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How Do You Get Six Meters of Epididymis Inside a Human Scrotum?

Review

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ABSTRACT: It is very clear that the epididymis plays a crucial role in the maturation of spermatozoa, and without a fully developed and functional epididymis, male infertility will result. We are especially interested in understanding the mechanisms that regulate the development of this important organ because disruptions to epididymal function will also arise as a consequence of abnormal development. Very little is known either of the process of epididymal development or the nature and causes of congenital defects that lead to male infertility. A major event during Wolffian/epididymal duct embryonic development is elongation and coiling and this short review outlines potential mechanisms by which these events occur. It is hypothesized that elongation is the result of cell proliferation coupled with directed cell rearrangements, the later regulated by the planar cell polarity signaling pathway. Coiling proceeds in a proximal to distal manner, with three-dimensional coiling beginning approximately embryonic day 16.5 to 18.5 in the mouse. The exact mechanisms of coiling are not known but we hypothesize that it involves an interaction between the Wolffian duct epithelium and the surrounding mesenchyme cells, such that the extracellular matrix is remodeled to allow coiling and growth of the duct. Cell proliferation in the Wolffian duct appears to be dependent on the presence of androgens and mesenchymal factors during embryonic development, but lumicrine factors play an additional role during postnatal development.

Key words: Androgen, reproductive tract, development, Wolffian duct.

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It is quite remarkable that in the mouse, the epididymis is just over 1 m (about a hundred times its body length); in the rat, it is 3 m; and in the human, it is 6 m (Maneely, 1959; Von and Neuhaeuser, 1964; Turner et al, 1990; Jiang et al, 1994; Stoltenberg et al, 1998). How does biology do this? How can a duct of such length elongate, coil, and fit into such a small space? Simply, the answers to each question are, it is not known. In this overview, ideas and hypotheses with some data will be used in an attempt answer the question as to how the developing epididymis (Wolffian duct) elongates and coils. The reason for such a long duct is presumably because without the appropriate length, sperm maturation would not occur. It is not possible to investigate the human Wolffian duct to answer the question posed in the title, and so the work presented is shown for the developing mouse Wolffian duct. The assumption is that the human Wolffian duct uses similar strategies to that of the mouse Wolffian duct to elongate and coil so that a proper functioning epididymis results. Without this, male infertility will result.

The formation of tubes is a fundamental biological process during the genesis of many organs—for example, kidney, intestine, brain, heart, and lungs (reviews by Hogan and Kolodziej, 2002; Lubarsky and Krasnow, 2003; Nelson, 2003; Neumann and Affolter, 2006; Andrew and Ewald, 2010). In fact, very few organs do not form tubular structures at some point in their development. Formation of the tubular structure is then followed by unique morphogenic events in each organ to generate their final adult structure. For example, the heart begins as a simple tube but folds and eventually forms the 4 chambers. In contrast, many organs, such as the kidney, the salivary gland, or the lung, begin as a simple tubular bud that ramifies into a very complex branched structure. The morphogenesis of the developing Wolffian duct exhibits a unique pattern. After the Wolffian duct forms as a simple tube, it then systematically elongates and coils dramatically, efficiently packing a tremendously long tube into the minimum of space.

How Does the Wolffian Duct Elongate?

There are several hypotheses by which the Wolffian duct could elongate (see also recent review by Joseph et

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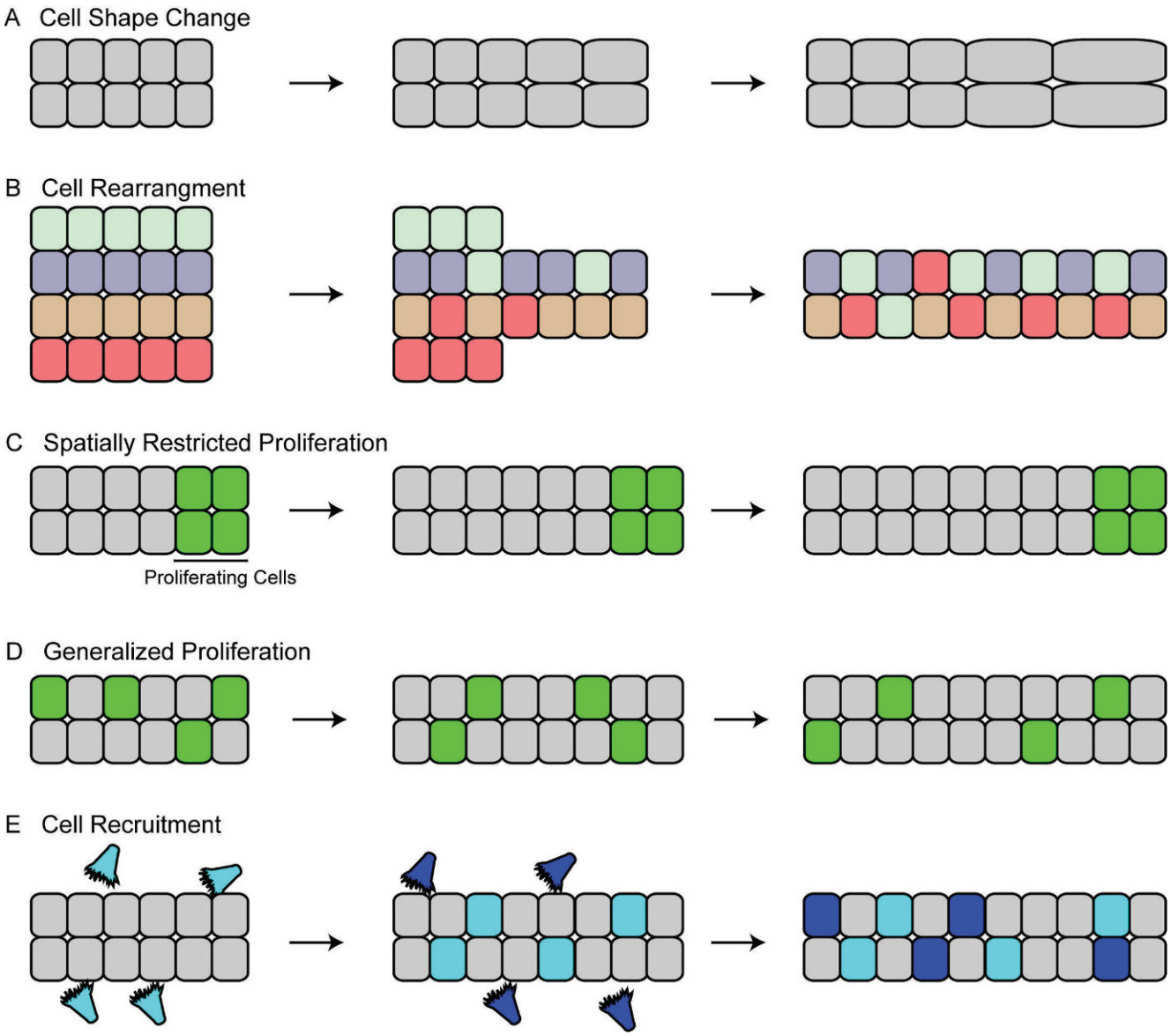

Figure 1. Figure legend taken from Andrew and Ewald (2010): Tubes can elongate by several distinct mechanisms. Cell number can remain constant and tubes can elongate as individual cells become longer **(A)**. Cell number can remain constant and tubes can elongate as cells rearrange **(B)**. Cells can divide either locally **(C)** or globally **(D)** to increase tube length. New cells can be recruited to elongate the tube. Reprinted from Andrew and Ewald (2010) with kind permission of Elsevier and Dr Andrew J. Ewald.

al, 2009) and include: 1) cell proliferation, 2) cell shape change, 3) cell rearrangements, such as cell intercalation, and 4) cell recruitment, such as mesenchymal-epithelial transition. Examples of different mechanisms by which the Wolffian duct can elongate are shown in Figure 1 (Andrew and Ewald, 2010). Cell proliferation is the most obvious because it is well known that the epithelium of the Wolffian duct and the postnatal epididymis is highly proliferative (Leeson and Leeson, 1964; Clermont and Flannery, 1970; Sun and Flickinger, 1982; Sujarit and Jones, 1991; Ramirez et al, 1999). Some preliminary studies in our laboratory (Xu, Yang, and Hinton, unpublished observations) have shown that from embryonic day 14.5 to postnatal day 1, the cells of the mouse Wolffian duct are proliferative and that rate of cell proliferation along the entire length of the duct is equal. Therefore, we suspect that the manner in which the Wolffian duct elongates through cell division is by generalized cell proliferation, but with equal rates along the duct (Figure 1D). At this moment, there is little evidence to suggest that the Wolffian duct elongates via spatially restricted proliferation (Figure 1C), although more studies are needed to test this hypothesis.

Cell proliferation can add both to the length and the width of the duct, depending on the orientation of the plane of division (Dyche, 1979). Cells dividing parallel to the axis of the duct, increase its length, whereas those that divide at right angles to the duct, increase the number of cells per cross-sectional area and so increase the diameter. Whether the polarity of cell division is biased during Wolffian duct development is not known, and more detailed studies are clearly needed to determine how this may contribute to overall elongation.

Duct elongation could also be the result of cell shape change. The cell could change its shape in a polarized manner, for example, along the proximal-distal axis (anterior-posterior) to increase the length of the duct. An example is shown in Figure 1A. It is not clear whether the cells of the Wolffian duct undergo cell shape changes to increase the length of the duct as suggested in Figure 1A, but a careful morphological analysis will answer this question. The *Drosophila* salivary gland is an excellent example of cell shape change contributing to tube elongation, and several key transcription factors and apically positioned membrane proteins such as Crumbs appear to play major roles in this process (see review by Andrew and Ewald, 2010).

Perhaps one of the most well studied aspects by which tissues and organs change shape during development is through directed cell rearrangements (Figure 1B; Keller, 2002), yet a role for cell rearrangements in the elongation of the Wolffian duct has not yet been established. Convergent extension is a process by which cells move between neighboring cells to lengthen the tissue in 1 dimension and narrow it in the other. The cellular mechanisms for this have been best described in mesenchymal tissues, where cells make protrusions only to their medial and lateral sides and exert traction on their neighbors to move between them. Protrusions on the anterior and posterior sides of the cell are specifically repressed.

Two different cellular mechanisms for epithelial cell rearrangement have been described. In the *Caenorhabditis elegans* hypoblast, oriented basolateral protrusions from 1 cell wedge between 2 adjacent cells, then widen and induce adherens junction remodeling and new cell-cell adhesions, in a process requiring intact microtubules and actin filaments (Williams-Masson et al, 1998). In contrast, epithelial cell intercalation during germ band elongation in Drosophila appears to depend primarily on apically driven changes. Cells undergo a polarized apical boundary adjustment in which the anterior/posterior boundaries shorten to a common vertex, thus bringing into contact dorsal and ventral neighboring cells, which then form cell-cell contacts and expand their region of interactions (Bertet et al, 2004; Blankenship et al, 2006, Bertet and Lecuit, 2009). Boundary adjustment occurs among pairs of cells (Bertet et al, 2004), as well as among larger numbers of cells, which shorten their boundaries to form rosettes, wherein all the cells meet at a common vertex and then exchange partners simultaneously (Blankenship et al, 2006).

Evidence suggests that convergent extension occurs during mouse gastrulation (Yen et al, 2009) and neural tube elongation and closure (Lu et al, 2004; Wang et al, 2006; Ybot-Gonzalez et al, 2007) and that the planar cell polarity (PCP)/noncanonical Wnt signaling pathway plays a major role in these events. Additionally, the protein tyrosine kinase 7 (Ptk7) is a critical regulator of convergent extension in the presomitic mesoderm (Yen et al, 2009), and our unpublished studies have also shown that Ptk7 may play a major role in Wolffian duct elongation, as well as cell intercalation.

Another potential mechanism to elongate the Wolffian duct is through cell recruitment (Figure 1E) by mesenchymal-epithelial transition. An example of this mechanism is the elongation of the renal tubules in *Drosophila* (Denholm et al, 2003). However, data from Mugford et al (2008) would suggest that cells from the mesenchyme do not contribute to the epithelium of the Wolffian duct. Using Cre/flox mice, these investigators were able to specifically label cells from the mesenchyme and those from the Wolffian duct. Labeled cells from the mesenchyme were not observed in the Wolffian duct during embryonic development, suggesting that mesenchymal-epithelial transition is not a mechanism by which the Wolffian duct elongates.

Evidence so far suggests that Wolffian duct elongation is primarily through a combination of cell proliferation and cell rearrangements, although cell shape change may also contribute.

How Does the Wolffian Duct Coil?

Few studies have focused on the mechanisms by which biological tubes coil, although the mechanisms of looping and coiling of the gut and heart are reasonably well known (Tsuda et al, 1996, 1998; Kurpios et al, 2008; Yin et al, 2010). From these studies, it is apparent that there is considerable extracellular remodeling and that this remodeling is asymmetric; that is, it is only observed where the looping or coiling takes place. For this to occur, considerable interaction between the epithelium and the mesenchyme takes place. Our gene microarray experiments (Synder et al, 2010) clearly show the expression of several genes that might be involved in extracellular matrix remodeling, cell adhesion, and cell movements and that some of these genes might be involved in coiling events, as shown for other tissues. At this time, the mechanisms of coiling of the Wolffian duct are not known, but we present some data describing this event.

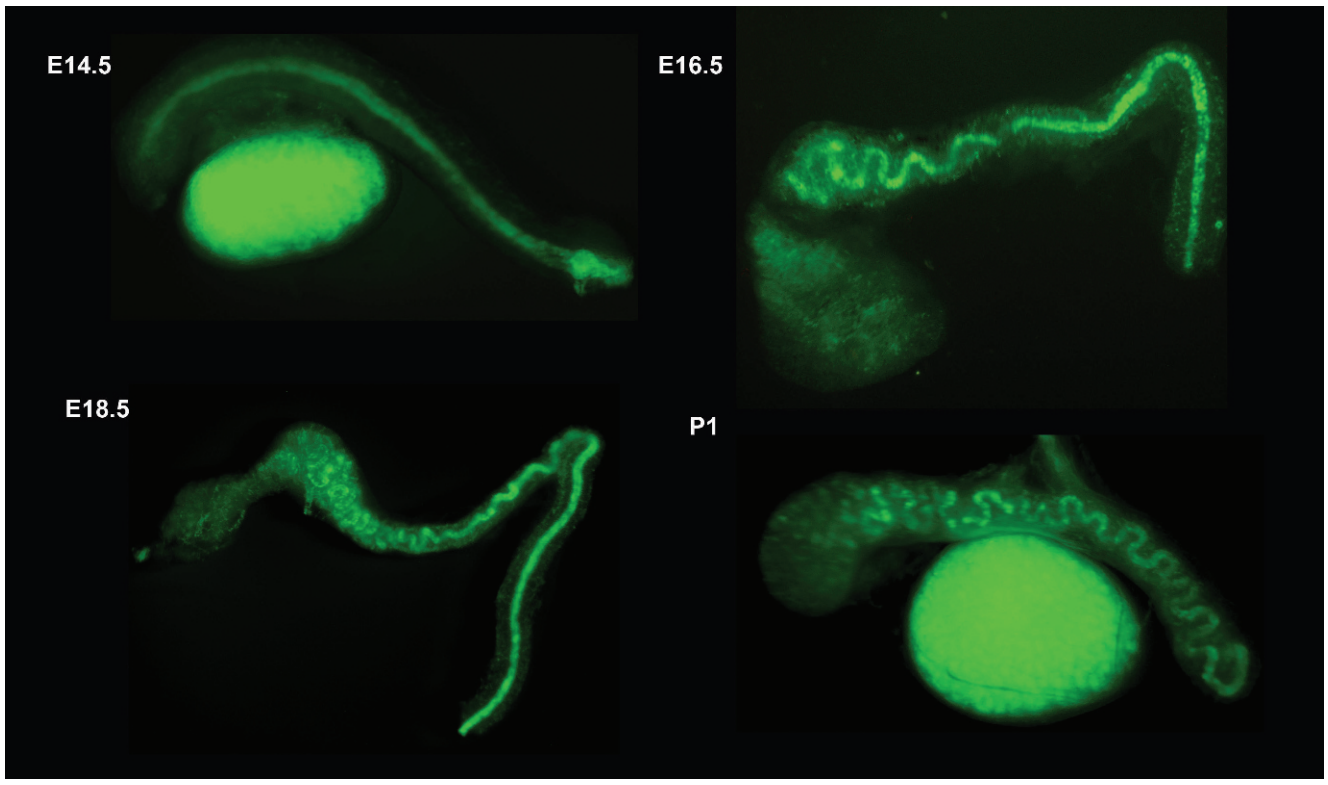

Figure 2. Wolffian ducts collected from hoxb7-GFP mice at different developmental stages, embryonic day (E)14.5, E16.5, E18.5, and postnatal (P) day 1 (P1). Note that coiling proceeds from proximal to distal. Coiling at E16.5 is initially two-dimensional, but by E18.5, three-dimensional coiling is observed.

Figure 2 shows Wolffian ducts removed from hoxb7-GFP mice at different stages of mouse embryonic development. These data show that 1) coiling moves from proximal to distal in a temporal fashion, 2) the initial stages of coiling are planar (ie, the coiling is at the two-dimensional level), and 3) three-dimensional (3-D) coiling does not begin until after E16.5. We and others have also shown similar findings in mouse Wolffian ducts cultured in vitro (Tsuji et al, 1991; Staack et al, 2003; Hannema and Hughes, 2007). Figure 3 shows a higher magnification of the proximal region of an E18.5 Wolffian duct, in which 3-D coiling is evident. On careful inspection, it can be seen that the duct does not show 360° coiling (ie, complete 360° loops are not observed). The appearance is one of switchbacks in a 3-D plane, such that when the duct seems to complete a 360° coil, the duct changes direction. How this is achieved is not clear, but we observed similar patterning from one animal to the next. One of the key points to be noted from this sequence of coiling events is that cells are proliferating at the same rate along the entire length of the duct. This may mean that coiling and cell proliferation, although intimately associated, are separately regulated. Furthermore, some regions of the Wolffian duct coil, for example, the initial segment, caput, corpus, and cauda; however, the vas deferens does not coil.

One factor regulating coiling in the vas deferens is hox gene action. In the absence of hoxa10 or hoxa11, the vas deferens undergoes coiling because of homeotic transformation of the cauda (Branford et al, 2000). It is

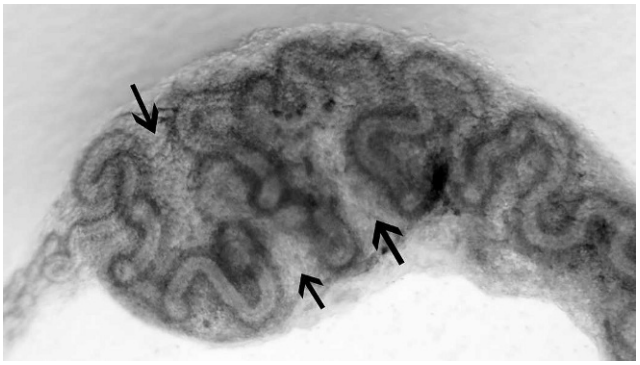

Figure 3. Initial segment and caput region of embryonic day 18.5 Wolffian duct. The tissue has been gently pulled apart to reveal the coiling pattern in greater detail. Also shown is the common efferent duct from the testis joining this region. Note in the coiling pattern that the appearance is similar to a three-dimensional switchback arrangement. Complete 360° coiling is not observed. Arrows mark the potential sites of septa formation.

possible that the multiple layers of smooth muscle surrounding the vas deferens physically prevent coiling. However, in the hoxa10 and hoxa11 knockout mice, it does appear that the vas deferens has retained its thickness, suggesting that the layers of muscle do not influence coiling in this region.

It should also be noted that at approximately E18.5, septa seemingly begin to appear (Figure 3, arrows) that will partition the epididymis into discrete segments. The septa are quite evident from approximately postnatal day 5. This partitioning is not trivial; studies show segment-specific gene expression in the adult epididymis (Johnston et al, 2005; Jelinsky et al, 2007). Although the exact role for each septum is unclear, it has been suggested that they may function to trap key regulatory molecules in the interstitium (Turner et al, 2003).

How the Wolffian duct elongates, coils, and then forms its characteristic shape and size is also of special interest. Shape and size could be dictated by several mechanisms. For example, the Wolffian duct is held proximally by a ligament attached to the kidney and is held distally by the gubernaculum. As the animal grows, forces could be exerted upon the Wolffian duct that will aid the shape and size of the tissue. In addition, members of the hippo kinase pathway, a pathway that has been shown to be involved in *Drosophila* wing size and shape, may play a role in the morphogenesis of the Wolffian duct (see Joseph et al, 2009, for further discussion). Many of the genes associated with the hippo pathway have been identified in our microarray analyses (Synder et al, 2010).

How is Wolffian Duct Elongation and Coiling Regulated?

Clearly, androgens play a critical role during Wolffian duct elongation (cell proliferation) and presumably coiling (Sujarit and Jones, 1991; Tsuji et al, 1991; Imanishi et al, 1992; Turner et al, 2003; Foster, 2006; Welsh et al, 2006, 2007; Hamzeh and Robaire, 2009; Rider et al, 2009; Welsh et al, 2009). Androgens could act either directly on the Wolffian duct epithelium or indirectly through the surrounding mesenchyme. Although testicular luminal fluid factors, or lumicrine factors (Hinton et al, 2000), play a role in the regulation of the adult initial segment region, lumicrine factors do not appear to play a role in Wolffian duct elongation and coiling. When E14.5 mouse Wolffian ducts are cultured in vitro (Figure 4), they elongate and coil without the testis, but androgens are necessary. However, in the postnatal Wolffian duct/epididymis, lumicrine factors are crucial for maintenance of cell proliferation (Xu et al, 2010). Data from our studies (Xu et al, 2010) suggests that cell proliferation in the postnatal initial segment is regulated by the extracellular signal-regulated kinase (ERK) pathway, the src family of kinases, and the phosphatase and tensin homolog. In the caput and corpus, we hypothesize that it is the ERK pathway and the phosphatase Dusp6. Cell proliferation continues throughout life in the epididymis of dusp6 null mice because of a loss of regulation of the ERK pathway (Xu et al, 2010). It is not clear at this time how cell proliferation is regulated in the cauda.

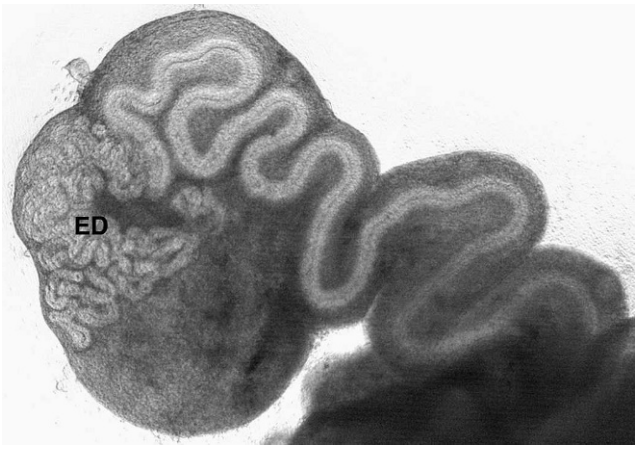

Figure 4. Embryonic day 14.5 mouse Wolffian duct that has been cultured in vitro for 2–3 days without the testis, similar to that described by Tsuji et al (1991). The media were supplemented with androgens and 1% insulin-transferrin-selenium. Note that coiling proceeds normally without lumicrine factors. ED indicates efferent ducts.

Other factors that also regulate cell proliferation/elongation and coiling during the embryonic period include members of the PCP/non-canonical Wnt pathway, as described above, as well as inhibin beta A (Tomaszewski et al, 2007). Loss of this subunit of inhibin results in a decline in cell proliferation and coiling.

It appears that Wolffian duct elongation and coiling is regulated during the embryonic period by androgens, members of the PCP pathway, and inhibin beta A, whereas during the postnatal period, lumicrine factors (eg, FGFs; Lan et al, 1998; Kirby et al, 2003; Cotton et al, 2008) and androgens play a major role.

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