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Journal of Anatomy

DOI:
10.1111/joa.13580

Published: 01/04/2022

Peer reviewed version

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Regulation of posterior Hox genes by sex steroids explains vertebral variation in inbred mouse strains

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Keywords

Vertebrae, homeotic transformation, sex steroids, Hox, endocrine disrupting chemicals

Abstract

A series of elegant embryo transfer experiments in the 1950's demonstrated that the uterine environment could alter vertebral patterning in inbred mouse strains. In the intervening decades, attention has tended to focus on the technical achievements involved and neglected the underlying biological question: how can genetically homogenous individuals have a heterogenous number of vertebrae? Here I revisit these experiments and, with the benefit of knowledge of the molecular-level processes of vertebral patterning gained over the intervening decades, suggest a novel hypothesis for homeotic transformation of the last lumbar vertebra to the adjacent sacral type through regulation of Hox genes by sex steroids. Hox genes are involved in both axial patterning and development of male and female reproductive systems and have been shown to be sensitive to sex steroids *in vitro* and *in vivo*. Regulation of these genes by sex steroids and resulting alterations to vertebral patterning may hint at a deep evolutionary link between the ribless lumbar region of mammals and the switch from egg-laying to embryo implantation. An appreciation of the impact of sex steroids on Hox genes may explain some puzzling aspects of human disease, and highlights the spine as a neglected target for *in utero* exposure to endocrine disruptors.

Background

In 1958, Anne McLaren and John Biggers published one of the most important papers in the history of reproductive and developmental biology, on the successful birth of live mice following a period of *in vitro* culture as early embryos (McLaren & Biggers, 1958). These experiments paved the way for mammalian experimental embryology, culminating in chimaeras, transgenic animals, and the identification of embryonic stem cells (Pedersen & Salter, 2007; Tam & Lovell-Badge, 2007), and were an important step in the development of human *in vitro* fertilisation and the first "test tube" baby twenty years later (Biggers, 1998; Johnson, 2019a, 2019b). What is often neglected in consideration of these experiments is the reason they were performed – as part of a wider project investigating vertebral variation in inbred mouse strains. Laboratory mice such as the widely-used C57BL6 strain typically have 7 cervical, 13 thoracic, 6 lumbar, 4 sacral, and ~30 caudal vertebrae, although this latter is variable, including with age (Hankenson et al., 2008). However, the C3HBi strain was known to have a high proportion of individuals with 5 lumbar

vertebrae (Green, 1962; McLaren & Michie, 1954b, 1955, 1956a, 1958b; Whitmore & Whitmore, 1985). Breeding experiments failed to identify the underlying cause, except to show that the trait tended to follow the maternal line (Green & Russell, 1951; McLaren & Michie, 1958b; Russell & Green, 1943). Through a painstaking series of embryos transfer experiments, McLaren and Donald Michie were able to show that the uterine environment was responsible for the reduction in the number of lumbar vertebrae (McLaren & Michie, 1958a, 1958b).

Regional numerical variation such as this can be achieved in two ways: either by a change in the number of segments formed during embryonic development, or by transformation of a segment. Bateson, in his 1894 study of variation defined changes that involved addition or subtraction of segments as *meristic* changes, and those that involved transformation of one body part to another as *homeotic* (Bateson, 1894). McLaren and Michie proposed that the 5 lumbar vertebrae phenotype of the C3HBi strain was through transformation of the sixth lumbar vertebra (L6) into the adjacent more posterior sacral type, through a process of sacralisation, i.e. a homeotic transformation. In some individuals this process might be incomplete, forming transitional vertebrae with characteristics of both types, and/or asymmetric vertebrae.

Whilst variation in the number of lumbar vertebrae in inbred mice may seem a rather niche subject, vertebral variation is a widespread phenomenon, and may be a common mammalian trait. Indeed, in some agriculturally important species such as pigs and sheep, numerical increases in vertebrae are desirable if they increase carcass length or are associated with an greater number of teats (Donaldson et al., 2013; Freeman, 1939; Mikawa et al., 2007; Zhang et al., 2017; C. Li et al., 2019). In other species, such as cats and dogs, vertebral variation and transitional vertebrae have been implicated in health issues such as *cauda equina* syndrome (Harris et al., 2019; Morgan et al., 1993; Newitt et al., 2008; Flückiger et al., 2006), and in thoroughbred race horses they may impact gait and performance (Haussler et al., 1999). Humans typically have 7 cervical, 12 thoracic, 5 lumbar, 5 sacral and 4 caudal vertebrae, except for the nearly 8% of humans that show numerical variation in the spine, and just over 3% of humans may have transitional vertebrae (Tins & Balain, 2016), which can underlie congenital scoliosis and lower back pain (“Bertolotti's Syndrome”) (Jancuska et al., 2015; Lee et al., 2015). Both numerical variation and the presence of transitional vertebrae have implications for determining the correct level for surgical intervention and injections (Konin & Walz,

2010). Vertebral variation is extremely common in deceased foetuses and infants, suggesting a high level of *in utero* negative selection (ten Broek et al., 2012). Finally, alteration of vertebral number, especially in the lumbar region, was a fundamental process in human evolution, as we shifted from a "long-backed" ancestor with a large number of lumbar vertebrae and a flexible trunk region, to a short-backed, more rigid morphology as required for a sustained upright posture (Machnicki & Reno, 2020; McCollum et al., 2010; Thompson & Almécija, 2017). But how is this vertebral variation produced?

Development and specification of mammalian vertebrae

Mammals, like all vertebrates, are segmented, and this segmentation is most apparent in the repeated arrangement of vertebrae in our spine. These vertebrae are not identical, and so our spine is also regionalised into cervical, thoracic, lumbar, sacral and caudal parts (Siomava et al., 2020). The problem of numerical variation in a specific vertebral type as seen in the C3HBi mice reflects a defect in this regionalisation process, so a vertebra that should assume characteristics of lumbar vertebrae instead takes on the characteristics of the adjacent sacral type. Vertebrae form from somites, blocks of mesoderm that sequentially bud off from the presomitic mesoderm in an anterior to posterior direction during embryonic development (Hirsinger et al., 2000; Pourquié, 2018; Saga, 2012), and somite identity (e.g. thoracic, lumbar, sacral) is determined by Hox genes, a family of transcription factors (Krumlauf, 1992; Mallo, 2018; Mallo et al., 2010). Mice and humans have 39 Hox genes, arranged in 13 paralogy groups (1-13) in four clusters (A, B , C, and D) in the genome, and the order of genes along the chromosome reflects the order of their expression along the anterior-posterior axis of the embryo (Figure 1). The four Hox clusters were produced by two instances of whole genome duplication in early vertebrate ancestry, where a single ancestral Hox cluster was duplicated to two, and then to four paralogous clusters (Garcia-Fernàndez & Holland, 1994; Holland, 1999; Wagner et al., 2003), and although few paralogy groups maintain four copies, most do consist of more than one paralog, and there is functional redundancy within paralogy groups.

Although Hox expression is maintained in multiple tissues throughout embryonic development, and even into adulthood (Alharbi et al., 2013; Kachgal et al., 2012; Rux & Wellik, 2017; Song et al., 2020), it appears that somite identity is set relatively early, in the presomitic mesoderm before the somites themselves even form (Carapuço et al., 2005). Each vertebra is composed of the posterior (caudal) half of one somite and

the anterior (rostral) half of the subsequent somite as a result of a resegmentation process (Remak, 1851) that appears to be an ancestral feature of jawed vertebrates (Criswell & Gillis, n.d.), and Hox gene expression boundaries that initially align with somite boundaries are therefore later found in the middle of a developing vertebra (Ward et al., 2017). Grafting experiments that moved blocks of several somites into different regions of the embryo showed that they retained their original patterning (Kieny et al., 1972), as do half-somites transplanted from the scapula-forming cervicothoracic boundary into the more anterior cervical region (Ehehalt et al., 2004), and this has generally been taken as evidence that the positional information (Wolpert, 1969) provided by the "Hox code" established in the presomitic mesoderm is fixed. Somites and somite cells do show evidence of developmental plasticity in certain situations however. For example, the most anterior somites normally form parts of the skull, but instead form vertebrae when transplanted into the developing trunk, seemingly in the absence of altered Hox expression (Kant & Goldstein, 1999). Chick-quail grafting experiments have shown that transplanted half-somites can contribute to more than one vertebra as cells move around post-transplant (Stern & Keynes, 1987), and this cellular "leakiness" seems to be the norm for fish (Morin-Kensicki et al., 2002). When transplanted somites are rotated, so that ventral cells are located on the dorsal side and dorsal cells become ventral, the cells differentiate according to their new orientation, whilst retaining their original positional information (Fomenou et al., 2005). Hox codes can also be rewritten, as evidenced by reprogramming of small clumps of transplanted cranial neural crest cells (Trainor & Krumlauf, 2000), tailbud progenitor cells (McGrew et al., 2008), or transplanted rhombomeres (Grapin-Botton et al., 1995). When dealing with homeotic transformations between adjacent vertebral types though, we may not actually need to invoke much plasticity or Hox code rewriting but rather temporal and/or spatial shifts in gene expression.

Somites can be considered to be pools of stem cells (Christ et al., 2007), and their ultimate fate is determined by the interplay of signals from neighbouring cells and structures. Embryonic development proceeds via activation of distinct but connected sets of modules (Kuratani, 2009; Wagner, 1996), and the switch between one module and another can be effected by relatively few genes. Indeed, what we now know as Hox genes were first discovered because of their ability to effect homeotic changes when mutated, and a single mutation in a single gene can be sufficient to transform one body part into another. A

homeotic change of one vertebral type to another may therefore not only require changes to few genes, but also relatively few cells. A single somite contains only 1,000-2,000 cells, and these few thousand cells are divided into two major compartments: the dermomyotome, which forms skeletal muscle and the dorsal dermis, and the sclerotome, which forms the vertebrae and proximal parts of the ribs (Stern & Piatkowska, 2015). There is further compartmentalisation within the sclerotome itself, reflecting an apparent underlying developmental modularity (Randau & Goswami, 2017). Cells from the ventral portion of the sclerotome migrate ventrally and medially from both the left and right sides of the embryo to surround the notochord and form the vertebral body or centrum; cells from the dorsal and central portions of the sclerotome move dorsally and medially from the left and right to surround the neural tube and form the neural arch, and cells from the lateral and central portions will form the neural arches and proximal ribs on each side (DeSesso & Scialli, 2018). These structures begin to ossify in the mouse after around 14.5 days of development, and neural arches ossify before the centra (Hautier et al., 2014). The presence of multiple developmental modules and ossification centres reflects the step-wise evolutionary history of vertebrae, where arch elements predate centra (Fleming et al., 2015), and it may be that reprogramming one of these developmental modules is sufficient to transform the whole vertebra.

In the case of the C3HBi mice, the boundary between the somites that will go on to form lumbar and sacral vertebrae is the site of interest. Mouse mutants show that Hox genes in paralogy group (PG) 10 (*Hoxa10*, *Hoxc10*, *Hoxd10*) are important for the formation of lumbar vertebrae, and genes in PG11 (*Hoxa11*, *Hoxc11*, *Hoxd11*) for sacral vertebrae (Carapuço et al., 2005; Davis et al., 1995; Morin-Kensicki et al., 2002; Wellik & Capecchi, 2003; Zákány et al., 1996) (figure 1). Lumbar vertebrae do not usually form ribs, but mouse triple mutants lacking all PG10 activity developed ribbed, thoracic-like vertebrae in the lumbar region (Mallo et al., 2010; Wellik & Capecchi, 2003) suggesting that PG10 genes suppress formation of ribs. Mouse triple PG11 mutants do not form the sacrum, and instead have an elongated ribless lumbar region (Mallo et al., 2010; Wellik & Capecchi, 2003), and so in both cases mutants possess an anteriorised phenotype. The sacrum does not form properly in PG10 mutants, showing that the action of both PG10 and PG11 genes is required for correct development of this structure, and it has been suggested that PG11 genes work to partially suppress the rib-suppressing function of PG10 genes, allowing the formation of modified rib-like

lateral projections that later fuse to form the mature sacrum (Wellik & Capecchi, 2003). Transgenic mice which overexpress *Hoxa11* in the presomitic mesoderm showed fusions between adjacent ribs, similar to the fusion of lateral projections that produces the mature sacrum, and an anteriorised sacrum, shifted forwards by one to three vertebra (Carapuço et al., 2005). The anterior shift of the sacrum in C3HBi mice must therefore reflect an anterior shift in the expression of one or more PG11 genes in the presomitic mesoderm. Because Hox genes show temporal collinearity, where genes at one end of the cluster are expressed first and in anterior structures, and genes at the other end of the cluster are expressed later and in more posterior structures, this shift may be temporal. The presomitic mesoderm is dynamic, with new somites budding off at the anterior end and continual extension at the posterior end, and so if PG11 genes are expressed too early, they will therefore end up in more anterior somites than they should, and because of posterior prevalence (Durston, 2012; Lewis, 1978), are dominant to more anterior genes in the same somite. What though might cause such a shift in gene expression?

McLaren and Michie demonstrated that the uterine environment could alter vertebral identity but these experiments were performed in the 1950's, and our knowledge of the molecular-level regulation of somite identity at the time was extremely poor. Although homeotic mutants had been known from the turn of the century, Hox genes themselves were not discovered until the late 1970's and early 1980's (Lewis, 1978; Nüsslein-Volhard & Wieschaus, 1980). However, by the late 1980's and early 1990's it was clear that not only were Hox genes involved in vertebral patterning, but that they could induce homeotic transformations when mis-expressed (Balling et al., 1989; Kessel et al., 1990; Le Mouellic et al., 1992). Around the same time, it was discovered that administration of the vitamin A derivative retinoic acid (RA) to pregnant mice 8 to 10 days post-conception could induce homeotic transformation of vertebrae in the offspring, and that this occurs via alteration of Hox gene expression (Kessel & Gruss, 1991). RA is clearly a promising candidate, as it is small and soluble and can cross plasma membranes. Indeed, Anne McLaren herself considered that RA might underlie the five lumbar vertebrae phenotype of the C3HBi strain in a letter to Robb Krumlauf in 1990 (now in the British Library). However, RA predominantly regulates expression of 3' Hox genes and therefore the development of anterior structures, and whilst it is true that RA treatment can reduce the number of lumbar vertebrae, these animals have 14 (or more) thoracic vertebrae (Kessel & Gruss, 1991)

and are the result of a transformation of the first lumbar vertebra (L1) to a thoracic morphology rather than sacralisation of L6. Clearly then we should look elsewhere for the molecular basis of the five lumbar vertebrae phenotype, and the answer may provide an intriguing link between two evolutionary novelties in therian mammals.

The lumbar region and mammalian evolutionary novelties.

Ribless lumbar vertebra originated early in the evolution of therian mammals, and their origin seems to coincide with the development of several adaptations for improved locomotion and respiration. The evolution of early mammals is characterised by reduced lateral movements of the vertebral column; reduction and subsequent loss of lumbar ribs; and the origin of a muscular diaphragm at the thoracic/lumbar boundary (Buchholtz et al., 2012; Hirasawa & Kuratani, 2013; Kemp, 2006). This period also coincides with increased functional divergence between the thoracic (primarily respiratory) and lumbar (locomotory) regions. As we have already seen, Hox genes in paralogy groups 5 to 9 pattern the ribcage, group 10 genes pattern the lumbar vertebrae, and group 11 genes pattern the sacrum (McIntyre et al., 2007; Wellik & Capecchi, 2003). These genes are also required in the development of the male and female reproductive tracts. In males, PG9 genes are expressed in the epididymis and vas deferens, PG10 genes in the caudal epididymis and vas deferens, and PG11 in the vas deferens (Brechka et al., 2017; Hannema & Hughes, 2007), and loss of function mutants shows anteriorised homeotic phenotypes. Spatial collinearity is also apparent in the developing female reproductive tract, with *Hoxa9* expressed in the oviduct, *Hoxa10* in the uterus, *Hoxa11* in the uterus and cervix, and *Hoxa13* in the cervix and upper portion of the vagina, and again loss of function mutants show anteriorised homeotic transformations (Kobayashi & Behringer, 2003). *Hoxa10* and *Hoxa11* also play important roles in the adult female reproductive system, especially endometrial differentiation and embryo implantation, and *Hoxc10*, *c11*, *d10*, and *d11* are expressed in the stromal cells of the adult endometrium (Du & Taylor, 2016). Innovation in the lumbar region in therian mammals therefore coincides with evolutionary innovation in the vagina and uterus, and a shift from egg-laying to embryo implantation (Mucenski et al., 2019; Wagner & Lynch, 2005). The role of these Hox genes in the embryonic and adult reproductive tracts suggests new candidates for homeotic transformation in the axial skeleton – sex steroids. Testosterone, progesterone and estradiol are small

lipophilic molecules, and can easily cross cell membranes. Their ability to freely move through the uterine environment is perhaps best demonstrated by the intrauterine position (IUP) effect, where the development of a given embryo is impacted by the sex of its neighbours (Ryan & Vandenbergh, 2002). This effect has been most actively studied in the uterine horns of rodents, where any given embryo can have 0, 1 or 2 neighbours of the opposite sex. A female embryo located between two male embryos is exposed to elevated levels of testosterone and will show masculinised anatomical, physiological and behavioural traits as an adult, and a male embryo located between two females will be exposed to elevated levels of estradiol and will show feminised traits as an adult. Not only do sex steroids readily move through the uterine environment, they regulate Hox genes.

Whilst retinoic acid acts most strongly on 3' Hox genes (those expressed in anterior structures), sex steroids seem to regulate more 5' (posterior) genes, including those acting at the lumbar/sacral boundary (Daftary & Taylor, 2006). Testosterone has been shown to downregulate *HOXA10 in vitro*, and increased levels of testosterone in women with polycystic ovary syndrome may underlie the lower expression levels of *HOXA10* in the endometrium and the resulting decline in fertility (Daftary & Taylor, 2006), and *HOXA10* and *HOXA11* show a dynamic temporal expression pattern in the endometrium in response to increasing levels of estrogen and progesterone through the reproductive cycle (Du & Taylor, 2016). Whilst all members of a paralogy group have to be removed to obtain a knock-out phenotype (Wellik & Capecchi, 2003), changes to the expression of a single member can result in altered phenotypes, as shown by the development of extensive regions of ribless (lumbar-like) vertebrae, or the anterior shift of the sacrum and altered vertebral morphology due to ectopic expression of *Hoxa10* or *Hoxa11* in the presomitic mesoderm respectively. *Hoxd11* expression is detectable in the tailbud of the mouse embryo from around 9 days of development, and delayed expression of *Hoxd11* alone has been shown to result in a posterior shift in the position of the sacrum in mouse mutants ((Zákány et al., 1997), see (Desanlis et al., 2020) and (Bolt et al., 2021) for other examples of gain-of-function phenotypes due to changes in single Hox genes). Sex steroid-induced changes to a single PG11 Hox gene, resulting in a gain of function would therefore be sufficient to explain the sacralisation of L6 in the C3HBi strain, but when might this be happening?

Earlier expression the presomitic mesoderm would result in the presence of PG11 transcript(s) in a more anterior region of the embryo than would normally be expected, and resegmentation and posterior prevalence (where segment identify is defined by the most posterior (5') Hox gene expressed) would result in transformation of the last lumbar vertebra to a sacral phenotype. The required shift could be relatively subtle, probably less than half a somite length. The lumbar/sacral boundary is located at somite 31 in the mouse, with the rostral portion contributing to the L6 vertebra and the caudal portion contributing to S1 (Chal & Pourquié, 2009) (figure 1), and this somite is formed at around 10-10.5 days of development (Theiler Stage 16) (Theiler, 1989). At the same time, levels of plasma progesterone decline, starting at day 8 and reaching the lowest circulating levels on day 10 (Murr et al., 1974; Naruse et al., 2014) figure 2, corresponding with the transition from pseudopregnancy to pregnancy, and the switch from pituitary to placental control of hormones (Choudary & Greenwald, 1969). If this decline in ovarian progesterone was not to occur, or to occur more gradually, levels of progesterone would be elevated relative to "normal", leading to inappropriate expression of *Hoxa10* and *Hoxa11*, and an anterior shift in the position of the sacrum through homeotic transformation of the last lumbar vertebra. Support for a role for the ovary in the 5 lumbar phenotype comes from ovary transplantation experiments in the 129 strain of mice, which also shows the 5 lumbar vertebrae phenotype. When 129 strain ovaries were transplanted into F1 hybrid 129 x BALB/c females that were mated with 129 males, the resulting offspring showed an increased prevalence of 5 lumbar vertebrae (Russell, 1948). There is some evidence for inter-strain variation in levels of progesterone during early pregnancy (e.g. see Murr et al vs McCormack & Greenwald (McCormack & Greenwald, 1974; Murr et al., 1974)), or differences due to maternal age (Holinka et al., 1979), but these comparisons are complicated by differences in the age of females used, their past breeding status, and whether the presence of a vaginal plug is counted as day 0 or day 1 of pregnancy.

Not every C3HBi individual has five lumbar vertebrae, and of those that do there seems to be a bias towards males (McLaren & Michie, 1954b). Variability between individuals might be explained by factors such as distance from the ovary and growth rate, but it is difficult to see how a general maternal effect would preferentially impact males if sacralisation is due only to changes to Hox gene expression in the presomitic mesoderm when the relevant somites are forming. Sacralisation in males must therefore be

exacerbated by intra-embryo processes, and the most obvious cause would be the development of the fetal gonads and onset of sex steroid production.

The male reproductive tract (vas deferentia, epididymides, seminal vesicles) develops from the Wolffian ducts, and the female reproductive tract (oviducts, uterus, upper portion of the vagina) from the Müllerian duct. All embryos initially form both Müllerian and Wolffian ducts, but in males Anti-Müllerian hormone (AMH) and testosterone promote regression of the Müllerian duct and differentiation of the Wolffian duct into vas deferentia, epididymides, and seminal vesicles. In the absence of these signals in females, the Wolffian ducts almost entirely degenerate and the Müllerian ducts differentiate to form the oviducts, uterus, upper portion of the vagina (Nef & Parada, 2000; Orvis & Behringer, 2007; Zhao et al., 2017). Testes and ovaries develop from a bipotential progenitor (the gonadal ridge), which develops after around 9.5-10 days of development (Tanaka & Nishinakamura, 2014; Yang et al., 2019). The female reproductive tract develops normally in the absence of estrogen signalling (Krege et al., 1998; Lubahn et al., 1993; Schomberg et al., 1999), but testosterone is essential for development of the male reproductive tract, and Müllerian duct regression in the mouse is apparent at around 13.5 days post-conception (Orvis & Behringer, 2007), with testosterone production detectable one to two days earlier (Livera et al., 2006; Migrenne et al., 2012), and the actual onset of fetal testosterone production likely precedes that. The formation of the modified rib-like lateral projections on sacral vertebrae requires suppression of PG10 function by PG11 (Wellik & Capecchi, 2003), and testosterone is known to downregulate *Hoxa10* (Cermik et al., 2003; Du & Taylor, 2016), and so increased prevalence of the five lumbar phenotype in male C3HBi mice is due to the combined effects of elevated circulating progesterone and the onset of testosterone production by the developing testis.

The Hox-hormone hypothesis

The hypothesis for the molecular mechanism underlying sacralisation of the last lumbar vertebra in the C3HBi strain can therefore be summarised as follows. The formation of ribless lumbar vertebrae requires the activity of PG10 Hox genes and the formation of sacral vertebrae requires the activity of both PG10 and PG11 genes, with PG11 genes acting to partially repress PG10 genes and allow the formation of lateral projections that fuse to make the sacrum. An anterior shift of PG11 expression (even a single paralog)

overwrites (through posterior prevalence) the Hox code of the last lumbar vertebra to a sacral type and activates sacral developmental modules, and such an anterior shift can be brought about only by earlier onset of PG11 expression. Posterior Hox genes are regulated by sex steroids under various other contexts, including progesterone, and the budding off of somite 31 from the presomitic mesoderm around 10-10.5 days of development and the initiation of PG11 expression a day or so earlier correlates with a decline in circulating levels of progesterone (as a result of reduced ovarian output) and the onset of a period of placental progesterone production. In the C3HBi strain, this ovarian decline either does not happen or is not so severe, and so progesterone levels are elevated compared to "normal". This elevated expression results in aberrant presence of and PG11 gene products (most likely *Hoxa11*) in somite 31 at a level sufficient to overwrite the lumbar Hox code. In males, testosterone production by the fetal gonad is detectable from around day 11.5 and likely precedes this at levels below current detection limitation. A higher prevalence of 5 lumbar vertebrae in males can therefore be explained by increased PG10 repression through combined activity of PG11 genes and testosterone in the newly formed somite as cells proliferate and arrange themselves into sclerotome and dermomyotome compartments. This hormone hypothesis can therefore not only explain the observed vertebral variation and increased prevalence in males, but also accounts for the observed correlation within litters, and fluctuation of the effect during a mother's lifespan (McLaren & Michie, 1954b, 1958b) .

A predisposition to develop five lumbar vertebrae is known from several strains of mice, including various C3H lines, the 129 strain, DBA2, and even C57BL6 at low frequency (Green & Russell, 1951; McLaren & Michie, 1954b, 1955; Russell, 1948; Russell & Green, 1943; Sengul & Watson, 2012), all of which can trace their origins to the formative early years of mouse strain development by Lathrop, Castle, Little, Strong, and others (Beck et al., 2000). A hormonal basis for this phenotype would suggest that many or all mouse strains have the ability to form five lumbar vertebrae instead of six under the right conditions. However, many of the reports of these altered vertebral morphologies are from the middle of the last century, and it may be that decades of selective breeding, introgression, founder effects, and genetic drift (Stevens et al., 2007) has resulted in the loss of the trait from some lines. More likely, it just isn't looked for. A brief survey of the DBA2J strain in summer 2020 showed that the five lumbar vertebrae phenotype is still prevalent (10

of 12 individuals examined). Such unappreciated variation has the ability to complicate experiments. For example, the classic experiments of Kessel and Gruss (Kessel & Gruss, 1991) that showed the effect of retinoic acid on vertebral patterning by alteration of Hox gene expression used males obtained from a C57BL6 x DBA cross, and 6 of 48 "wildtype" animals had five lumbar vertebrae rather than the expected six. More recently, attempts to identify the function of *HOTAIR* (Hox Antisense Intergenic RNA, a long non-coding RNA), at the lumbar/sacral boundary has been complicated by inter- and intra-strain variation (Amândio et al., 2016; L. Li et al., 2013, 2016; Selleri et al., 2016).

Beyond mice

The widespread use of inbred mouse strains is likely to be a major factor in the prevalence of a phenotype based on a relatively minuscule shift in gene expression, as inbred strains have long been known to show greater variability than outbred or hybrid strains, presumably due to a loss of robustness related to increased or total homozygosity (Biggers et al., 1958; Galis et al., 2014; McLaren & Michie, 1954a, 1956b). But the lumbar/sacral boundary is also potentially an inherently flexible and evolvable region (Jones et al., 2018). The number of cervical vertebrae is to all intents and purposed fixed at 7 across mammals, and alteration to cervical vertebrae appears to be subject to strong selection *in utero* (ten Broek et al., 2012). The thoracic region is intimately associated with respiration; the thoracic/lumbar boundary is linked to positioning of the diaphragm (another mammalian innovation) (Perry et al., 2010), and the sacral region correlates with positioning of the pelvis and hind limbs. It should not be too surprising that the lumbar region might show increased morphological disparity and evolutionary rates compared to these other regions of the mammalian vertebral column, and in primates alone this region can contain as many as 7 (e.g. macaques) or as few as 3 (e.g. Gorilla) vertebrae (Thompson & Almécija, 2017; Williams et al., 2016; Williams & Russo, 2015). Alteration of Hox gene expression by sex steroids during early development may underlie at least some of the observed intra-species variation in this region in therian mammals, and susceptibility of this region to exposure to sex steroids may also render it susceptible to changes as a result of *in utero* exposure of developing embryos to endocrine-disrupting chemicals (Diamanti-Kandarakis et al., 2009; Kahn et al., 2020) Such chemicals have received great attention for their roles in formation of hypospadias (Sinclair, Cao, Baskin, et al., 2016; Sinclair, Cao, Shen, et al., 2016; van der Horst & de Wall,

2017), impaired male and female fertility, reduced semen quality, polycystic ovarian syndrome, endometriosis, and breast cancer (Kahn et al., 2020). The synthetic non-steroidal estrogen Diethylstilbestrol (DES) was widely used from the late 1940's until the early 1970's in an attempt to reduce pregnancy loss or complication, but was withdrawn from use once it became apparent that girls exposed to DES in utero ("DES daughters") had higher incidence of clear cell adenocarcinoma of the vagina and cervix (Herbst et al., 1971), as well as defects of reproductive tract development such as T-shaped uterus. Similarly, boys exposed to DES in utero ("DES sons") suffer a range of health issues, including defects of genital development (Klip et al., 2002; Palmer et al., 2009; Schrager & Potter, 2004). To date, there has been no investigation of a possible association between environmental endocrine disruptors or pharmaceutical agents like DES and defects in vertebral patterning, but it should not be surprising if such a link exists, and consideration should be given to this hypothesis in the emerging fields of Evolutionary-Developmental-Anthropology (Evo-Devo-Anth): Evolutionary-Developmental-Pathology-and-Anthropology (Evo-Devo-P'Anth) (Diogo et al., 2015).

Mayer-Rokitansky-Kuster-Hauser (MRKH) syndrome affects roughly 1 in 5,000 females, and is characterised by absence or hypoplasia of Mullerian duct derivatives such as the uterus, cervix and upper vagina (Herlin et al., 2020; Patnaik et al., 2015). Occurrence is usually sporadic, and there is no evidence for Mendelian inheritance. No candidate genes have been associated with MRKH syndrome. In addition to reproductive tract defects, patients with MRKH also commonly exhibit renal and vertebral issues (e.g. fused and asymmetric vertebrae), and, less frequently, cardiac, hearing, and digital anomalies (Guerrier et al., 2006). Hox genes are implicated in the development of all affected structures and so the reason that there have been no candidate mutations linked to MRKH syndrome may be that it is the result of altered Hox gene expression by perturbed steroid hormones.

The regulation of Hox gene expression by sex steroids has typically only been considered to be significant for development of the male and female reproductive systems and in cancer progression (B. Li et al., 2019). However, given the diversity of roles for Hox genes in embryonic development in mammals and the modular nature of gene regulatory networks, it is clear that this phenomenon is likely to be of much wider

significance, and that *in utero* exposure to perturbed sex hormones warrants both fuller investigation, and greater appreciation as a force in evolution, development, and disease.

Acknowledgements

I would like to thank Richard Behringer, Susan Evans, Ryan Felice, Olivier Pourquie, Claudio Stern, and Jozsef Zakany for comments on drafts of this manuscript. I'd also like to thank Bridget Moynihan for sharing her transcript of the 1990 letter from Anne McLaren to Robb Krumlauf where she discussed her ideas about a possible role for RA, and of course the Michie family for providing access to this and other resources through the British Library. I would also like to thank Rui Diogo and an anonymous reviewer for their helpful comments on the manuscript.

Funding

None.

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