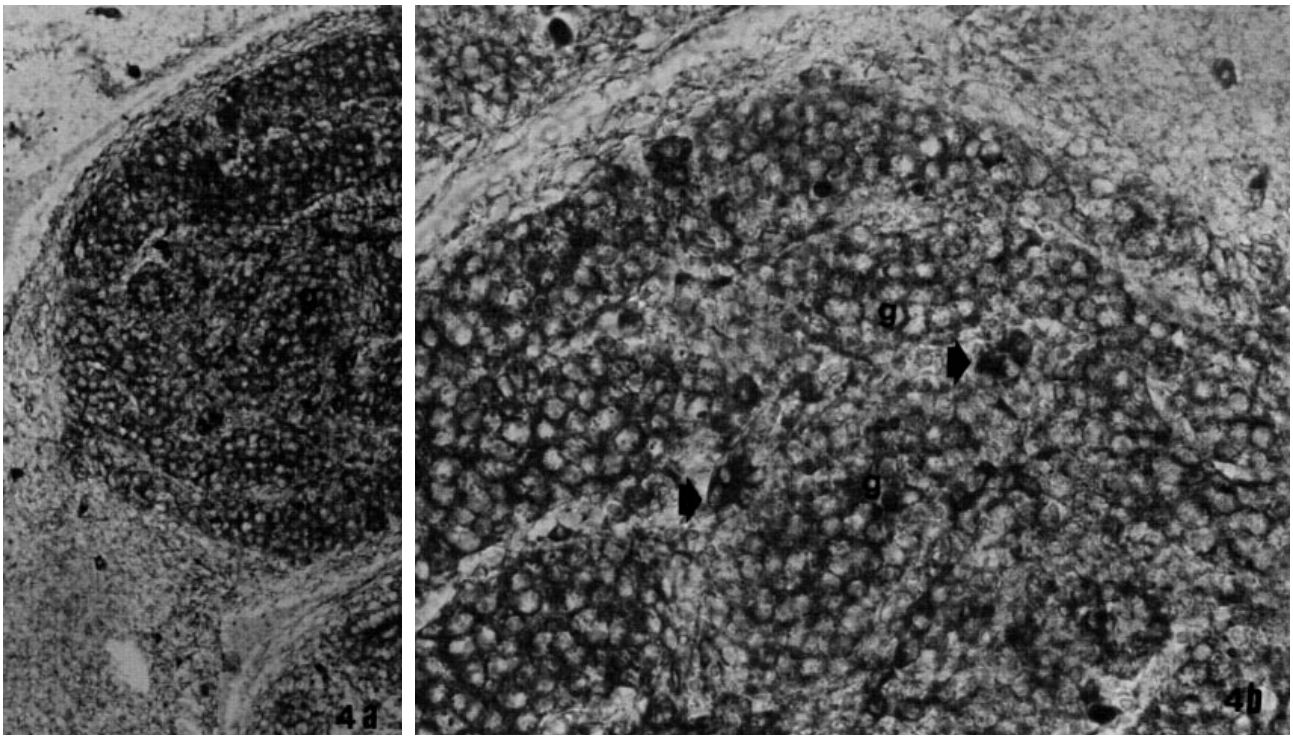


Figure 4a, b. Shows a strong EGFR immunoreactivity in interstitial Leydig cells (◗) and a medium staining in gonocytes (g) of the seminiferous tubules in 18-day-old foetal testes. a: X200, b: X400.

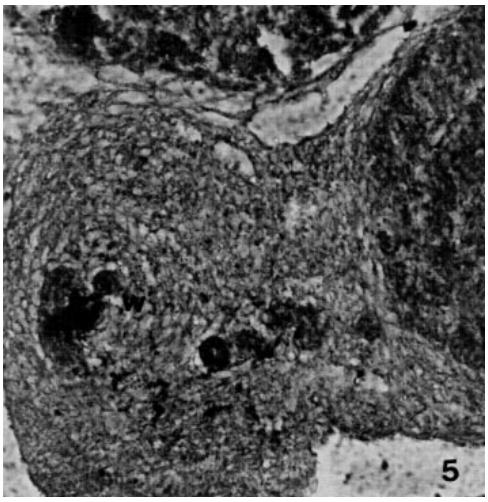


Figure 5. Demonstrates a strong EGFR immunolocalization in the proximal part of the Wolffian duct (W) neighbouring to the developing testes on prenatal day 18. X400.

Posnatal 10th Day and Adults

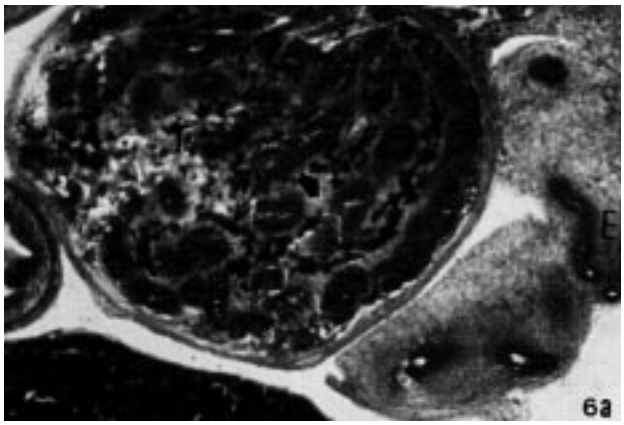
While there was no EGFR immunoreactivity in the gonadal cell lines of seminiferous tubuli having apparent lumina, the interstitial Leydig cells demonstrated a strong staining in their cytoplasm (Fig. 7). Epididymal epithelial cells had a strong EGFR immunoreactivity (Fig. 8).

Mature testicular tissue contained seminiferous tubular germinal cell lines without EGFR immunostaining, whereas a decreased amount of interstitial tissue had strongly EGFR immunostained Leydig cells (Fig. 9). Similar to the postnatal embryos, the mature epididymal epithelium showed strong EGFR staining (Figs. 10a, 10b).

Discussions

Gonadal development in mammals starts to occur with the migration of primordial germ cells to the gonadal ridge formed by the proliferation of the coelomic epithelium and condensation of the underlying mesenchyme (8,9). The gonadal ridge is located in the medial region of mesonephric duct.

Although the sexual determination of an individual occurs genetically during fertilization with the type of sex chromosome (X or Y) of the spermium, there is an indifferent stage during which the sex of an individual is not apparent. While the indifferent stage occurs until the 6th or 7th week of pregnancy in humans, it until occurs about 14th day of pregnancy in rats (9).



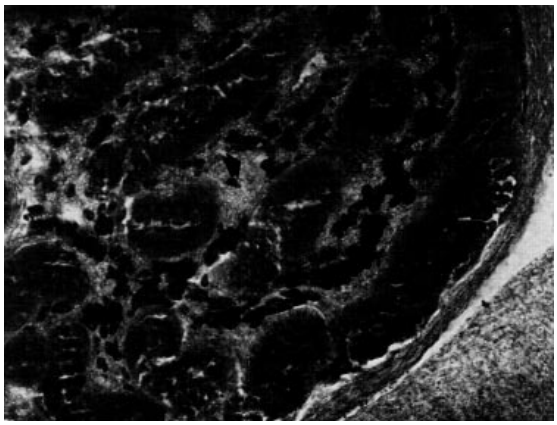


Figure 6a, b. Strong EGFR immunoreactivity in the neonatal rat testes (T) and differentiating epididymis (E) were observed (a) X100. EGFR immunolocalization in the interstitial Leydig cells (◗) is stronger than the gonocytes (g) in the seminiferous tubules in neonatal testes (b). X 200.

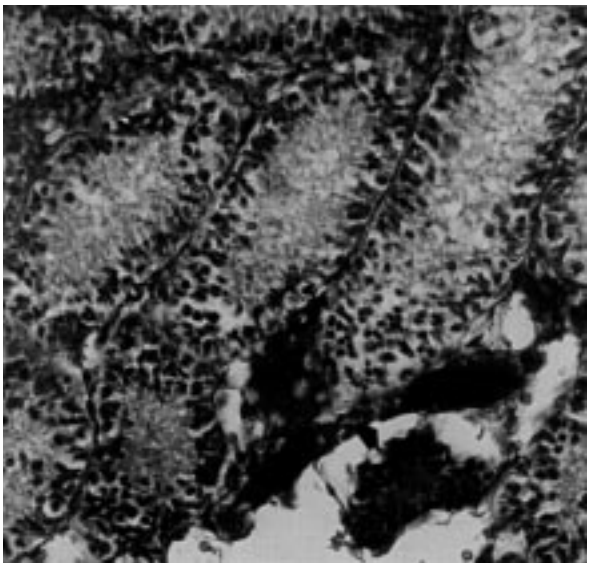


Figure 7. Strong EGFR immunoreactivity is seen in interstitial Leydig cells (◗) on postnatal day 10. X400.

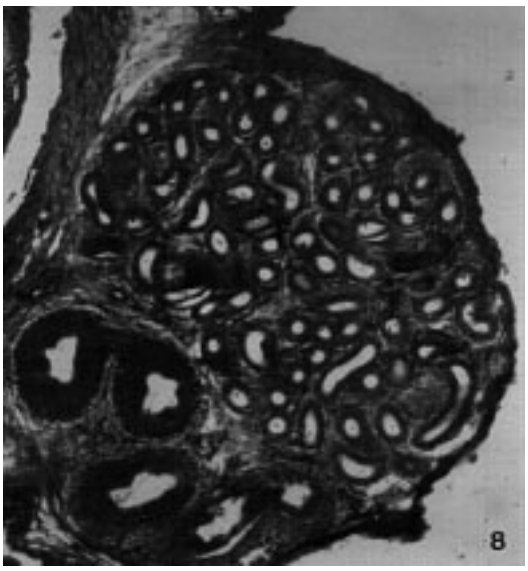


Figure 8. Shows the strong EGFR immunoreactivity in the epithelial region of epididymis (E) and there is no immunostaining in the epididymal interstitial region. X100.

It has been suggested that growth factors such as EGF, IGF and TGF-α might be important as well as genetic factors in the developmental and differentiation stages of the testes and the excretory ducts, and studies on the effects of growth factors on this subject are still being carried out (6, 10, 11).

EGF is a 53 - amino - acid polypeptide which was found firstly in the mouse submaxillar gland (12). Although it plays a key role in the proliferation and preservation of tubular germ cells, androgen aromatization, lactate secretion and inhibin synthesis from Sertoli cells, and androgen synthesis and secretion from interstitial Leydig cells, the modulation mechanism and testicular target cells of EGF remains to be understood (11,13).

The present study investigated whether or not EGF influences testicular and excretory duct development and differentiation. For this purpose, EGFR immunolocalization was studied at different stages of embryonic development and in mature testes and excretory ducts.

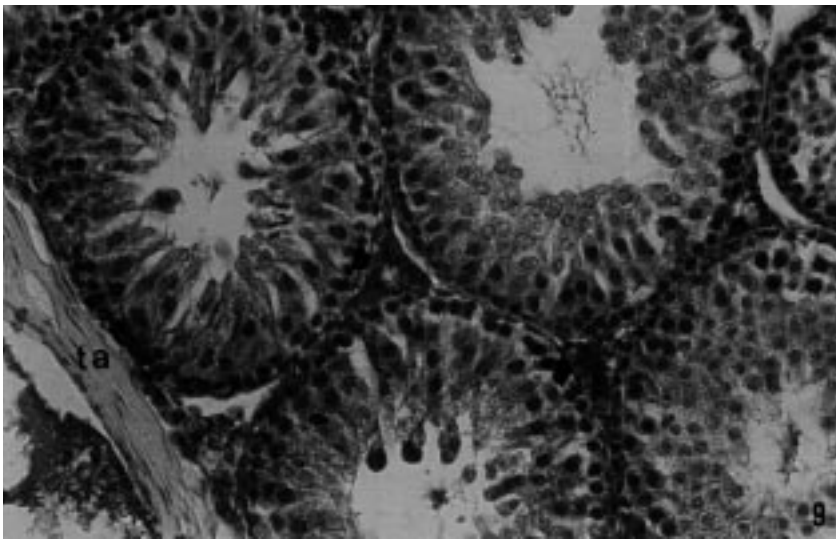


Figure 9. While there is strong EGFR localization in adult testicular interstitial Leydig cells (◗), germinal cells and Tunica albuginea (ta) show no immunoreactivity. X400, Haematoxylene counterstaining.

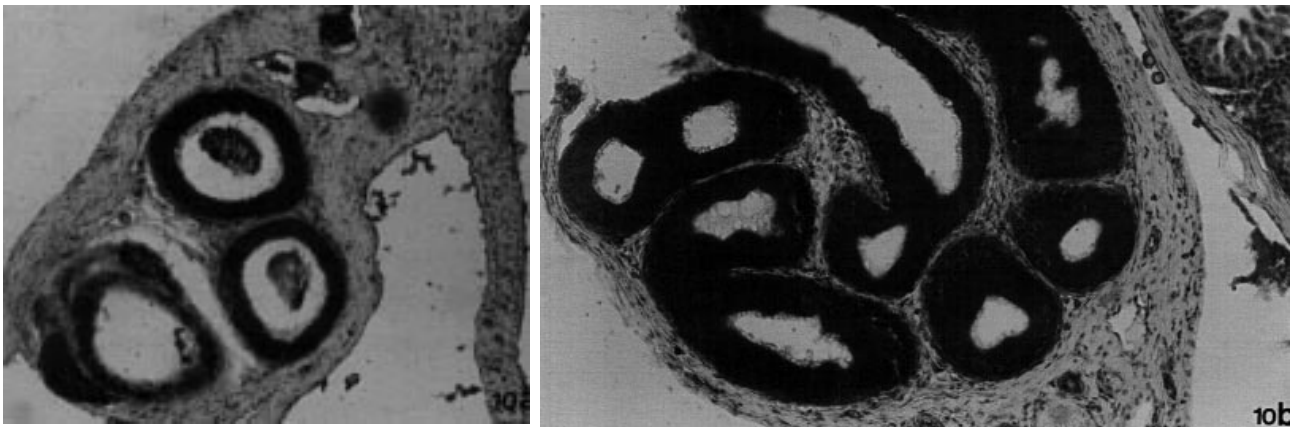


Figure 10a, b. Shows strong EGFR immunostaining in the mature rat epididymal epithelium and no immunolocalization in the interstitial region. a: X100, b: X200.

EGFR immunostaining was localized in the mesonephric duct epithelium and in the coelomic epithelium surrounding the gonadal ridge on prenatal day 13. It may be that EGF affects gonadal development, while there is no sexual differentiation in the embryo on this day. EGFR immunoreactivity was present in various testicular compartments and in excretory ducts throughout the pre- and postnatal periods and mature rats. This immunoreactivity was maximum in differentiated Leydig cells and in excretory ducts, whereas it was weaker in tubular germ cells. Moreover, the EGFR localization in these regions was stronger during the immature period than the mature period.

The findings of the present study are consistent with those of Mulaney and Skinner (1992) in that EGFR gene expression during the early testicular development was higher than that found in the pubertal period (1). Suarez-Quian and co-workers (1990) showed EGFR immunoreactivity only in testicular somatic cells (Leydig and Sertoli cells) (7), whereas others suggested strong EGFR immunostaining also in testicular germ cells, indicating a responsive capacity of those cells to EGF during spermatogenesis (6). In addition, while EGFR immunostaining was detected in Sertoli, Leydig and peritubular cells of non-human primates (4), it was found only in Leydig cells in humans (13), indicating a crucial role of EGFR in steroidogenesis.

Although there have been conflicting data on target cells of EGF in testicular and excretory duct tissues, it has been clearly demonstrated that the number of spermatocytes in the preleptoten and pakiten stages, round spermatids and mature spermia decreased following sialoadenectomy (removal of submandibular glands) in mice (14). In addition, EGF treatment of those mice (100 µg/kg/day) resulted in a number of spermium produced similar to that observed in the control group, suggesting the stimulation of meiotic phase spermatocytes by EGF (14).

Although the previous studies suggested different EGFR immunolocalizations in testicular tissue such as Leydig cells, Sertoli cells and germinal cell lines, Leydig cells were the only EGFR immunoreactive cell type shown by all authors, possibly indicating an alternative indirect mechanism of the EGF effect on spermatogenesis through influencing Leydig cell steroid production.

Three distinct waves of Leydig cell development were found in the pig testis, which occur during the foetal, perinatal and prepubertal periods. The proliferation of Leydig cells is primarily regulated by luteinizing hormone (LH). More recently, however, it has been suggested that the effect of LH on proliferation of immature Leydig cells is mediated by specific growth factors, such as EGF and IGF (15). The proliferation and differentiation mechanisms of immature Leydig cells may occur in a similar way in rats (15). Kerr and Knell (1988) demonstrated 25 000 Leydig cells in 17-day-old rat foetal testes, whereas it reached 90 000 in 21-day-old rats (16). While Gondons and co-workers (1974) described the majority of Leydig cells in the hamster testicular interstitium as being differentiated on day 14 of pregnancy (17), differentiated Leydig cells in rat testicular interstitial tissue were present on day 18 of pregnancy, forming 3-8 cell groups localized in the peritubular and perivascular regions (18).

The present study demonstrated strongly EGFR immunostained Leydig cell populations in 18-day-old embryo testes, and this strong immunoreactivity seemed to be preserved in 10-day-olds whereas it was found to be relatively weaker in the mature rat Leydig cells. It may be that the proliferation and differentiation of Leydig cells is mediated by EGF since the strong EGFR immunoreactivity coincided with the proliferation and differentiation of those cells. The activity of EGF may be LH/hCG dependent (15). On the other hand, EGF may induce the increase of intracellular calcium level, which functions as a gonadotropic stimulus (19).

Gonocytes filling the central regions of the seminiferous tubules during the early stages of testicular development undergo mitosis and migrate onto the basal laminae in the neonatal period (18, 20). The present study found no EGFR immunoreactivity in the germinal cells of the seminiferous tubules on postnatal 10th day and mature testes which were positively immunostained for EGFR on prenatal day 18 and in neonate testes. EGFR immunoreactivity observed in the germinal cells during the early stages of development could be due to the effect of EGF on the proliferation and migration of gonocytes. The findings of the present study are also consistent with those of a study that investigated the germinal cell development following various degrees of testicular damage, in which a potential effect of EGF on gonocyte development was suggested (11).

Anti-Mullerian hormone (AMH) and testosterone, secreted from the developing testes, affect the differentiation of the Mullerian and Wolffian ducts. While AMH, secreted from the foetal Sertoli cells, inhibits the development of the Mullerian ducts (21, 22), testosterone induces Wolffian duct development, which then forms the excretory ducts (21). More recent studies have demonstrated a possible role of EGF in the differentiation of the Wolffian ducts (5, 23). Gupta (1996) determined higher EGFR levels in the male rat reproductive tract than those found in females on prenatal day 18 (5). The author also suggested a higher EGFR expression induced by testosterone. On the other hand, while EGFR was located in the luminal and basolateral borders of the epididymis and vas deferens, there was no immunoreactivity in interstitium and smooth muscle in non-human primates (4).

In the present study a strong EGFR immunoreactivity in the Wolffian duct started on prenatal day 16, went on during the postnatal period and became stronger in the mature epididymis. There was strong immunostaining in the epididymis, especially in the luminal region, whereas there was no EGFR positive staining in the interstitial region. The findings of the present study indicate that EGF has a mediating effect on epididymal luminal epithelial function while inducing Wolffian duct differentiation during the developmental stages.

In conclusion, since EGFR localized in testicular interstitial tissue (especially in Leydig cells) and in the excretory ducts throughout the developmental periods, it may have a regulatory autocrine/paracrine effect on the development and differentiation of these organs.

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