Blackwell Publishing

*Asian J Androl 2007; 9 (6): 751–759*
DOI: 10.1111/j.1745-7262.2007.00320.x

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·*Original Article*·

# Androgen receptors are expressed in a variety of human fetal extragenital tissues: an immunohistochemical study

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**Abstract**

**Aim:** To investigate the expression of androgen receptors in the extragenital tissues of developing human embryo. **Methods**: Using immunohistochemistry, we investigated the distribution of androgen receptor (AR) in the extragenital tissues of paraffin-embedded tissue sections of first trimester (8–12 weeks gestation) human embryos. Gender was determined by polymerized chain reaction. **Results:** There were no differences in the expression and distribution of AR in male and female embryos at any stage of gestation. AR expression was seen in the thymus gland. The bronchial epithelium of the lungs showed intense positive staining with surrounding stroma negative. Furthermore, positive staining for androgen receptor was exhibited in the spinal cord with a few positive cells in the surrounding tissues. Cardiac valves also showed strong positive staining but with faint reactivity of the surrounding cardiac muscle. There was no staining in kidney, adrenal, liver or bowel. **Conclusion:** This study demonstrates that immunoreactive AR protein is present in a wide variety of human first trimester fetal tissues and shows the potential for androgen affecting tissues, which are mostly not considered to be androgen dependent. Moreover, it implies that androgen might act as a trophic factor and affect the early development of these organs rather than simply sexual differentiation. *(Asian J Androl 2007 Nov; 9: 751–759)*

**Keywords:** human androgen receptor; extragenital tissues; tissue distribution; fetal tissues; immunohistochemistry

## 1 Introduction

Androgens have a great variety of effects on many target tissues [1, 2]. They induce the development and physiological function of male accessory sex organs, such as the prostate and seminal vesicles, and later in life they control the functional activity of target tissues. Androgen action in these organs and tissues is believed to be mediated by androgen receptor (AR) [2].

The AR belongs to the super family of ligand-responsive transcription regulators, which includes the retinoic acid receptors, the thyroid hormone receptors and several steroid hormone receptors. Immunohistochemical techniques have become the predominant

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Received 2007-01-30 Accepted 2007-06-11

 

method of characterization of both cellular and subcellular distribution of the AR because of the sensitivity, specificity and ease of methods [3].

Using immunohistochemical techniques, AR has been clearly demonstrated in nearly all human adult tissues [3]), including fetal tissues from second and third trimester gestation, suggesting that AR is involved in early development [4]. Similarly, AR has been observed in a variety of animal tissues. We have previously studied the expression of AR in the upper reproductive tract [5] and urogenital tissues [6] of first trimester fetal tissues.

The present study has been designed to detect immunoreactive AR expression in fetal tissues, other than genital organs, many of which are not considered to be androgen dependent.

## 2 Materials and methods

### *2.1 Patient population*

The research protocol for the present study was approved by the Liverpool Research Ethics Committee. Human fetal tissues (8–12 weeks gestation) were obtained after elective therapeutic termination of pregnancy at Liverpool Women's Hospital. Informed consent was obtained from women prior to elective termination. As ultrasound scans were not performed prior to the termination, the gestational age of the fetuses was estimated by the last menstrual period (LMP) and the foot length measurement (in mm), as described previously [7]. Throughout the present paper, the gestational age used to date the specimen is based on LMP rather than postovulatory weeks.

Terminations were performed by suction and curettage of the uterine cavity under general anaesthetic. The evacuation container contained 100 mL of phosphate-buffered saline (PBS) (pH 7.6) containing 2 500 IU of heparin (CP Pharmaceuticals, Wrexham, Clwyd, UK). This was added to the container prior to the evacuation procedure to prevent the specimen from clotting. Samples were then washed in PBS to reduce the blood contamination and help in the identification of the fetal parts for assessment. The selected fetal tissues were placed in processing cassettes (BDH, Poole, Dorset, UK) that hold the tissue specimens during the embedding process, which were then fixed in 4% buffered paraformaldehyde (BDH) for 24 h at 4ºC. These tissue containing cassettes were processed in a tissue processing machine (Shandon Scientific, Cheshire, UK) for 24 h for dehydration and mounting in paraffin. 5-μm sections were cut on a microtome (Microm, Walldorf, Germany) and mounted on microscopy slides that had been coated for 10 min with 10% Poly-*L*-Lysine (Sigma-Aldrich, Poole, Dorset, UK). Sections were dewaxed in xylene and rehydrated in graded ethanols prior to staining.

After sectioning, the fetal organs were identified under the microscope. Not all samples contained all the organs to be examined because of destruction of tissues during the collection process. A total of 109 fetal samples were used.

### *2.2 DNA extraction*

The gender of the fetal samples used in the present study was determined by sex karyotyping using DNA extracted from paraffin embedded sections of the samples. Polymerase chain reaction (PCR) was used to detect the presence of X and Y chromosome material at the Amelogenin (AMXY-specific for X chromosome) and SRY (for Y chromosome) loci. Details of this method and the primers used in this study have been described previously [5].

### *2.3 Immunohistochemstry*

The immunohistochemistry was performed as previously described [5]. The primary antibody, mouse anti-human androgen receptor antibody (AR 441; Dako, Cambridgeshire, UK) was incubated with the sections in a humidity chamber for 30 min. These sections were then washed with the buffer and incubated with secondary rabbit anti-mouse immunogloulins (Z0259; Dako, Cambridgeshire, UK) in a dilution of 1 in 25 for 30 min. Following this, the sections were washed three times with Tris-buffered saline (TBS) and incubated for 30 min with mouse monoclonal peroxidase-antiperoxidase (P0850; Dako, Cambridgeshire, UK) diluted to 1:100 in TBS. Finally, after further washing with TBS, visualization was carried out using 3,3'-diaminobenzidine tetrahydrochloride (DAB) (Sigma-Aldrich, Poole, Dorset, UK). DAB acts as a chromogen and the sites indicating antibody binding become brown in the sections following this treatment, suggesting AR-positive cells. After checking the staining intensity, the sections were washed in water and couterstained with Harris haematoxylin (Merck, Poole, Dorset, UK). These were dehydrated in ascending grades of ethanol and then, after clearing the slides with xylene, they were mounted on DPX mountant (Merck, Poole, Dorset, UK).

For all tissues examined using immunohistochemistry, mouse IgG was used as a negative control. Slides were examined by two independent observers who were blinded to the sex and gestational age of the samples. The percentage of positively stained cells for each tissue layers was examined under high power field (× 400) and the intensity of the positively stained cells was classified into six different groups, including all cells positive, strong, moderate, mild and occasional staining and none for no positive staining. All cells meant that 100% of the cells in the high power field were strongly positive. Strong staining had 60%–90%, moderate 30%–60%, mild 10%–30% and occasional staining had less then 10% of positively stained cells in that particular tissue layer. None meant that no positively stained cells were identified.

## 3 Results

In the present study, 109 samples of gestational age 8–12 weeks were examined. Samples of this gestational age were used because most of the organs can be identified at these times. PCR karyotyping demonstrated that 59 samples were female and 50 were male. From these samples, the following tissues were examined: thymus ($n$ = 15), lungs ($n$ = 36), spinal cord ($n$ = 35), heart ($n$ = 23), kidneys ($n$ = 43), adrenals ($n$ = 32), liver ($n$ = 14) and bowel ($n$ = 31).

For evaluation, the immunohistochemical localization and relative intensity of the positive staining of AR was expressed in terms of three relative intensities: high intensity (+ + +) with more than 80% of cells being positively stained, moderate intensity (+ +) with 10%–80% of cells positively stained, and low intensity, when 10% of cells positively stained cells were labeled as (+), as shown in Table 1. Those samples with no AR positive cells were labeled as negative. The relative intensity of tissue was determined by comparison with the positive control, for which prostate cancer sections were used.

The immunostaining was observed in thymus, lungs, spinal cord and heart tissue, whereas the kidney, adrenal gland, liver, rectum and bowel were devoid of any immunoreactive AR staining.

No difference was observed in the number of AR positive cells for all organs studied between the male and female samples. Similarly, no gestational age difference was observed in the expression and distribution of AR. For all tissues examined, mouse IgG was used as a negative control (Figure 1C, D).

### *3.1 Thymus*

In the present study, 15 thymuses were examined, comprising male ($n$ = 8) and female ($n$ = 7) samples. In the thymus, both the lobes showed strong AR immunoreactivity (Figure 1A, B) (Table 1). The intervening tissue between the two thymic lobes showed no positive staining (Figure 1A).

### *3.2 Lungs*

A total of 36 samples were analyzed, with 20 female and 16 male specimens. AR was localized within the epithelium of the developing and branching bronchi (Figure 1E, F, Table 1). These epithelial cells surrounding the bronchial lumen were tightly packed together and all epithelial cells were stained positive for AR, whereas the bronchial lumen (Figure 1F) and the surrounding mesenchymal stroma showed no positive staining (Figure 1E, F).

Table 1. Summary of the immunohistochemical localization and relative intensity of positive staining of androgen receptor in the cells of first trimester fetal tissues stained with androgen receptor antibody. The designation appears as high (+ + +), moderate (+ +), low (+), or no (–) relative positive staining.

| Tissue | Relative staining |
|---|---|
| Thymus | + + + |
| Lungs | |
| Alveolar epithelium | + + + |
| Surrounding stroma | + |
| Spinal cord | |
| Epithelium | + + + |
| Stroma | + |
| Heart | |
| Valvular region | + + + |
| Cardiac muscle | + |
| Kidney | |
| Glomerulus | – |
| Tubules | – |
| Adrenal gland | |
| Cortex | – |
| Medulla | – |
| Liver | – |
| Bowel | |
| Epithelium | – |
| Stroma | – |

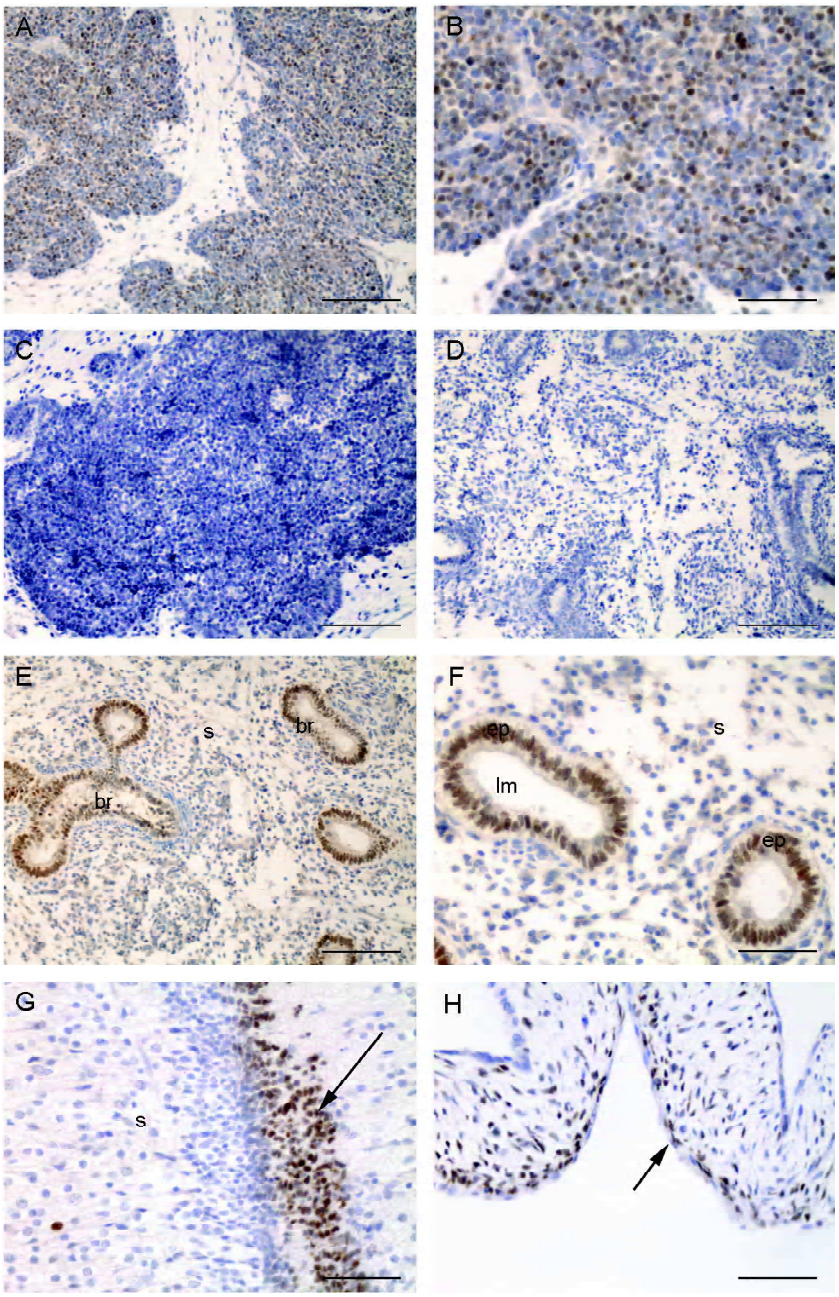


Figure 1. Immunohistochemical localization of androgen receptor (AR) in first trimester human fetal thymus and lungs. Immunopositive cells for AR appear brown as a result of diaminobenzidine tetrahydrochloride (DAB) colorimetric reaction. (A): AR staining in a 10-week female thymus showing cells in both the lobes of the gland to be closely placed but all the cells not uniformly stained (× 200) (bar = 150 μm). Intervening tissue between the two lobes shows absence of staining. (B): AR positive staining in a 10-week female thymus gland (high power view of (A). All the cells are closely packed together in the thymus and approximately half of the cells in the gland show positive reactivity (× 400) (bar = 300 μm). (C): Negative control (IgG) of an 11-week male thymus gland (× 200) (bar = 150 μm). (D): Negative control (IgG) of an 11 week male lung (× 200) (bar = 150 μm). (E): Low power view of 11 week male lung showing bronchial (br) branching with similar AR positive staining (× 200) (bar = 150 μm). (F): Higher magnification of (E) showing strong AR positive staining of the epithelial cells (ep) surrounding the bronchial lumen (lm). The surrounding mesenchymal stroma (s) lacks any AR positive cells (× 400) (bar = 300 μm). (G): Low power view of spinal cord showing the whole spinal cord intensely stained for AR shown by arrow. Surrounding stroma (s) shows faint staining (× 200) (bar = 150 μm). (H): High power of cardiac valve in Figure 2B showing strong staining of the cardiac valves for AR (short arrow) (× 400) (bar = 300 μm).

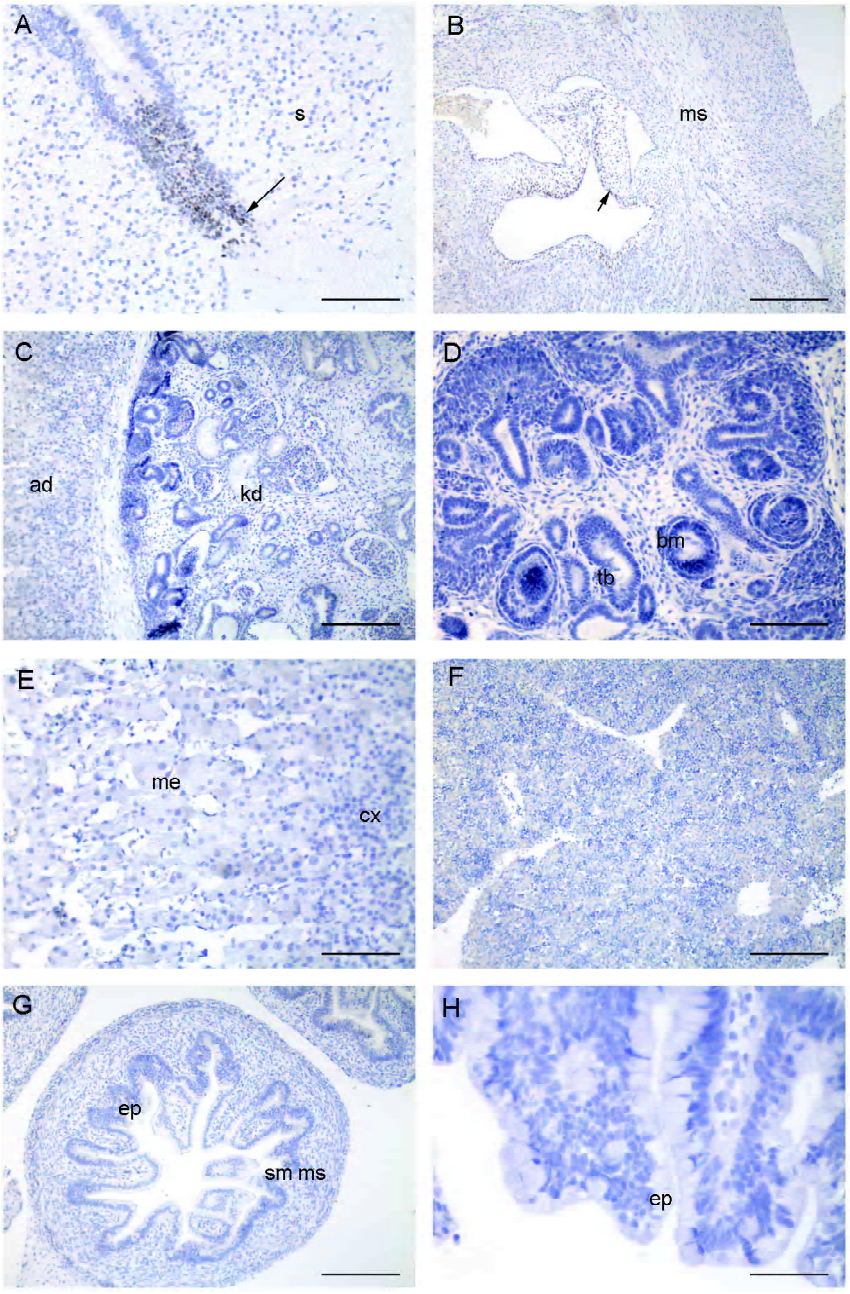


Figure 2. Immunohistochemical staining of AR in first trimester (8–12 weeks) spinal cord and heart tissues, whereas kidney, adrenal and hepatic tissue show negative reactivity. (A): Spinal cord of 11-week female (high power view of Figure 1G) showing intense immunostaining of AR in the lower end of the spinal cord. Distal end of the sacral region showing cluster of positively stained cells (shown by the arrow), but the surrounding stroma (s) shows only few positive scattered cells (× 400) (bar = 300 μm). (B): AR positive staining in an 11-week female with intense immunoreactivity staining in the valves (shown by the short arrow), but only faint reactivity in the surrounding cardiac muscle (ms) (× 200) (bar = 150 μm). (C): Eleven-week male kidney (kd) and adrenal junction (ad) showing no AR detectable reactivity in this area (× 200) (bar = 150 μm). (D): Eleven-week male kidney showing the whole organ to be negatively stained, no detectable reactivity is seen in either the Bowmans capsule (bm) or the tubules (tb) (× 200) (bar = 150 μm). (E): Eleven-week female adrenal gland showing no detectable immunoreactivity in either the cortex (cx) or the medulla (me) (× 200) (bar =150 μm). (F): A 10-week male liver without detectable reactivity in the hepatic cells (× 200) (bar =150 μm). (G): Low power view of 11-week female showing cross-section of bowel with no AR staining in the epithelium (ep) or in the surrounding smooth muscle (sm ms) (× 100) (bar = 75 μm). (H): No AR staining observed in 11-week male rectum. This high power view shows absence of staining in the epithelium (ep) (× 400) (bar = 300 μm).

*3.3 Spinal cord*

We examined 35 fetal specimens with spinal cord tissue for AR immunoreactivity, including 22 male and 13 female samples of 8–11 weeks gestation. No 12-week samples of either sex were available. The spinal cord tissue showed strong immunoreactivity in the matrix zone (ventricular), which is in proximity to the lumen of the spinal cord. Where longitudinal sections were available, the whole length of the spinal cord stained strongly positive (Figure 1G, 2A, Table 1). The surrounding stroma showed occasional positive stained AR cells (Figure 1G).

*3.4 Heart*

We examined 23 samples of heart tissue comprising 11 male and 12 female tissues with gestation varying from 8–12 weeks. Occasional positive staining was seen in the myocardial tissues examined (Figure 2B), whereas moderate AR immunoreactivity was seen in the valvular tissue (Table 1, Figure 1H, 2B).

*3.5 Other tissues*

No AR positive staining was found in any of the tissue constituents of the kidney (Figure 2C, 2D) or adrenal (Figure 2E, Table 1). Similarly, no positive AR immunoreativity could be detected in the hepatic parenchymal cells (hepatocytes) (Figure 2F, Table 1).

All of the 31 samples of fetal tissue, which had the bowel or rectum present, showed no AR positive staining in either the epithelial and smooth muscle of the bowel (Figure 2G) or the epithelial tissue of the rectum (Table 1, Figure 2H).

## 4 Discussion

This study demonstrates localization of AR by immunohistochemical analysis in first trimester human fetal tissues, several of which are not generally regarded as potential targets for androgens. As well as biochemical assay, immunohistochemical techniques can provide reliable information on the cellular/subcellular distribution of AR in a wide range of tissues [1].

ARs have been shown previously using immunohistochemical techniques, in human adult tissues [1, 3] and animal tissues (rats, mice) [1]. AR has also been described in adult tissues using biochemical ligand binding assays and autoradiography. High levels of immunoreactive AR positive cells were found in fetal thymus, lungs, spinal cord and heart. Our hypothesis is that the presence of AR in different organs in the first trimester fetal tissues could be responsible for the growth and development of these organs.

*4.1 Thymus*

The presence of specific ARs in the human thymus has been reported previously in second and third trimester gestation foeti and mature stillborn fetuses [8] and childhood thymi (7 months–6 years) [9]. The exact site of action of androgen in the thymus is presently unclear. In some studies, AR expression has been reported to be restricted to the thymic epithelial cells [8], whereas others have presented data in support of expression of AR in developing thymocytes [9].

In addition, previous investigations in the rat and mouse thymi have demonstrated androgen receptors in thymus, although their cellular localization has been disputed [11]. They have been reported to be present in the thymocytes [11] as well as in the thymic epithelial cells [12].

In the present study, the human fetal thymus examined were from 8–12 weeks gestational age. We observed that the cells in the thymus were closely placed in both the lobes of the gland and approximately half of the cell population of the thymus were AR positive. It was not possible to differentiate between the epithelial or the thymocytes staining in the thymus because of the tightly packed position of the cells in this gland. We worked on the lymphocyte differentiation markers CD45 to stain the lymphocytes and to differentiate between the two population cells but no difference in the staining was observed because of the compact and overcrowded nature of the cells in the gland. Khylostova *et al.* [13] demonstrate that the first lymphocytes in the human thymus primordium appear at 7.5–8 weeks gestation. In the thymus, the lymphocyte begins to invade the epithelial stroma, and various thymic hormones stimulate the thymus, causing proliferation of the thymocytes to become competent T lymphocytes by 14–15 weeks of gestation. A major implication of these observations is that androgenic steroids might be capable of exerting immuno-modulatory effects on the maturing lymphocytes within the thymic environment and, upon maturing, the cells might no longer express the androgenic receptor protein. This observation is further supported by the absence of AR in circulating lymphocytes, mature T cells from the thoracic duct [9] and in the peripheral organs of the immune system [1].

Androgens are thought to play a role in thymic involution in animals and appear to exert antiproliferative effects on thymic tissue both *in vivo* and *in vitro*, thereby exerting considerable influence on the size and composition of the thymus. Removal of androgens by castration of adult male animals results in remarkable thymic enlargement and increase in the cell population [14], whereas these results are reversed by androgen replacement. This data can, therefore, be interpreted as further evidence that the effects of androgens on thymus apoptosis are dependent on expression of a functional AR.

*4.2 Lungs*

Nuclear receptors and their ligands are known to play important roles in lung development. Various factors, including the signals through nuclear receptors, have been proposed to contribute to the branching morphogenesis of developing human lung. In the present study, AR immunoreactivity was detected during the critical time period of human pulmonary development, as fetal lung branching morphogenesis commences at 5 weeks gestation. Previous studies have reported the expression of AR to be significantly higher in the second trimester fetal lung than in the adult lung [15]. To our knowledge, this is the first study to demonstrate the expression of immunoreative AR in the epithelial cells of the branching bronchi in the human fetal lung as early as 8 weeks. The presence of AR at this early gestation could suggest the role of androgen in the early development of human lung as rapid division of the branching bronchi occurs in the first trimester.

The activation of androgens on lung morphogenesis is similar to its developmental regulation of other target tissues, where it stimulates cell proliferation in androgen sensitive tissues, such as the seminal vesicles and prostate. In the developing prostate and seminal vesicle, the androgens help in cell proliferation. Similarly, the lungs undergo rapid branching and proliferation in the first trimester and, therefore, AR might be responsible for this phenomenon.

*4.3 Spinal cord*

Our results confirm the presence of the AR within the matrix (ventricular) zone of the spinal cord. This layer of epithelial cells is closest to the central canal (lumen) of the neural tube and undergoes mitosis, ultimately becoming the ependyma, a columnar epithelium that lines the ventricular system and the central canal of the nervous system. This finding supports our hypothesis that AR might be involved in the growth of the neuroepithelial cells.

Studies have identified AR in both ventral and dorsal spinal cord of male and female rats [16]. However, further investigations, such as the application of immunoelectron microscopy, is be required to study the accurate intracellular localization of these receptors in the human spinal cord.

*4.4 Heart*

In the myocardial tissues tested, strong staining was mostly confined to the cardiac valves, whereas the myocardial tissue surrounding the valves had fewer stained cells. Similar findings have been demonstrated in animal tissues by autoradiographic, biochemical ligand binding and immunohistochemical analysis [1]. However, Ruizeveld de Winter *et al.* [3] showed positive AR in adult male human myocardial tissues, whereas the equivalent female tissues were negative for staining. However, only four specimens were examined in this work.

*4.5 Other tissues*

Some of the tissues (kidneys, adrenal, liver, large bowel and rectum) were negative for AR in the present study, whereas previously reports have demonstrated positive AR expression in the fetal mid-trimester (14–22 weeks gestation) and in adult tissues [17]. It is difficult to compare these results directly, as these studies used different techniques (Western blot analysis) and considered different population groups (i.e. second trimester fetal tissues [17].

The inability to detect AR immunohistochemically in fetal renal and adrenal tissues might be attributed to either an absence of AR or a very low AR content of these tissues in early fetal life. Later in adult life, the AR in androgen producing cells might be involved in an autocrine function [1]. The effects of androgens on the kidney has been examined extensively in murine kidney but little data on the fetal kidney is available. Our immunostaining failed to show any AR staining in the liver.

The absence of immunostaining of the intestinal tissues (bowel, rectum) is in contrast to positive findings in these tissues in adult specimens with Western blot analysis. The inability to detect AR immunohistochemically in early development stages might be attributed to the absence of AR content of these tissues in early gestation. These changes might appear later in the development or

in adult tissues. In all the tissues examined from 8–12 weeks gestation, similar staining was observed in embryos of both sexes.

Female predominance exists for autoimmune disease, such as systemic lupus erythematosis and rheumatoid arthritis (RA). This is because oestrogen in women appears to promote more exuberant immune responses and a heightened risk of autoimmunity, whereas androgen exerts generally suppressive effects [11]. However, in our study, we did not find any difference in the expression of AR in male and female thymic tissue. It is not known if these differences might appear later in gestation or in the adult.

Although human fetal androgen production begins at about 6–8 weeks gestation with maximum concentration reached at the end of the first trimester [18], no sex difference was observed in the expression and distribution of AR in lung tissues at any gestation [19]. This suggests that androgen affects the early lung development rather then sexual differentiation. Moreover, it is possible that expression of AR in the developing tissue might be modulated by several different co-activaor and co-repressor proteins [20]. Therefore, changes in the expression level and pattern of steroid receptor coactivators or corepressors, or mutations of their functional domains can affect the transcriptional activity of the steroid hormones and, hence, cause disorders of their target tissues. Such ligand-dependent activation of AR has been described in prostate cancer.

In the present study, both the male and female spinal cord showed similar patterns of expression of AR in both sexes and at all gestational periods examined. This is in accordance with studies using both light and electron microscopy, yet in those studies no qualitative or quantitative difference of immunoreactivity has been observed in male and female rats.

The male and female adrenal gland and renal tissues showed the same pattern of staining with no difference evident in the expression at any gestational age and between the two sexes. Similar results have been described in immunohistochemical analysis of adult tissues. Both male and female tissues showed similar patterns of expression at all gestation examined (8–12 weeks) in the hepatic and intestinal tissues.

The present study demonstrated the diversity of androgen effects on many target tissues. It reveals that AR might play an important role in the early development of many organs rather than just sexual differentiation.

## Acknowledgment

We wish to thank Ms Michelle Bates for her valuable time and helpful assistance in the photography.

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