Mayer-Rokitansky-Kuster-Hauser Syndrome

by

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STATEMENT BY AUTHOR

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Abstract

Mayer-Rokitansky-Kuster-Hauser Syndrome is a congenital disorder of the female reproductive tract due to impaired Müllerian duct development. There are three known categorical presentations: isolated, atypical, and MURCS association. Several developmentally significant factors including inappropriate AMH/AMHR interaction, and mutations in the WNT gene family and HOXA7-13 cluster have been studied. There has also been investigation into an autosomal dominant pattern of inheritance in families with multiple cases of the syndrome. Due to the presence of multiple subsets of patients with similar genetic abnormalities, it seems unlikely that a single etiology will be discovered.

Introduction and Overview

MRKH Defined

Mayer-Rokitansky-Kuster-Hauser syndrome, otherwise known as MRKH, is a congenital disorder caused by the incomplete differentiation of the Müllerian ducts. These are paired structures of the urogenital system that run laterally down the side of the urogenital ridge in the posterior abdomen and terminate at the mullerian eminence in the primitive urogenital sinus. They develop into the fallopian tubes, uterus, and upper vagina in females (Valappil et. al., 2012). MRKH is categorized by the incomplete development of the reproductive system in a female with normal secondary sexual characteristics and a 46, XX karyotype (Morcel et. al., 2007). About 1 in every 4500 females are affected worldwide, but it is thought that the syndrome is under-diagnosed, making it difficult to determine MRKH's actual frequency in the general female population.

MRKH is divided into three subtypes: MRKH Type I, also called typical MRKH or CAUV- **C**ongenital **A**bsence of the **U**terus and **V**agina, is isolated to defects in the vagina, cervix, and uterus; MRKH Type II, commonly referred to as atypical MRKH, which consists of associated defects in the ovaries and/or kidneys; and MURCS- **MU**llerian duct aplasia, **R**enal aplasia, and **C**ervical **S**omite anomalies- association, which consists of associated defects in the renal, skeletal, and cardiovascular systems (Guerrier et. al., 2006). Of these three subtypes, typical MRKH presents in a majority of cases.

Discovery of MRKH

The syndrome was first described in 1829 by German histologist August Mayer following his analysis of four stillborns. During post-mortem examination, each body presented with an underdeveloped uterus as well as a stunted vaginal canal. Each examination also confirmed the presence of limb, cardiac, and/or renal abnormalities, but at the time no connection was made between them (Patnaik et. al., 2014). Later, in 1838, Austrian pathologist Carl Rokitansky found nineteen cases of uterine and vaginal aplasia, three of which also had renal deformities. He became the first scientist to make the connection between partial Müllerian agenesis and developmental defects in genital organs such as the vagina, uterus, fallopian tubes, and cervix (Patnaik et. al., 2014). In 1910, German gynecologist Hermann Kuster described the first case of MRKH in a living patient and reported cases with associated defects of the musculoskeletal system. He also summarized the previous findings and case studies with his own in a review paper that provided the beginnings of MRKH syndrome (Patnaik et. al., 2014).

Then in 1961, German gynecologist G.A. Hauser clarified the differences in karyotype that present in Müllerian agenesis and testicular feminization. This is a genetic disorder in which XY individuals are insensitive to androgens and appear female despite the presence of a normal XY karyotype, which differs from the XX karyotype seen in patients with Müllerian agenesis. In addition, he gave MRKH its name and was the first to compile a complete definition of MRKH syndrome, including the variety of defects seen in phenotypic expression (Patnaik et. al., 2014). These four physicians provided the foundation for the clinical presentation now being used to diagnose MRKH. One example is described as follows:

Example Case Study

A sixteen-year-old female presented with primary amenorrhoea- a diagnosis that is classified by the absence of menses by age fifteen in the presence of normal growth and secondary sexual characteristics. Family history revealed that her father has renal agenesis on the left side. A pelvic exam, ultrasound, and abdominal MRI revealed the absence of both the uterus and upper vagina- also described as uterine or vaginal aplasia. The patient's ovaries were intact, but had not descended into their proper anatomical position and instead were located in the abdominal cavity, superior to the pelvis. This could have resulted from failure of development of the inferior ovarian ligaments- paired structures that form in the intermediate mesoderm near the Müllerian ducts and connect the ovaries to the lateral surface of the uterus (Ogata et. al., 2000). Patient history revealed the presence of a horseshoe kidney with unilateral multi-cystic dysplasia on the right side, which prompted removal of the affected portion at ten weeks. Clinical observation and patient history confirmed a diagnosis of atypical MRKH with associated renal defects.

This is just one of many presentations seen amongst patients with MRKH which often makes the diagnostic process more convoluted. Although there is abnormal differentiation of the Müllerian duct in all MRKH patients, defects in this structure can manifest as a variety of abnormalities in the female reproductive tract, the reason for which is still unknown. In addition, a greater emphasis has historically been placed on the development of more efficient diagnostic techniques and advancements in corrective measures for patients with MRKH as opposed to advances in the understanding of MRKH at a molecular level. Although various subsets of a small number of patients with

similar genetic defects have been identified, the multifactorial nature of MRKH means it is unlikely that only one defect in the genome will be found that is consistent amongst all patients.

The Female Reproductive System

Normal Embryogenesis and Anatomy

During embryonic development, every organ system in the body develops from some combination of the three primary germ layers: the ectoderm, endoderm, and mesoderm. The urogenital system develops primarily from a sub-division of the mesoderm called the intermediate mesoderm. A part of this layer called the urogenital, or gonadal, ridge is longitudinally elevated along the posterior abdominal wall. It gives rise to three sets of tubular structures: the pronephros, mesonephros, and metanephros (See Figure 1) (Moore, 2008). The pronephros begins to form around embryonic week three and gives rise to a network of tubules that mostly regress by week five. The mesonephros is the part of the gonadal ridge that develops into the mesonephric tubules and Wolffian duct (See Figure 1). In addition, the mesonephros performs the functions of the renal system from embryonic week six until the development of the metanephros into the adult kidney. During this time, there is also development of the paramesonephric ducts lateral to the already formed mesonephric ducts (Healey, 2010).

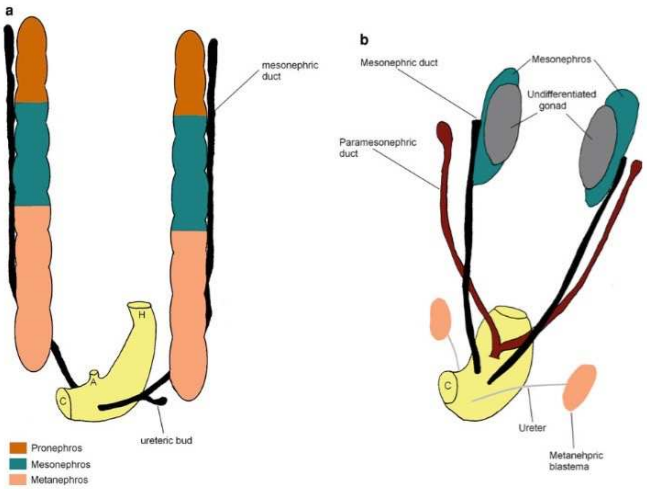

Figure 1: A) The urogenital tract at embryonic week 4 showing the presence of the pronephros, mesonephros and metanephros as well as the mesonephric ducts.
B) The urogenital tract at embryonic weeks 6-8 showing undifferentiated gonads, mesonephric and paramesonephric ducts. From Healey et. al., 2010.

Gonadal development begins at around week five through a thickening of the mesothelium along the medial side of the mesonephros. The proliferation of epithelial tissue creates a bulge which becomes the undifferentiated gonad. At this time, there is also formation of the primary sex cords which are then exerted into the supporting mesenchyme, thereby giving the undifferentiated gonad both a cortex and a medulla (Healey, 2010). After this occurs, primitive germ cells migrate from the yolk sac and into the primary sex cord. Gonadal differentiation does not occur until week seven, at which time the presence of either two X chromosomes or one X and one Y chromosome dictate the development of ovaries or testes respectively. In the male, the cortex of the undifferentiated gonad regresses while the medulla develops into the testis due to the presence of the SRY gene which is found only on the Y chromosome and expresses testis determining factor (TDF). In the female, the absence of a Y chromosome and its associated genes allows for regression of the medulla of the undifferentiated gonad and development of the cortex into the ovary (Healey, 2010).

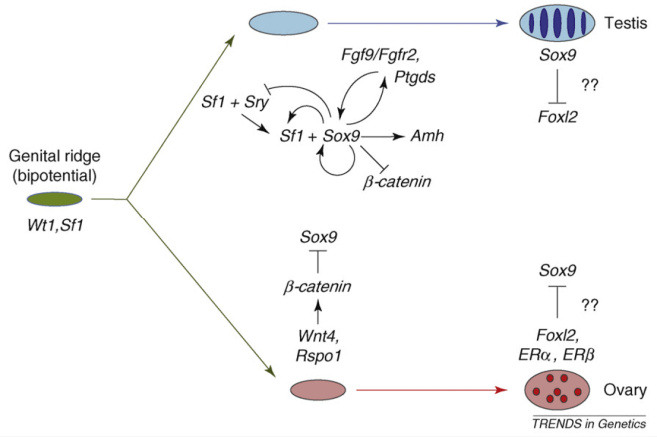

Figure 2: The pathway of gonadal differentiation based on the presence of XX or XY chromosomes as well as other factors. From Sekido, et. al. 2009.

Until week nine of development, there are still two pairs of genital ducts- the mesonephric and paramesonephric ducts (Moore, 2008). In addition to other genes and

factors, development of only one set of ducts is determined by the presence of either ovaries or testes. In males, the presence of the SOX9 gene up regulates the production of Anti-Müllerian Hormone (AMH) which, along with testosterone, promotes the development of the mesonephric ducts as well as the regression of the paramesonephric ducts (Healey, 2010). In females, the WNT4 gene is responsible for the promotion of the DAX1 gene which acts as an antagonist to SRY by inhibiting SOX9 (See Figure 2).

During normal development in males, Anti-Müllerian Hormone, or AMH, mediates the degeneration of the paramesonephric, or Müllerian, duct through its interaction with its receptor. AMH receptors are found on the surface of cells that make up the Müllerian duct. When AMH binds to its receptor, it triggers the apoptosis of those cells thus allowing for the degeneration of the Müllerian duct, something that normally only occurs in males. In females, a lack of androgen and AMH production causes regression of the Wolffian duct and maintenance of the Müllerian duct (See Figure 3) (Moore, 2008). The paramesonephric ducts develop laterally to the gonads and mesonephric ducts. They run parallel to both of these structures until they reach the future pelvic region in the fetus where the ducts converge and fuse together to form the uterovaginal primordium (Robbins et. al., 2015).

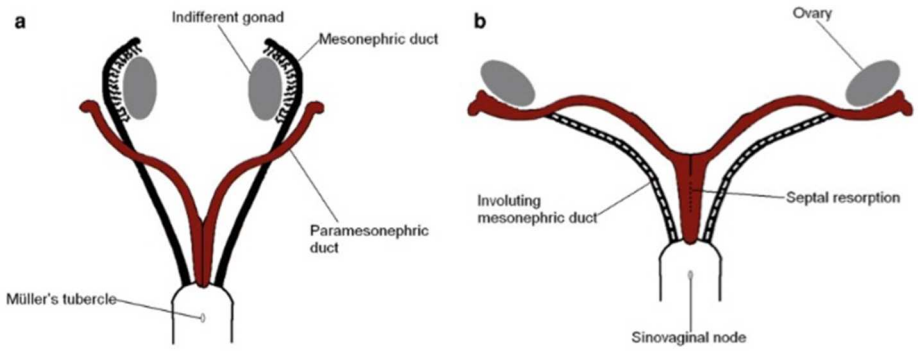

Figure 3: In females, lack of AMH promotes the degeneration of the mesonephric ducts and the maintenance of the paramesonephric ducts. From Healey et. al., 2010.

Once this occurs, the now fused duct develops in three distinct stages which are described as follows: 1) The anterior aspect of the duct develops into the right and left oviducts while the caudal regions of the duct develop into right and left sections of the uterus, cervix, and upper vagina. 2) There is midline fusion of the left and right segment of each structure in the caudal region. 3) There is degeneration of the fused segments, resulting in a distinct uterus with upper, middle, and lower regions, as well as a single cervix and vaginal canal (Robbins et. al., 2015).

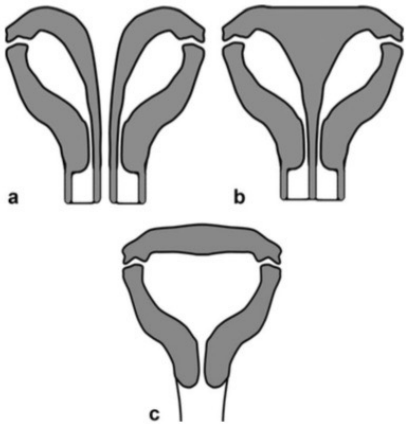

Figure 4: The three stages of reproductive tract development following fusion of the paramesonephric ducts. From Robbins et. al., 2015.

Anatomical Differences in MRKH Patients

In patients with MRKH, regardless of subtype, Müllerian duct development is altered on its posterior end. This results in normal development of the oviducts and alterations in the development of caudal structures including the uterus, cervix, and upper vagina. Although exact mechanisms for alterations are not fully known, abnormal Müllerian duct development can result in a variety of defects in the female reproductive system (See Figure 5).

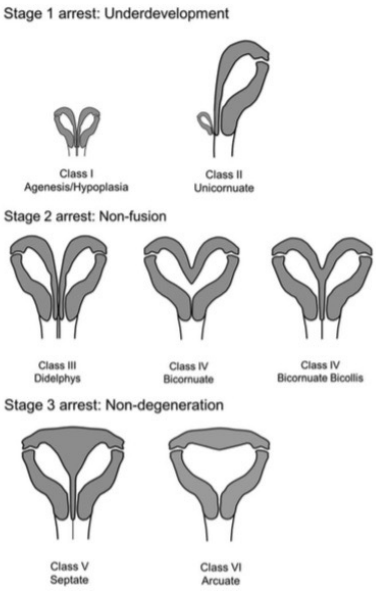

Figure 5: The various anatomical abnormalities associated with defects in each stage of Müllerian duct development following fusion. From Robbins et. al., 2015.

Anatomical presentations seen in MRKH patients come as a result of a defect during the first stage of Müllerian duct development (See Figure 5). Phenotypically, this can present as a rudimentary uterus resulting in a hypo-plastic horn or bud, complete aplasia of the uterus, or a unicornuate uterus on either the left or right with or without a rudimentary horn on the opposite side (Berger et. al., 2013). There is also typically agenesis of the cervix and upper vagina resulting in a shortened vaginal canal formed only by the outer portion, often referred to as a vaginal dimple. Although there is much known about patterning of the Müllerian duct from anterior to posterior, it is not yet known what would cause abnormal let to right patterning like that seen in MRKH patients with a unicornuate uterus.

Potential Molecular Mechanisms of MRKH Development

Further investigation into the etiology of MRKH did not begin until years after the initial findings were made and categorized, when most studies were conducted in the form of clinical observation and the primary focus was on the connection between congenital Müllerian duct malformations and the physical abnormalities they produce. Early investigation into potential genetic abnormalities as causative agents for Müllerian duct aplasia included a cohort of candidate genes including WNT4, WT1, PAX2, PBX1, and HOXA genes 7-13, each of which plays a role during embryonic development of the urogenital system (Burel et. al., 2006). It was thought that, due to the phenotypes seen in mutant mice, a sequence mutation in one or a number of genes in this group could be identified in patients with MRKH.

MRKH and Anti-Müllerian Hormone

One of the first hypotheses for a molecular mechanism of MRKH proposed a role for hormone receptors in the production of AMH in females (Arango et. al., 1999). It was thought that an activating mutation affecting the Anti-Müllerian Hormone receptor, or AMHR, either in the AMHR gene or in the receptor itself was present. It was hypothesized that in either case, a defect in the receptor could bind AMH with a higher affinity. If this occurred in tandem with inappropriate production of AMH, it would result in at least partial regression of the Müllerian duct in females during fetal development (Arango et. al., 1999). With regards to this hypothesis, there seem to be problems with the argument. In females, it would not matter if there was a defect in the AMH receptor, unless it was accompanied by a separate mutation which up-regulated the production of

AMH. It seems more likely that an activating mutation in AMHR could cause cell apoptosis in the Müllerian duct without the presence of AMH.

Another hypothesis involved the effect of estrogen on the production of AMH. In patients with MRKH, testing on rudimentary uterine tissue still present from early development confirmed hypo-methylation on the gene encoding the estrogen receptor ESR1 (Oppelt et. al., 2004). This may cause over expression of the ESR1 gene resulting in an abnormally high level of estrogen-mediated signaling during fetal development. A third hypothesis was that a similar result could have been caused by in utero exposure to an abnormally high level of E2 estrogen (Oppelt et. al., 2004). Since it is known that estrogen regulates the expression of the AMH gene in males, it was hypothesized that over-exposure to estrogen in females could cause an increase in AMH promoter activity (Oppelt et. al., 2004). It has been shown that low level expression of E2 (17beta-estradiol) can up regulate the expression of estrogen receptor alpha, thus indirectly causing an increase in the promoter activity of AMH (Oppelt et. al., 2004). If this increase in promoter activity included the inappropriate activation of AMH, it is possible that abnormal expression of E2 could cause adverse effects during embryogenesis by way of partial Müllerian duct regression.

A third hypothesis for increased AMH expression in the female could involve aberrant expression of transcription factors WT1 and GATA4, both of which play a role in the regulation of the AMH gene in males. WT1 and GATA4 genes were hypo-methylated in MRKH patients, potentially causing higher levels of the transcription factors as well as activation of the AMH gene (Miyamoto et. al., 2008). If this hypothesis proved to be accurate and it was determined that abnormal methylation in either WT1

and/or GATA4 caused the inappropriate activation of AMH, it would be advantageous to study the methylation patterns in both of these genes in MRKH patients.

MRKH and WNT Genes

The WNT4 gene is one of sixteen known genes in the WNT family that is responsible for patterning during embryogenesis. An initial study found that WNT genes 4, 5A, and 7A were expressed at high levels in the epithelium of the uterus with WNT4 also being expressed in the vagina of mice (Miller et. al., 1998). WNT4 knockout mice showed a failure in the postnatal development of the Müllerian duct as well as its subsequent differentiation into reproductive tract structures. Failure of duct development can be caused by either of the following: a failure in the initial development of precursor ducts, or an over expression of duct repressors causing their regression following their initial formation. Although the mechanism of failed Müllerian duct development is not explicitly stated, it is likely that it is a result of regression of the duct following its initial formation in utero.

These mice also had elevated androgen levels and ectopic expression of Anti-Müllerian Hormone (AMH). In male reproductive development, AMH is produced by Sertoli cells while testosterone is produced by Leydig cells, both of which are cells found in the testes. These cells work in tandem to inhibit the development of the paramesonephric ducts and stimulate the development then differentiation of the mesonephric ducts into the structures of the male reproductive tract (Al-Attar et. al., 1997). As this relates to females with a mutation in the WNT4 gene, this mutation could result in the ectopic expression of AMH. If the ectopic production of AMH directly affects gonadal androgen levels in females, this in turn could lead to masculinization of

the ovaries due to their inability to repress testosterone synthesis. This mechanism was probably acting in humans wherein a loss of function mutation in WNT4 was detected in patients with an MRKH-like phenotype and elevated levels of testosterone and other androgens (Miller et. al., 1998).

Ultimately these studies lead to the conclusion that WNT4 is involved in the initial formation and regulation of Müllerian duct development as well as the regulation of androgen synthesis in the ovaries by way of the repression of testosterone synthesis (Biason-Lauber et. al., 2004). Despite this, WNT4 is no longer considered to be a gene of interest in MRKH. This was determined when sequence analysis of the gene in nineteen MRKH patients without elevated testosterone revealed no sequence mutations in the signal peptide region of the WNT4 protein like that seen in patients with elevated androgen levels, indicating the presence of two separate syndromes with different genotypes and phenotypes (Philibert et. al., 2008). Instead the subset of patients with both an MRKH-like phenotype and elevated androgen levels should be categorized under a different syndrome called WNT4 deficiency.

There is evidence, however, that suggests a connection between WNT and HOX genes during initial development of the Müllerian duct, specifically WNT genes 5A and 7A and HOX genes A11 and A13. Both of these WNT genes are responsible for development and subsequent differentiation of structures along the caudal portion of the anteroposterior axis of the Müllerian duct, specifically the cervical and vaginal regions (Miller et. al., 1998). Even though there is a strong correlation between the proper function of WNT genes and Müllerian duct development, they are not considered to be candidate genes in MRKH. A sequence mutation in either the WNT5A gene or WNT7A

gene would result in other, more severe phenotypes in the limbs in addition to the more mild Müllerian duct malformations like those seen in MRKH (Woods et. al., 2006; Yamaguchi et. al., 1999). This was confirmed when genome analysis in MRKH patients did not reveal sequence mutations in the coding regions of either gene. However, expression of either WNT5A or WNT7A could be altered by way of a mutation that effects enhancer control of gene expression. Enhancer sequences are regions in the DNA with the ability to increase gene expression by serving as binding sites for transcription factors (Uchikawa et. al., 2003). It is reasonable to predict that a mutation, if present in either an enhancer sequence or binding site could adversely effect the rate of WNT5A or WNT7A gene expression. Despite this, neither gene has been analyzed for this in MRKH patients most likely because limb deformities, such as aplasia or hypoplasia, that are found in patients with WNT5A and WNT7A mutations are not consistent with the various associated phenotypes in MRKH.

Although WNT genes are no longer being explored as candidate genes in MRKH, similar phenotypes in the Müllerian duct have been reported in both WNT5A and HOXA13 deficient mice, suggesting that they may act along the same pathway during development (Mericskay et. al., 2004). In addition, WNT7A knockout mice and those with a mutation in HOXA11 show an inverse relationship between the function of these genes during embryonic development. When WNT7A is non-functional, there is normal expression of HOXA11, but its expression is not maintained and when HOXA11 is non-functional, there is normal expression of WNT7A, but its expression is not maintained. This suggests that while each of these genes is activated independently, both are needed in order to maintain the expression of the other (Miller et. al., 1998). The relationship

between WNT and HOX genes provides insight into the importance and function of the HOXA gene cluster that is responsible for Müllerian duct development. It is for this reason, that HOX genes have become a focus of study in MRKH patients.

MRKH and HOX Genes

HOX genes are a group of genes that play a crucial role in the early development of the anterior-posterior, or head-tail, axis of an embryo (Lappin et. al., 2005). They were first discovered in fruit flies and studies have since found a high level of genetic conservation of HOX genes in all complex animals including mice, chickens, and humans. In humans, there are 39 HOX genes that are divided into four clusters, each of which is found on a separate chromosome. The HOXA cluster is found on chromosome 7, HOXB on chromosome 17, HOXC on chromosome 12, and HOXD on chromosome 2 (Mallo et. al., 2010). Following the formation of embryonic segments, HOX proteins, which function as transcription factors, are responsible for the determination of segments into different types of structures, but not actual segment formation. HOX genes are spatially and temporally additive during expression. This means that their expression occurs one after the other in an anterior to posterior direction and once a HOX gene is initially expressed, its expression will not stop during continued development. In addition, there is an overlap in HOX cluster expression, meaning that HOX genes of the same number, but from different clusters can play a part in the development of a given body segment (Deschamps et. al., 2005).

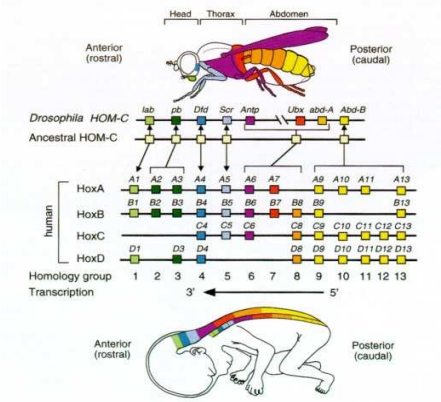

Figure 6: The homology of HOX genes between Drosophilia and humans. From Mark et. al., 1997.

It was found that HOX genes A9, A10, A11, and A13 are all expressed along the head-tail axis of the paramesonephric duct in mice- HOXA9 in the fallopian tubes, HOXA10 in the uterus, HOXA11 in the uterus and uterine cervix, and HOXA13 in the upper vagina (Taylor et. al., 1997). However, this same study also found that HOX gene expression was only spatially, not temporally, regulated. In mice, expression of these HOXA cluster members begins at embryonic day 15.5 and expression of HOX A9, A10, A11, and A13 commenced simultaneously (Taylor et. al., 1997). Because this study was conducted in mammals, there is a high probability that the location and function of this section of these HOXA genes will be conserved in humans and indeed, the same study confirmed a similar spatial pattern of HOXA expression in human reproductive tissues obtained by hysterectomy (See Figure 7).

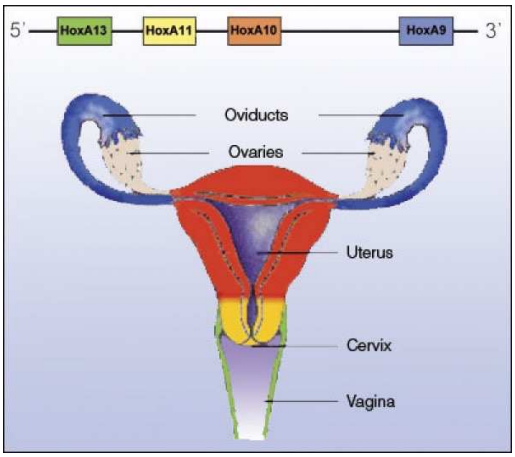

Figure 7: The spatial expression of HOXA genes 9-13 in the Müllerian duct from news.sciencemag.org. Based on Taylor et. al., 1997.

The prolonged expression of the HOXA cluster suggests that its function may be required to regulate structural and functional changes to the female reproductive system that occur at different stages of life (Taylor et. al., 1997). In order to accommodate the cyclical changes seen over the course of a woman's reproductive life, cells in the reproductive tract must retain their plasticity (Ono et. al., 2008). It was postulated that prolonged HOXA gene expression is one way this is done, providing a unique pattern of HOX expression which has thus far only been found in the female reproductive tract.

Two separate studies focused specifically on mutations in the HOXA cluster of genes as possible candidates for malformations found in MRKH. However, genetic analysis of each of these candidate genes did not find any definitive evidence supporting their involvement in the etiology of MRKH, because no sequence mutations or polymorphisms were present in coding regions (Lalwani et. al., 2007; Liatsikos et. al., 2010). This study seemed incomplete primarily because only coding regions of each gene were sequenced without looking for mutations in non-coding regions or alterations in protein expression. A sequence mutation, if severe enough and in an important region of the DNA, would likely cause total failure of Müllerian duct development, resulting in a complete aplasia of reproductive organs like that seen in mutant mice. However, in patients with MRKH, development is altered, not absent, indicating a more mild defect has occurred. This is evident in the presence of normal oviducts as well as remnant uterine structures that are seen in a majority of patients.

If sequence mutations were responsible for the presentation of MRKH, there would have to be significant mutation present in each of the HOXA genes responsible for Müllerian duct development. This does not seem likely but it is possible that a sequence

mutation outside of the coding region, if present or a post-translational modification such as methylation would adversely affect HOX protein expression and also present in the form of the more mild phenotypes that are seen in the reproductive tract of MRKH patients. It has also been shown that in normal HOX expression regulation, there are two regulatory modules between the 3' and 5' ends of the HOX gene cluster which are responsible for the anterior and posterior domains of the cluster respectively. It may be possible then that there is a defect in the second regulatory module which is adversely affecting HOX expression in the Müllerian duct. It is for this reason that the HOXA cluster continued to be the primary focus of subsequent studies. Later it was found that HOXA genes are very likely responsible for the more mild anatomical features of the reproductive system that are found in a subset of patients with MRKH. It was determined that uncontrolled regulation, whether in the form of up-regulation or down-regulation, of HOX protein expression- specifically the HOXA cluster- could be responsible for the incomplete differentiation of the Müllerian duct in a subset of patients with MRKH (Rall et. al., 2011).

2000 Diethylstilbestrol Study

Much of the continued interest in HOXA cluster as candidate genes was based on the result of a study conducted in 2000 that, until the early 1970s, tested in utero exposure to diethylstilbestrol, or DES- a synthetic, non-steroidal estrogen that was used to treat many pregnant women who were considered to be at risk for adverse pregnancy outcomes. DES is part of a group of estrogen-like endocrine disruptors called xenoestrogens and was a common treatment until 1971, when it was banned after studies showed an increase in adenocarcinoma of the vagina in young females who were exposed

to the drug in utero as well as a high incidence of genital abnormalities similar to those now considered part of the MRKH phenotype (Block et. al., 2000). This study was based on the thought that DES was a potential cause of hypo-methylation and over expression of HOXA9, even though at the time HOXA9 was not considered a candidate gene for MRKH. In comparison with the control group, DES exposed mice were found to have increased posterior HOXA9 expression in their uterine tissue which in turn caused similar alterations in the expression of both HOXA10 and A11. As a result, these mice showed a caudal, or posterior, shift in HOXA expression leading to developmental disruption throughout the reproductive tract (Block et. al., 2000). Although the administration of DES during pregnancy may have caused MRKH-like symptoms in these studies, this mechanism is unlikely to be the direct cause in any patient with MRKH in recent years. However, this study still provides insight into the relationship between hormone production and HOX gene expression. It is possible that MRKH may ultimately be caused by endocrine disruption during the point in embryogenesis at which HOX genes determine specific structures (Taylor et. al., 2009). This argument was supported by observations that another xenoestrogen methoxychlor (MXC) also affected the female reproductive tract in a manner similar to DES (Taylor et. al., 2009). Specifically in mice, MXC acts to permanently repress the expression of HOXA10 in a process mediated through the estrogen response element of HOXA10 on a dose dependent basis. As shown with both substances, excess estrogen production alters Müllerian duct development even if its exact mechanisms are still to be determined.

2007 Bisphenol A Study

A later study examined the effects of bisphenol A (BPA)- another xenoestrogen used in the production of polycarbonate plastic- on epigenetic variations, or post-translation modifications which affect gene expression, in utero, particularly DNA methylation. DNA methylation is the process by which methyl groups modify the function of DNA by being added to CpG sites- specific regions of the DNA categorized by a cytosine nucleotide next to a guanine nucleotide in the linear sequence. Two possible mechanisms currently exist as explanations for the inverse relationship between methylation and gene expression. The first is that methylation inhibits the binding of transcription factors to their specific recognition sites, each containing its own CpG site. The second mechanism is the binding of proteins or protein complexes to CpG sites, effectively preventing the binding of the necessary transcription factors. By way of either mechanism, a hypo-methylated CpG site would result in increased transcription and over expression of the gene while a hyper-methylated CpG site would result in decreased transcription and under expression of the gene (Costello et. al., 2001). It was found that exposure to BPA resulted in hypo-methylation of nine different CpG sites found in the promoter region of the Agouti gene in mice. This mis-regulation ultimately promoted the over expression of various genes resulting in obesity, diabetes, tumorigenesis, and lighter fur color (Dolinoy et. al., 2007). In addition, that same study tested the effects of maternal nutrition supplementation in combination with continued exposure to BPA. Testing on mice confirmed that the administration of prenatal supplements successfully containing methyl donors counteracted hypo-methylation of each CpG site that occurred as a result of exposure to BPA. This is another indication of a possible environmental connection

and inductively supports the relationship between endocrine disruption and gene expression found in the DES study (Taylor et. al., 2000). In addition, understanding the effect of BPA on DNA methylation provided further insight into the etiology of MRKH. Although the bulk impact of exposure to these kinds of compounds in the developing reproductive tract has yet to be determined, there have been instances where the effects of a particular xenoestrogen have been demonstrated to alter the expression of HOX genes as shown above (Taylor et. al., 2009). It would be interesting to see if the abnormal methylation patterns seen with exposure to BPA are applicable to HOX genes and if so, whether or not there are similar defects in Müllerian duct development. Considering what is already known about the structure and function of BPA, research that studies its interaction with CpG sites of HOXA genes 9-11,13 would determine if similar methylation patterns are seen and thus provide insight into a specific mechanism responsible for the genital defects consistent with MRKH.

2011 Tubingen Study

Each of the studies described above served as a basis for a study conducted in Tubingen, Germany which used a whole-genome approach to find differences in transcription and methylation between the remnant uterine tissue of MRKH patients and that of non-MRKH controls (Rall et. al., 2011). Relevant genes which showed altered expression and abnormal methylation were identified in order to find potential mechanisms and their underlying role in MRKH. Of the potential candidate genes that had abnormal methylation and expression, two are of particular interest: HOXA5 and HOXA9. Both were found to have hypo-methylated CpG sites resulting in over expression of each gene (Rall et. al., 2011). With regard to HOXA5, it was hypothesized

that its hypo-methylation resulted in ectopic expression at the 5’ end of the HOXA cluster, meaning that the gene was being expressed in the wrong location. Since HOX gene expression occurs from the 3' to 5' direction, the expression of HOXA5 would prevent normal development of the Müllerian duct because its expression would impede that of HOX genes responsible for its development (See Figure 7). In addition, HOXA5 plays a significant role during the development of the cervical and upper thoracic regions of the skeleton which could explain the associated malformations of the spine and middle ear consistent with MRKH Type II and MURCS association subtypes. However, the connection between abnormal methylation and phenotype as well as that between abnormalities in HOXA5 and the HOXA genes of the Müllerian duct remains unknown.

In addition, it was hypothesized that hypo-methylation on HOXA9 was due to either the exposure to a substance similar to DES, possibly another xenoestrogen, or a deficiency in HOXA10 resulting in anterior transformation (Rall et. al., 2011). Anterior homeotic transformation has previously been described as a process wherein an abnormal HOX gene causes its corresponding body segment to develop characteristics of the segments anterior to it, resulting in the improper expression of those anterior genes (Carroll et. al., 1995). In fruit flies, this results in very serious defects, however in humans and other vertebrates, the redundancy found in HOX clusters very likely lessens the phenotypic severity (Taylor et. al., 1997). Studying the effects of anterior homeotic transformation, or a process similar to it, in the HOXA cluster in the Müllerian duct could provide insight into whether or not there a causal relationship between this mechanism and phenotypes consistent with MRKH patients. As this relates to HOXA9, it is possible that hypo-methylation on the second CpG site of the HOXA9 gene is the result of

abnormal gene expression of the posterior regions of the HOXA cluster, specifically HOXA10 just like that seen in the 2000 DES study (Taylor et. al., 2000). In order for this to be a plausible explanation, however, there would also need to be insufficient HOXA10 expression in MRKH patients, something that was not found.

Another element that should be considered is the unique nature of simultaneous and prolonged HOXA expression in the Müllerian duct. As opposed to other HOX clusters where expression occurs one after the other, the genes that make up the posterior HOXA cluster that is responsible for differentiation into the Müllerian duct are all expressed at once. It has also been shown that HOXA expression is maintained past development in areas of the female reproductive tract, particularly uterine tissue, in order to maintain cell plasticity through different stages of life. Because HOXA genes in the Müllerian duct are only regulated based on the location of their expression, a mis-regulation of one HOXA gene might affect the expression of the others. The Tubingen study found that all five CpG islands in the HOXA9 gene are over expressed in the uterine tissue MRKH patients. This location shows that the over expression of HOXA9 was partially ectopic, meaning the primary HOXA gene being expressed in the uterine, cervical, and vaginal regions of the Müllerian duct would be HOXA9 as opposed to HOXA 10, 11, and 13 respectively. If hypo-methylation of HOXA9 caused competition in gene expression with the posterior HOXA genes, at least during initial development, Müllerian aplasia found in MKRH patients could have resulted. This warrants further study in mouse models. Testing should be done to determine whether or not the over expression of HOXA9 could be ectopic and if so, if this would result in competition with HOXA 10, 11, and 13 thereby repressing their expression in the developing reproductive

tract. This connection might be able to provide a specific developmental gene which could serve as a target in the diagnosis of MRKH in utero. It could be tested by an assay for HOXA expression in the uterus and/or upper vagina to determine if HOXA9 is being expressed in those areas and if its expression is greater than HOX genes A10-13. If this hypothesis proved true, it could provide a causal relationship between HOXA expression and Müllerian duct development as well as provide a mechanism responsible for a subset of patients with MRKH and hypo-methylation on HOXA9.

MRKH and MUC1 Gene

A recent study used a whole genome approach to analyze differences in gene expression between sixteen MRKH patients, ten with MRKH type I and six with MRKH type II, and five healthy controls to investigate the relationship between gene regulation and expression and phenotypic presentation. Whole genome microarray analysis was done using vaginal tissue in eight of the sixteen MRKH patients as well as all five healthy controls. Of the 275 delineated genes found during gene profiling in at least one patient with MRKH type I or type II, six genes were selected for further study using microarray analysis in eight MRKH patients based on their fold change, frequency of abnormal expression in a significant number of MRKH patients, or their known relevance to embryologic development (Nodale et. al., 2014). Real time PCR was then done in all sixteen MRKH patients to confirm the result found by microarray. Of the six genes that were studied, the Mucin 1, or MUC1, gene showed great promise due to its significant upregulation in all eight MRKH patients using microarray analysis as well as a similar amount of upregulation in fifteen of sixteen MRKH patients using PCR.

Several studies have shown that MUC1 affects WNT signaling and AMH and AMHR activation through its interaction with beta-catenin, a protein that functions as a regulator of degradation (Allard et. al., 2000). Because of this, it was hypothesized that over expression of MUC1 indirectly causes improper activation of AMH with its receptor (Nodale et. al., 2014). It has also been shown that MUC1 acts only during activation, not maintenance of AMH. Therefore in this case it is plausible that activation of AMH and maintenance of its activity could result in at least partial regression of the Müllerian duct by way of induced apoptosis of the cells that make up the duct, thereby potentially leading to phenotypes consistent with those seen in MRKH. Because of the results of this study, further analysis of the regulation patterns of the MUC1 gene should be done in a larger cohort of MRKH patients. It would also be advantageous to determine if any overlap or cooperation exists between MUC1 and HOXA genes 9-11,13 during embryonic development. This could be done using mice with a knockout MUC1 gene and determining the affects, if any, on the regulation and/or expression of the HOXA cluster of the Müllerian duct.

Isolated Cases of MRKH

Micro-Deletion at Chromosome 17q12

It is probable that a genetic mutation exists at least in a subset of patients with a phenotypical presentation of MRKH. Twenty two females previously diagnosed with MRKH, none of whom were related, underwent genomic analysis to determine the presence of a common genetic mutation. In two of these patients, array- comparative genomic hybridization found a micro-deletion 1.5Mb in length at chromosome 17q12. This mutation included the deletion of sixteen genes including TCF2 and LHX1, both of which code for transcription factors and are significant in the development of the renal and urogenital systems (Bernardini et. al., 2009).

Clinically, patient one presented with a congenital absence of the uterus and vagina, polycystic left ovary, and normal kidneys. In addition, a normal karyotype, audiogram, ECG, and cardiac sonogram were found with this patient. This presentation of MRKH is consistent with that found in type I, isolated MRKH. Patient two presented with congenital bilaterally, small-sized multi-cystic kidneys with poor differentiation of the cortex and medulla as well as agenesis of the upper and middle thirds of the vagina and a right unicornuate uterus. This presentation is consistent with that of type II, atypical MRKH with renal malformations (Bernardini et. al., 2009).

The twenty patients without the micro-deletion at 17q12 were tested for mutations in their TCF2 and LHX1 genes as possible explanations for their various physical manifestations of MRKH defects as well as any associated malformations, but no significant mutations were found (Bernardini et. al., 2009). TCF2 mutations are more commonly found in patients with maturity-onset diabetes; however, because of its

expression in renal metanephroi during the pre-glomerular stages in the development of the kidneys, mutations in the TCF2 gene are also seen in many individuals with renal defects. LHX1 is a gene that encodes a member of a LIM domain family protein. The LIM domain is a cystine-rich zinc-binding domain found in transcription factors, some of which are active during the development of renal and urogenital systems. The deletion of LHX1 was found to potentially act as a causative agent of Müllerian duct abnormalities after studies in mice found that the gene was involved in the developmental processes of the urinary and genital tracts. As this pertains to the two patients with the micro-deletion at 17q12, even though they presented with different MRKH subtypes, defects were found in the gonads and kidneys. In mice with a mutant LHX1 gene, both the kidneys and gonads are absent, a topic that will be discussed in a later section. (Tsang et. al., 2000)

Mutations in LHX1 Gene

A study performed in 2011 tested for mutations in the LHX1 gene in a separate subset of patients with MRKH. Of the sixty-two patients with a clinical diagnosis of MRKH consisting of congenital aplasia of the uterus and upper vagina, twenty three of them had type I MRKH while the remainder had type II MRKH. The only significant mutation found during sequence analysis of the LHX1 gene was a frameshift mutation leading to a stop codon in one patient with unilateral renal agenesis and type II MRKH (Ledig et. al., 2012). A similar phenotype was also found in one MRKH patient with the same micro-deletion at 17q12 as well as 5 additional patients with novel variants in the LHX1 gene (Sandbacka et. al., 2013).

As further support for the significance of the LHX1 gene in MRKH, it was found that LHX1 is expressed in both the Müllerian and Wolffian ducts during development

and that it is a critical component in the formation and maintenance of the female reproductive tract. Mice with a conditional knockout in the LHX1 gene had shortened oviducts and a complete lack of uterus, cervix, and upper vagina. This was because the absence of the LHX1 gene prevented the elongation of the Müllerian duct, meaning the posterior end of the duct was absent (Huang et. al., 2014). This was most likely due to an increase in cell death and decrease in cell proliferation of Müllerian epithelial cells in both the trunk and tip regions of the duct. As previously stated, LHX1 acts as a transcription factor during urogenital development but its regulation of downstream targets is still unknown.

So far, the micro-deletion on 17q12, as well as mutations in the LHX1 gene have only been reported in a small number of MRKH patients, meaning the likelihood of 17q12 or LHX1 gene mutations as the primary cause in a significant number of MRKH cases is very low. There does seem to be a connection between these studies, however, which could be an indication that LHX1 is the gene of interest in patients with a 17q12 micro-deletion, particularly in patients with renal defects. Demonstrating that there is a causal relationship between the mutations discussed above and MRKH could bridge the gap between MRKH patients with either a mutation in LHX1 or a micro-deletion in 17q12 by showing that the gene of interest in this subset of patients is LHX1. If analysis of future patients with MRKH reveals a similar mutation in the LHX1 gene or a deletion on the 17q12 chromosome, this could indicate a connection between individuals in a specific subset with type II MRKH. The next step would be to study the genomes of patients who present with MRKH type II to look for the presence of any mutations in their LHX1 genes to determine if such a connection exists. At that time more research

could be done to determine if there are any similarities, either in environment or genetics, which could increase the likelihood of a mutation in the LHX1 gene.

It is known that LHX1 and HOXA 9-11,13 genes play a role in the development of the Müllerian duct and its subsequent structures. There is not enough evidence to support the theory that LHX1 affects the expression of HOXA genes or vice versa. A reasonable argument can be made, however, in support of a model in which mutations, or other factors, resulting in abnormal expression of LHX1 or HOXA 9-11,13, could produce phenotypes that are consistent with MRKH syndrome. This would mean that there are multiple genes and pathways that can be affected in MRKH patients. Since the development of the Müllerian duct is a complex process involving the cooperation of many different genes and pathways it is also possible that there is cooperation between LHX1 and HOXA 9-11,13 at some point during development.

Study into the involvement of LHX1 during Müllerian duct development, particularly its downstream affects, would be the next step in determining causality between the LHX1 gene and MRKH patients with a defect in it. In addition, analysis of the methylation and expression of HOXA 9-11,13 should be done MRKH patients with an LHX1 defect. An experiment could be done in mice that alters or inhibits LHX1 expression, then studies its effect on the expression of HOXA 9-11,13. Furthermore, if future studies indicate that LHX1 had a downstream affect, whether direct or indirect, on the HOXA cluster of the Müllerian duct during development, this could indicate that the primary target gene in MRKH is the posterior HOXA cluster in a larger subset of patients and thus warrants further study. It would also be advantageous to complete similar

analyses in patients with an LHX1 deletion or mutation to determine if there is any overlap between the LHX1 and MUC1 genes during Müllerian duct development.

Familial Occurrence of MRKH

Early research explored the possibility of a genetic component among families in which more than one member has been diagnosed with MRKH. However, when genomic analysis of candidate genes found no sequence mutations, the focus switched to alterations in epigenetic factors such as methylation and gene expression as a likely cause of MRKH. Although this continues to be the main focus, there is still the possibility that genetics play a role in certain families, suggesting the possibility of an autosomal dominant pattern of inheritance in a specific subset of patients (Morcel et. al., 2007). An autosomal dominant disorder means that only one copy of the affected gene is needed for phenotypic expression of the disorder in subsequent familial generations. To date there have been 35 studies looking at a total of 67 families with a familial occurrence of MRKH classified by either two or more females with MRKH or one female with MRKH and a non-MRKH relative with at least one associated anomaly of the kidneys, heart, or skeleton (Herlin et. al., 2014). Although several studies have suggested either an autosomal dominant or multi-factorial/polygenic etiology, none have found a definite molecular genetic defect consistent throughout the pedigree that would explain MRKH or any of its associated anomalies. This is most likely because thus far studies have taken a candidate gene approach to genomic analysis, using genes that are known to contribute to MRKH in only a very small percentage of patients. More information might be discovered with the introduction of genome-wide analysis and this approach should be used whenever possible in future studies.

With the suggestion of an inherited genetic mutation within families, problems have also arisen due to the nature and physical manifestation of MRKH. Women who have been diagnosed with MRKH are unable to get pregnant, meaning they cannot have their own children without the use of in vitro fertilization in a surrogate, a relatively new practice. This begs the question as to whether or not a similar condition could also present itself in males by way of the regression or abnormal development of the Wolffian duct in a manner similar to that seen in the Müllerian duct of affected females (McGaughran, 1999). The Wolffian duct is the structure that connects the primitive kidney to the cloaca during embryogenesis and serves as the beginning of male reproductive organs including the epididymis, vas deferens, and seminal vesicles. Several case studies have shown males with Wolffian duct agenesis, resulting in unilateral or bilateral defects in the development of the vas deferens, both with and without associated defects of the kidneys, heart, or skeleton (Meschede et. al., 1998). Although severe defects in the Wolffian duct early in development would likely result in bilateral kidney agenesis and death, it is possible that a more mild phenotype could be the result of a failure of the Wolffian duct to properly differentiate into the structures of the male reproductive tract, much like that seen in the Müllerian duct of MRKH patients (Wellesley et. al., 1995). However, further research would need to be done in order to determine if such a possibility could occur.

Due to the variability in symptom presentation resulting from different degrees of expressivity, it is very possible that males would lead a normal life without ever being aware of defects resulting in hypoplasia or aplasia of the vas deferens, especially if fertility was never an issue. Therefore, the frequency of Wolffian duct agenesis may be

relevant to the understanding of familial MRKH (Wellesley et. al., 1995). In addition, it is also possible that females in the family could be carriers of the affected gene, yet display incomplete penetrance and/or variable expressivity of MRKH. It is difficult, however, to find a definitive connection not only because of the variability in expressivity that presents in MRKH and the different physical manifestations that would present in males and females, but also because the etiology of MRKH is still unclear.

It would be interesting to complete genetic testing on the parents of the MRKH patients who presented with either the same micro-deletion at 17q12 or frameshift mutation in the LHX1 gene to see if any genetic similarities were present in these candidate genes. If similar mutations were found in either parent, this could provide insight into the etiology of MRKH in this particular subset of patients. If a mutation was found, phenotypic presentation of MRKH-like defects in the father may provide support for the theory of an autosomal dominant pattern of inheritance of a mutated gene which can affect both male and female reproductive tract differentiation.

Presence and Significance of Associated Abnormalities in MRKH

Analysis of Associated Abnormalities and Their Frequencies

Despite the progress that has been made on the understanding of MRKH in recent years, there is still much to be discovered. It is known, for example, that associated malformations are present in a majority of cases, meaning that MRKH cannot be exclusively referred to as a genital malformation, even though it is generally classified as such. A meta-analysis conducted in 2005 analyzed 53 patients with MRKH for the presence of any associated defects (Oppelt et. al., 2005). Of these 53 patients, 25 were of the typical presentation, 11 presented with atypical MRKH, and 17 presented with MURCS association, meaning that 53 percent of patients in this cohort had at least one defect in a body system other than the reproductive tract (Oppelt et. al., 2005). The most prevalent defect was found in the renal system presenting in 36 percent of tested patients with unilateral renal agenesis occurring in nearly half of those patients with renal defects. Moreover, these results were compared to those of sixteen previous studies. Out of the total 521 patients studied, 127 presented with MRKH type II and another 61 presented with MURCS association, combining for a total of 36 percent of patients. In addition, 166 of the total 521 MRKH patients presented with renal malformations, accounting for 32 percent of all defects (Oppelt et. al., 2005). Similar results were found in a second study wherein 53 of 128 patients with associated defects were found to have defects in the renal system, with unilateral renal agenesis occurring the most frequently (Oppelt et. al., 2012).

Due to the high frequency of patients with MRKH syndrome as well as at least one of the described associated malformations, finding the connection between these defects will be vital to a better understanding of MRKH type II and MURCS association.

These defects could be epigenetic, environmental, or a combination, resulting in reproductive tract defects exclusive to MRKH regardless of the specific subtype. It is interesting that the most prevalent abnormality seen in MRKH patients is found in the renal system. As previously stated, the most common renal defect is unilateral renal agenesis which is defined as the presence of only one kidney at birth due to the failure of the other to develop. Other, less common defects include ectopia, or incorrect positioning, of one or both kidneys; and horseshoe kidney, which occurs when the patient's kidneys fuse together at the midline and remain connected by a band of fibrous tissue.

Overall, abnormalities associated with typical MRKH as well as those seen in both atypical presentations, are most likely the result of a defect that occurs during the development of the intermediate mesoderm, affecting proper development. The higher frequency of renal defects as opposed to those in the heart and skeleton in patients with MRKH could be attributed to the proximity of the urinary and genital systems throughout fetal development. Each of these systems develops from the intermediate mesoderm during embryogenesis whereas the cardiovascular and skeletal systems develop from the lateral plate and paraxial mesoderm respectively.

Connection to LHX1 Gene Mutations

One gene that could potentially be of interest is that which codes for Lim1, otherwise known as LHX1. As previously stated LHX1 is a gene that encodes for a transcription factor which is part of the LIM domain and important during the primitive stages of embryogenesis (Shawlot et. al., 1995). Groups conducting two different studies found that LHX1 was an important component in the patterning of the anterior-posterior

axis of the embryo as well as the development of the intermediate mesoderm (Tsang et. al., 2000). It was found that LHX1 is initially expressed in the precursors of the lateral mesoderm, then subsequently in the nephric ducts of the intermediate mesoderm (Kinder et. al., 2001). In mice with a mutant LHX1 gene, both the gonads and kidneys were missing, demonstrating the genes importance during embryogenesis (Tsang et. al., 2000). Even though its importance is clear, the mechanism of action of LHX1 is still unclear. A better understanding of this mechanism during the early stages in development would allow us to determine if LHX1 is responsible for the initiation of urogenital system development or for subsequent differentiation of the nephrogenic mesoderm and urogenital ridges.

There may also be a connection between the role of LHX1 during early embryogenesis and defects in this gene in a subset of patients with MRKH (Bernardini et. al., 2009; Ledig et. al., 2012; Sandbacka et. al., 2013). In these cases, one presented with a polycystic left ovary, another had two small sized multi-cystic kidneys with poor differentiation of the cortex and medulla, and the final patient presented with unilateral renal agenesis. This is consistent with earlier studies wherein a mutant LHX1 gene in mice resulted in the absence of gonads and kidneys. This suggests alterations in LHX1 are at least partially responsible for defects in the reproductive tract as well as those in the gonads and/or kidneys. If this is the case, then an absent LHX1 gene in humans would produce milder phenotypes when compared to those seen in mice. However, it is also possible that a mutant LHX1 gene in humans could result in a less functional protein, or one that is being expressed at the incorrect place and/or time. This is most likely due to the complexity of urogenital development including the presence of multiple genes and

pathways which overlap and cooperate with each other throughout embryogenesis. In order to provide more conclusive evidence that a defect in LHX1 would play a role like that described above, it would be beneficial to study the gene in a cohort of patients with MRKH Type II for the presence of any defects or alterations. If it is discovered that a larger number of MRKH patients with either gonadal or renal defects have some kind of mutation in their LHX1 gene, there would be more evidence backing the importance of this gene and the phenotypic effects of its mutant forms. Even if no sequence mutations are found in the LHX1 gene, defects may still arise as a result of abnormal LHX1 expression during early fetal development, much like that seen in the HOXA genes of MRKH patients. Although it is unlikely that expression levels could be tested in humans, it might be possible to conduct studies in mice by altering LHX1 expression during gastrulation and studying its effects on the mice embryos.

Another area that can be studied is the relationship between LHX1 and HOX genes throughout development. It is known that both genes play a role in the patterning of the anterior-posterior axis during embryogenesis; however it would also be beneficial to study the interaction of LHX1 and HOX genes at this stage if there is one. To achieve this, a better understanding of HOX gene regulation will be necessary. The problem is that not all HOX genes have been directly linked to the development of specific structures due to the tremendous amount of overlap of each HOX cluster. For example, although HOXA genes 9-11,13 have been directly linked to the development of the fallopian tubes, uterus, cervix, and upper vagina (Taylor et. al., 1997), HOX genes responsible for the development of the heart, spine, and middle ear are yet to be definitively identified (Simpson, 2000). In addition, studies have shown overlap between

HOX genes on the differentiation of various structures during development. Evidence also suggests that multiple HOX clusters work together during limb differentiation and HOX genes A11, B11, and C11 may each play a role during the differentiation and development of the kidneys (Lappin et. al., 2006). Further study into the mechanism of action of LHX1 as well as its interactions with HOX genes would provide insight into its role in MRKH as well as potentially explain the varying degrees of phenotypic expressivity that are present in the urogenital system.

Discussion

Future Work

As shown throughout this paper, MRKH is a multifactorial syndrome with a variety of potential molecular causes and resulting physical manifestations which affect multiple organ systems. It would be beneficial to obtain a more thorough understanding of the genes whose defects contribute to MRKH as well as the mechanisms and pathways through which these genes act. Currently, one of the biggest problems is that patients who present with the physical symptoms of MRKH do not appear to have common gene defects upon sequence analysis of their genomes. Sequence defects in any of the proposed candidate genes have yet to be identified amongst a significant number of MRKH patients whose genomes have been extensively analyzed.

One explanation for this could be the effect of various epigenetic mechanisms that have been accurately identified in one or more candidate genes. Some progress has been made with a candidate gene approach, but this has resulted in the identification of genetic changes in only a very small number of patients. In order to gain more insight into this syndrome, future studies should focus on a whole genome approach. This might provide insight into genes which had not been previously considered.

It has been shown that HOXA genes 9-11,13 are responsible for the differentiation of reproductive tract structures of the Müllerian duct and abnormalities in any of these genes is promising. Determining the effect of HOXA9 methylation and gene expression on the expression of HOXA genes 10, 11, and 13 as well as its mechanism of action on the Müllerian duct would allow for a better understanding of one piece of the etiology of MRKH.

MUC1 is another gene that shows tremendous promise and warrants further study in MRKH patients. Due to the occurrence of abnormal MUC1 gene regulation in almost 94% of patients thus far, it is worth completing a microarray analysis similar to the initial study of MUC1 from 2014 in a larger cohort of MRKH patients to see whether or not these results can be recreated. It would also be beneficial to determine if a connection exists between MUC1 and HOXA genes 9-11, 13. This might allow for a better understanding of the role of MUC1 during Müllerian duct development which in turn would provide a more clear picture of at least part of the etiology of MRKH.

A third promising gene is LHX1 which has been found to play a role during early stages of embryogenesis. Due to its importance in the development of the urogenital system defects in LHX1 may be specific to patients with MRKH Type II and warrants further study in these patients. In addition, further study into the potential cooperation between and/or overlap of LHX1 with both MUC1 and HOX genes during embryogenesis could help determine if a connection between LHX1 and either of these two genes exists.

In addition, as previously stated, associated anomalies can also occur in the heart and skeleton, even though they were not discussed. Although each of these structures arise from the same primary germ layer as the reproductive tract, it has yet to be determined whether or not these defects arise from a defect that would also explain alterations in the reproductive tract like what has been reported in cases with renal defects. Finally, there have been several reported cases of a familial occurrence of MRKH in females as well as associated anomalies in male relatives. This evidence does suggest a genetic component may need to be considered in MRKH, however, no genes

have been identified conclusively as causative agents of the syndrome and further research must be done.

Environmental Factors

In many cases of patients with MRKH, genetic defects are not found in the sequence, but occur through post-translational modifications, specifically DNA methylation. As evidenced in the Bisphenol A study, mice exposed to BPA who were also given nutrient supplementation in the form of methyl donors such as folic acid were found to have successful counteraction of BPA induced hypo-methylation (Dolinoy et. a., 2007). Based on this evidence, surveying mothers of MRKH patients for their dietary habits and use of prenatal supplements while pregnant could provide insight into a potential environmental cause for MRKH. If it can be shown that methyl donor groups have similar counter effects on genes that have been implicated in MRKH, it might be possible to prevent Müllerian duct abnormalities in a large number of potential MRKH patients. In addition, although maternal supplement use is becoming common practice amongst pregnant women, it may simply be a matter of dosage or other dietary factors that are causing abnormal development of the Müllerian duct in utero. It would be advantageous to determine the extent of counteraction of methyl donor groups so prenatal care can be modified accordingly.

Psychosocial Significance

Due to the complexity of MRKH syndrome as well as the very nature of congenital disorders, it seems much more probable to develop methods of earlier diagnosis, even if measures cannot be taken to prevent or reverse the developmental malformations, as a way to increase the patient's quality of life. Although there are many

physical abnormalities associated with MRKH, arguably one of the most challenging aspects of an MRKH diagnosis is the potential for psychological or emotional trauma (Bean et. al., 2008). For many patients, MRKH is not diagnosed until they are in their teens or early twenties when they present with primary amenorrhea, making the adjustment to a life with MRKH challenging. Often patients, upon learning of the diagnosis, question their identities as women. In addition, many experience feelings of isolation and confusion regarding their social and sexual roles, often resulting in social anxiety, severe depression, and sometimes even atypical and/or erratic behavior, ultimately leading to a lower quality of life (Bean et. a., 2008). To date there has been one reported suicide of an MRKH patient: a nineteen year old female from New Delhi, India (Gupta et. al., 2012).

In addition to the necessary emotional and psychological support, correction of the vaginal hypoplasia by way of both surgical and non-surgical methods is often done in order to give MRKH patients a relatively normal sex life (Bianchi et. al., 2011; Ismail-Pratt et. al., 2007). Measures to improve the mental and physical health have greatly improved the quality of life in MRKH patients, but the ramifications of infertility and the challenges that must be overcome in order to maintain a sense of normalcy can create a lasting negative psychological effect. Even though it seems that prevention or reversal of a congenital disorder like MRKH is highly unlikely, or at the very least far off into the future, the development of painless and relatively simple methods of earlier diagnosis could help alleviate the emotional and psychological stress put on both the patients and their families by allowing for a slower, more gentle transition into a life with MRKH.

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