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Revisiting old vaginal topics: conversion of the Müllerian vagina and origin of the "sinus" vagina

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ABSTRACT Vaginal development has been a longstanding controversy, which hampers studies on vaginal diseases as well as cervical and uterine diseases. Most concerns center on: why is the vaginal epithelium different from the uterine epithelium; and where does the vagina originate from? It is commonly held that the rodent vagina has a dual origin: the cranial part is derived from the Müllerian duct (Müllerian vagina) and the caudal part derived from the urogenital sinus ("sinus" vagina). This concept was deduced from morphological observations. However, it cannot explain the difference between the Müllerian vagina and the uterus. Moreover, accumulating new data from genetic and molecular studies contradicts the urogenital sinus origin of the "sinus" vagina. The present review summarizes previous morphological observations and new findings from genetic and molecular studies, and addresses molecular mechanisms underlying the origin and organogenesis of the vagina in rodents. It provides evidence to show that the whole vagina is derived the Müllerian duct. BMP4 reshapes the intermediate mesoderm-derived Müllerian duct into the vaginal primordium. The latter thus exhibits different features from the uterus, including the stratified squamous epithelium and insensitivity to anti-Müllerian hormone. The "sinus" vagina is formed by extrinsic BMP4-mediated caudal extension of the Müllerian duct. The present review thus shows how a century of controversy over the origin and organogenesis of the vagina has been resolved. This new understanding will provide additional insight into genetic diseases and tumors of the female reproductive tract.

KEY WORDS: *vagina, Mullerian duct, androgen, estrogen, prostate*

Introduction

In 1830, Johannes Müller first traced and recognized development of the Müllerian duct. Since then, an intense controversy on the vaginal development was raised and has continued to the present. Most concerns center on: why is the vaginal epithelium different from the uterine epithelium? and what does the vagina originate from? Rodents play an important role in biology and medicine. Today it is commonly held that the rodent vagina has a dual origin: the cranial part is derived from the Müllerian duct (Müllerian vagina) and the caudal part derived from the urogenital sinus ("sinus" vagina). This concept was deduced from previously morphological observations that the Müllerian vagina is initially lined with pseudostratified columnar epithelium, while the epithelium in both the Müllerian and "sinus" vagina converts to the stratified squamous epithelium later (Forsberg, 1965, Forsberg, 1973). However, it is yet unable to explain how the columnar epithelium in the Müllerian vagina converts to the stratified squa-

mous epithelium. Particularly, with advances in molecular biology and genetics, this conventional concept clearly contradicts the new findings on the Müllerian duct. Accumulating data from genetic and molecular studies show that the Müllerian duct determines the vaginal development. The present review summarizes previous morphological observations and new findings from genetic and molecular studies, and will aim to address molecular mechanisms on the origin and organogenesis of the vagina in rodents.

The vaginal development requires the Müllerian duct

Current genetic knockout studies have provided direct evidence for a Müllerian duct origin of the vagina. If the development of the Müllerian duct is disrupted, the vagina is absent. 1) If Wnt4 is mutated, the whole Müllerian duct is absent and no female internal

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genital organ is thus formed in the female mutant mice (Vainio *et al.*, 1999). 2) Wnt5a, which is expressed in the mesenchyma of the uterus, cervix and vagina, is required for posterior growth of the female reproductive tract (Mericskay *et al.*, 2004). In Wnt5a$^{-/-}$ mutant mice, the cervix and whole vagina are not formed (Mericskay *et al.*, 2004). 3) Wnt9b, acting upstream of Wnt4 in Müllerian duct formation, is essential for the caudal extension of the Müllerian duct and consequently Wnt9b$^{-/-}$ mutant female mice lack a uterus and vagina (Carroll *et al.*, 2005). 4) Pax-2 is a dorsal mesoderm factor (Patel and Dressler, 2004), and a urogenital developmental transcription factor expressed in the Wolffian and Müllerian ducts during embryonic stage. Pax-2$^{-/-}$ mutants lack the Wolffian and Müllerian duct derivatives, including the vagina, whereas the urogenital sinus still develops into the bladder and urethra (Torres *et al.*, 1995). 5) Lim1, another dorsal mesoderm signal (Capel, 1998), is also required for female reproductive tract development. While female Lim1$^{-/-}$ neonates have ovaries, they completely lack oviducts, a uterus, cervix and vagina (Kobayashi *et al.*, 2004). Therefore, the Müllerian duct is required for vaginal development.

BMP4-induced conversion of the Müllerian vagina

BMP4 expression in the vaginal primordium

Hoxa genes, including Hoxa9, Hoxa10, Hoxa11, and Hoxa13, specify the anterior-posterior patterning of the Müllerian duct system (Kobayashi and Behringer, 2003). Among them, Hoxa13 determines development of the caudal Müllerian duct. Hoxa13$^{-/-}$ mice lack the caudal Müllerian duct (Warot *et al.*, 1997). In newborn and mature rodents, Hoxa13 expression is restricted to the cervical and vaginal tissues, but not in the uterus (Taylor *et al.*, 1997, Post and Innis, 1999).

Sato and Iguchi (Sato and Iguchi, 2004) stated that in 2- or 15-day-old female mice, mRNA expressions of BMP4 and Hoxa13 were abundant in the vagina in comparison with the uterus. Their observations in 2-day-old mice are in accordance with previous observations by other groups. 1) The Hoxa13 expression in the female reproductive tract of newborn mice is in accordance with Post and Innis' study (Post and Innis, 1999). Their *in situ* hybridization results showed that Hoxa13 was expressed in vagina and cervix but not in uterus in newborn mice. 2) Lamm *et al.*, (Lamm *et al.*, 2001) observed in neonate mice that BMP4 expression remained diffusely distributed in the urogenital sinus region caudal to the uterus. In neonate mice, the vaginal morphology clearly develops, and is distinct from the uterus. Moreover, its inside is differentiated into the cranial part (Müllerian vagina) and the caudal part. Since the vagina was not their target, Lamm *et al.*, didn't mention the vagina in the article. Nevertheless, their data clearly showed that at postnatal one day, BMP4 expression was highest in the female urogenital sinus. There was a decreasing gradient in BMP4 expression in the Müllerian duct from the urogenital sinus to the uterus, in which the vagina was located. BMP4 was absent in the uterine horns.

Although there is no either further confirmation or contradiction for BMP4 expression in the vagina of 15-day old mice, it is indeed supported by indirect evidence. 1) At this stage, the uterus and vagina undergo organ differentiation under the induction of different Hoxa genes. Hoxa13 is strongly expressed in the vagina and cervix, but not in the uterus at two weeks of age (Taylor *et al.*, 1997). It is consistent with Sato and Iguchi's observation. 2) Hoxa13 can

activate BMP4 promotor activity (Suzuki *et al.*, 2003). It is thus reasonable that BMP4 remains higher in the vagina in comparison with the uterus. In addition, a series of phenomena take place in the Müllerian vagina but not in the uterus, for instances, expression of p63 and stratified squamous differentiation in the vaginal epithelium. They are associated with BMP4 as discussed, indirectly supporting BMP4 effects on the vaginal differentiation. Therefore, it is confirmable that BMP4 is expressed in the vaginal primordium.

BMP4 converts the intermediate mesodermal nature of the vaginal primordium

BMP4 plays an important role in specifying ventral mesodermal fate and dorsoventral patterning of mesoderm in mice (Jones *et al.*, 1991). It induces ventral mesoderm but represses dorsal mesoderm signals. Wnt7a is an important dorsal signal (Parr and McMahon, 1995, Kengaku *et al.*, 1998). BMP4 disrupts Wnt7a-induced signaling in chicks (Hirsinger *et al.*, 1997, Marcelle *et al.*, 1997) and mammals (Cossu and Borello, 1999, Viti *et al.*, 2003). In addition, BMP4 also suppresses expression of other early intermediate mesoderm-specific markers, including expression of Lim1 and Pax2 in the mesenchyma (Kim and Dressler, 2005).

The Müllerian duct originates from the intermediate mesoderm, which forms in the back of the embryo along the spine and belongs to the dorsal structures. Thus, BMP4, whose expression undergoes an ascending increase in the vaginal primordium, plays roles in conversion of the intermediate mesoderm nature of the Müllerian duct.

Loss of Wnt7a and appearance of BMP4 and p63 in the vaginal primordium

Wnt4 initiates invagination of coelomic epithelium to form the Müllerian duct, as well as induces expression of Wnt7a (Vainio *et al.*, 1999). Therefore, Wnt7a is initially expressed throughout the Müllerian tract (Miller and Sassoon, 1998). However, Wnt7a expression gradually declines in the Müllerian vaginal epithelium after birth, and is undetectable in the vaginal epithelium by 10 days after birth (Miller *et al.*, 1998).

As discussed, BMP4 at birth shows an opposite expression pattern, which displays the highest in the female urogenital sinus, then a descending gradient in the Müllerian duct from the urogenital sinus to the uterus, and absent in the uterine horns.

p63 is a downstream of BMP4 as discussed. Its expression has the same caudal-to-cranial descending pattern in the vaginal primordium around birth. At embryonic day 18.5 (E18.5), p63 is highly expressed in the caudal or "sinus" vagina, while is detected in small numbers of epithelial cells of the Müllerian vagina (Kurita *et al.*, 2005).

The timing of the loss of Wnt7a from the vagina corresponds to the timing of vaginal cytodifferentiation, which coincides with establishment of a spatial Hoxa axis in the Müllerian duct (Miller *et al.*, 1998). Initially, Hoxa9, Hoxa10, Hoxa11, and Hoxa13 are expressed at uniform levels throughout the length of the Müllerian duct. At around birth, their expressions become spatially restricted in the differentiating female reproductive tract (Taylor *et al.*, 1997). Hoxa13 is restricted to and strongly expressed in the vagina and cervix at birth (Taylor *et al.*, 1997, Post and Innis, 1999). Hoxa13 can activate BMP4 promotor activity (Suzuki *et al.*, 2003). Accordingly in the vagina, BMP4 is gradually expressed in a caudal-to-cranial pattern. Consequently due to the inhibition by BMP4, Wnt7a
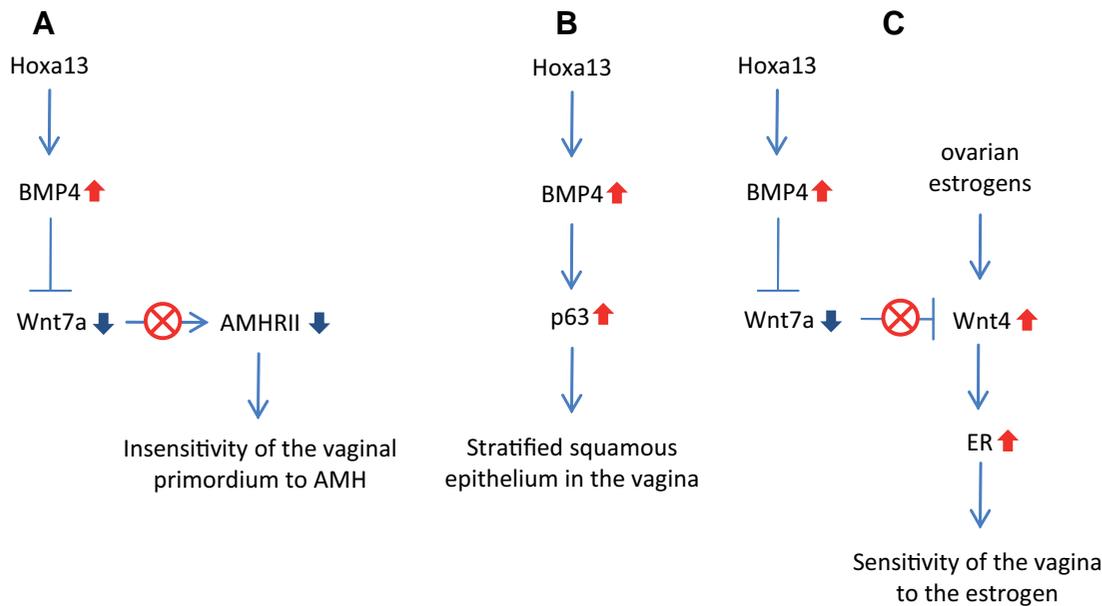
A

Hoxa13
↓
BMP4 ⬆
⊣
Wnt7a ⬇ → ⊗ → AMHRII ⬇
↓
Insensitivity of the vaginal
primordium to AMH

B

Hoxa13
↓
BMP4 ⬆
↓
p63 ⬆
↓
Stratified squamous
epithelium in the vagina

C

Hoxa13   ovarian
↓   estrogens
BMP4 ⬆   ↓
⊣
Wnt7a ⬇ → ⊗ ⊣ Wnt4 ⬆
↓
ER ⬆
↓
Sensitivity of the vagina
to the estrogen

Fig. 1. BMP4 induces the conversion of the Müllerian duct-derived vagina. (A) *BMP4 represses Wnt7a signaling, and in turn down-regulates AMHRII expression. It makes the vaginal primordium insensitive to AMH.* **(B)** *BMP4 induces p63 expression, and generation of the stratified squamous epithelial cells in the vagina.* **(C)** *BMP4 represses Wnt7a signaling, and in turn up-regulates Wnt4. Wnt4 enhances ER expression, which makes the vaginal primordium more sensitive to the estrogen than the uterine primordium.*

expression in the vaginal primordium gradually disappears in the same caudal-to-cranial pattern. Accordingly, Wnt7a determines the proper differentiation of the uterus (Miller and Sassoon, 1998, Parr and McMahon, 1998). Wnt7a⁻/⁻ mutation affects the uterus, but not the vagina (Miller and Sassoon, 1998). Therefore, BMP4 expression causes loss of Wnt7a in the vagina.

Insensitivity to anti-Müllerian Hormone

Anti-Müllerian hormone (AMH) is involved in male sex differentiation. It induces regression of the Müllerian duct structures in the male. However, the caudal Müllerian duct is insensitive to AMH. 1) In *in vitro* organ culture of the Müllerian duct from 14.5- and 15.5-day-old rodent fetuses, the cranial portion regresses almost completely during the 3-day culture period in the presence of AMH, whereas the caudal half to third remains intact but tapers to a fine point cranially (Tsuji *et al.*, 1992). 2) In AMH transgenic female mice, the oviduct and uterus fail to form due to the inhibition by AMH, whereas the vagina develops (Behringer *et al.*, 1990). 3) The vagina also forms irrespective of AMH in rodent males with testicular feminization (Tfm) and human males with complete androgen insensitivity syndrome (CAIS).

AMH induces regression of the Müllerian duct by binding to a specific AMH type II receptor (AMHRII). Mutation in AMHRII in male mice disrupts signaling, producing male pseudohermaphrodites that possess oviducts and uteri (Mishina *et al.*, 1996). Wnt7a drives the expression of AMHRII; the Müllerian duct in Wnt7a mutant mice thus fails to regress, but rather persists (Parr and McMahon, 1998). As discussed, BMP4 inhibits Wnt7a signaling in the vaginal primordium. It is able to explain the fact that AMHRII expression in the caudal part of the Müllerian duct is less than the intermediate part (Xavier and Allard, 2003). Therefore, BMP4-induced conversion makes the vaginal primordium insensitive to AMH (Fig. 1A). Taken together, BMP4 convert the intermediate mesoderm nature of the vaginal primordium, though it is derived from the Müllerian duct.

BMP4 reshapes the Müllerian vagina

BMP4 in the vaginal primordium not only converts the interme-

diate mesoderm nature, but also exerts effects on the vaginal differentiation. For instance, the Müllerian vagina becomes distinct from the uterus during organ differentiation, though both are derived from the Müllerian duct exhibit a similar epithelial phenotype at birth. Both the cranial part and caudal part of the vagina eventually possess the same histologic features.

Generation of stratified squamous epithelium

It is first found in *Xenopus* that BMP4 induces differentiation of epidermis or stratified squamous epithelium (Wilson and Hemmati-Brivanlou, 1995). BMP4 induces p63 expression via Smad4 and Smad5 signaling pathway in zebrafish and *Xenopus* (Bakkers *et al.*, 2002, Tribulo *et al.*, 2007). In rodents, BMP4 is also able to induce stratified squamous epithelial differentiation of embryonic stem cells; p63 is activated and plays a key role in this process (Aberdam *et al.*, 2007). p63 is a basal cell marker, and plays a crucial role in regulation of stem cell commitment in squamous epithelium (Yang and McKeon, 2000, Pellegrini *et al.*, 2001). It is also essential for squamous epithelial differentiation in the vagina. p63-null vaginal epithelium reverts to the epithelial phenotype similar to the uterus (Kurita and Cunha, 2001, Kurita *et al.*, 2004, Kurita *et al.*, 2005). Moreover, in the vagina, both BMP4 and p63 expression patterns have remarkably similar temporospatial characteristics. Therefore, BMP4-induced conversion generates the stratified squamous epithelial cells, which replace the columnar epithelium initially exhibited in the Müllerian vagina (Fig. 1B).

Sensitivity to estrogen

Wnt4 controls the expression of the genes encoding estrogen receptors ((ERs); the estrogen receptor α (ERα) is downregulated in Wnt4-null mice (Heikkila *et al.*, 2005). Because Wnt7a inhibits Wnt4 signaling (Miller and Sassoon, 1998), an inverse association is found between Wnt7a and ERα (Li *et al.*, 2001). Thus, Wnt4 signaling is enhanced in the caudal Müllerian tissue because of suppression of Wnt7a signaling by BMP4. 1) The ER signaling is consequently enhanced in vaginal primordium. Nuclei of the epithelial and stromal cells in the vagina and of the stromal cells in the uterus show strong ER expression on the day of birth (Sato *et al.*,

1996), whereas the uterine epithelium of the neonatal animal is devoid of estrogen-binding activity (Bigsby and Cunha, 1986, Bigsby *et al.*, 1990, Sato *et al.*, 1996). The ER exhibition pattern is consistent with the expression pattern of Wnt4, which is expressed in the stroma subjacent to the luminal epithelium of the uterus and the vagina (Miller *et al.*, 1998). But it is opposed to the expression pattern of Wnt7a, which is expressed only in the uterine epithelium but not uterine stroma and vagina (Miller and Sassoon, 1998). 2) It is also supported by a phenomenon that the vagina is more sensitive to estrogenic stimulation than the uterus. Under estrogenic stimulation, almost all ovariectomized mice exhibit stratification of vaginal epithelium, while few take place in the uterus (Mori *et al.*, 1992). ERα is required for E2-induced cornification and normal epithelial stratification (Buchanan *et al.*, 1998).

On the other hand, under BMP4 stimulation, Smad4 and Smad1 physically interact with the ER. BMP4 and estrogens act through overlapping intracellular signaling mechanisms. They have additive effects at low concentrations, mediating proliferation (Paez-Pereda *et al.*, 2003). Therefore, BMP4-induced conversion makes the vaginal primordium more sensitive to the estrogen than the uterine primordium (Fig. 1C).

Müllerian origin of the "sinus" vagina

In the mouse, the Müllerian duct begins to form as an invagination of the surface epithelium of the mesonephros at E11.5 and continues to elongate until it reaches the urogenital sinus at E13.5 in both male and female embryos (Kobayashi and Behringer, 2003). During the rest of gestation, the vaginal primordium continues its down-growth to the vestibule.

At birth the cranial portion of the vagina has a lumen lined with pseudostratified columnar epithelium indicating the Müllerian origin, while the caudal portion of the vagina has a solid epithelial cord. With time the caudal portion of the vagina canalizes and the cranial portion stratifies, so that eventually the entire vagina is lined by a stratified epithelium. According to the morphological observations, Forsberg (Forsberg, 1965, Forsberg, 1973) believed that the rodent vagina consisted of a cranial Müllerian part and a caudal part derived from the urogenital sinus: the "sinus" vagina.

The "sinus" vagina originates from the Müllerian duct
Do the different epithelial phenotypes between the vagina and uterus mean different origins other than the Müllerian duct origin?

In contrast to Forsberg's deduction, the following provide evidence that the urogenital sinus epithelium is unable to replace the vaginal epithelium. Actually, Forsberg (Forsberg and Olivecrona, 1965) himself found no signs of invasion of sinus epithelium into the Müllerian vagina. Additionally, later studies confirmed that the growth rate of urogenital sinus epithelial cells was lower than both caudal and cranial epithelial cells in the vagina (Boutin and Cunha, 1996). Later, Forsberg also did a transplantation experiment to prove that the epithelium in the Müllerian vagina performed the phenotypic transformation by itself. At E14.5, the Müllerian vagina analogue was removed and transplanted into the thigh muscle of newborn mice. After 12-day growing, stratification transformation of the Müllerian epithelium (including a superficial zone and a basal zone) occurs (Forsberg and Norell, 1966).

In addition, the stratified squamous phenotype does not support roles of the Wolffian duct in the vaginal development. For instance, the normal vagina is lined by stratified squamous epithelium, while persistent Gartner's duct, a vestigial Wolffian duct, is lined by columnar epithelium.

Finally, the stratified squamous epithelial phenotype is unique to the Müllerian duct-derived vagina. 1) The stratified squamous epithelial phenotype is determined by the vaginal stroma (Cooke *et al.*, 1987, Boutin and Cunha, 1997). The urogenital sinus mesenchyma fails to induce it on either the urogenital sinus epithelium or the vaginal epithelium under estrogenic conditions (Cunha and Young, 1992). 2) As discussed, under the induction of BMP4, the Müllerian duct-derived vaginal primordium itself generates the squamous epithelial phenotype. The epithelium in the vaginal primordium did not need other sources, but spontaneously achieved the conversion from uterus-like columnar to stratified squamous epithelium. Thus the stratified squamous phenotype is unable to support the possibility of other origins than the Müllerian duct.

Does the vagina in Tfm mice originate from different origins other than the Müllerian duct?

The conventional concept on the "sinus" vagina was previously supported by the presence of a shortened "sinus" vagina in androgen-insensitive Tfm mice. The vagina in Tfm mice was believed to be derived from the other tissues than the Müllerian ducts because it is resistant to the anti-Müllerian effect of AMH. It was previously plausible because at that time the Müllerian duct was commonly believed to regress in the male due to the inhibition by AMH. However, this view contradicts the following fact. Normal vaginal epithelium exhibits cyclical changes in both the morphology and the histology during the estrus cycle. These changes are variously caused by several steroidal hormones (Jones and Edgren, 1973). If the "urogenital sinus-derived" vagina (or "sinus" vagina) could display hormonal responses, other urogenital sinus derivatives should also have this feature. The answer is negative as the bladder and the urethra (the urogenital sinus derivatives) do not have a menstrual cycle like the uterus and the vagina.

Then what about the vagina in Tfm mice? The adult Tfm vaginal epithelium responds steroid stimulations like Müllerian-derived and not urogenital sinus-derived epithelium, indicating its nature of the Müllerian duct (Boutin and Cunha, 1996). Moreover, reconstruction analysis of the vagina in Tfm mice further confirms that the caudal Müllerian duct participates in vaginal formation (Drews *et al.*, 2002).

In fact, under BMP4 induction, the vaginal primordium is insensitive to AMH induction as discussed. The caudal Müllerian tissue consequently persists in the male and retains the ability to form the vaginal structure. A question thus arises why no vagina forms in the normal male. 1) The caudal Müllerian derivatives including the uterus and vagina are androgen target organs. The androgen receptor (AR) is constitutively expressed in both the mesenchyma surrounding the caudal Müllerian duct (Drews *et al.*, 2001) and its mature derivatives (Pelletier *et al.*, 2004). Strikingly, animal studies show that the caudal Müllerian tissue is more potent than the urogenital sinus in the conversion of testosterone to DHT. In rats, at fetal day 22, the activities of 5alpha-reductase in the vagina (67 pmol/h per mg protein) and the uterus (50-70 pmol/h per mg protein) is as high as in the differentiated prostate (41 pmol/h per mg protein), while the activity in urinary bladder is low, a baseline level (approximately 10 pmol/h per mg protein) (George, 1993). The androgen induces the caudal Müllerian tissue to participate in

prostate development (Cai, 2008). 2) In rats, the window of sensitivity to AMH is E14-15 days. After that, exposure to AMH does not cause Müllerian duct regression (Tsuji *et al.,* 1992). However, the fetal testes produce the androgen after that time. As discussed, the androgen represses BMP4 effects and thus suppresses vaginal organogenesis in the male. Therefore, under androgenic induction, the caudal Müllerian tissue fails to form the vagina, while it in turn participates in prostate development in the normal male. If the androgen signaling is disrupted, such as in Tfm mice, it forms the vagina rather than the prostate. Taken together, the "sinus" vagina is not derived from the urogenital sinus. On the contrary, the whole vagina is derived from the Müllerian duct.

The "sinus" vagina forms due to BMP4-mediated caudal extension of the Müllerian duct

The Müllerian duct extends downwards to the vestibule

Early view held that the vagina developed in a caudal-cranial direction, deduced from morphological observation of the sinovaginal bulbs (Koff, 1933). It seemed plausible because it seemed to be in agreement with Grunwald's observations on early development of the Müllerian duct. In early embryonic stage, the Wolffian duct first reaches the urogenital sinus. Under its guidance, the Müllerian duct reaches the urogenital sinus too (Grunwald, 1941). However, this is the case for early development of the Müllerian duct, but not for the formation of the caudal vagina. According to the position relationship between vaginal bud and orifice of major vestibular gland, Witschi (Witschi, 1970) observed that the vagina progresses down the dorsal wall of the urogenital sinus on its way to a separate opening in the vestibule. It is confirmed by Drews (Drews, 2007), who generated intersex mice composed of androgen insensitive X^{Tfm} and normal X^+ cells. In feminised intersexes the AR defective cells formed a vagina and the androgen responsive cells male sex organs. Similar to the Tfm/Y mice, the AR-mutated vagina descends and enters the penile bulb (an analogue of female vestibule). Strikingly, the AR+/+ Wolffian ducts, which normally open on the urethra in wild type mice, also descend with the vagina and enters the penile bulb. Therefore, the Müllerian duct constitutively possesses the ability to extend downwards and it can even drive the Wolffian duct to extend caudally together.

Extrinsic BMP4-induced caudal extension of the Müllerian duct

At birth, the caudal part of the mouse vagina is solid. Thus, a question arises why the caudal part is solid, but cranial part remains patent at birth. BMP4 can induce the epithelial-mesenchymal transition (EMT) (Endo *et al.*, 2002, Theriault *et al.*, 2007, Molloy *et al.*, 2008). It induces expression of Snail (Chiba *et al.*, 2005, Cazillis *et al.*, 2006, Theriault *et al.*, 2007), which represses E-cadherin expression and triggers the EMT (Davidson and Sukumar, 2005). Therefore, solidification of the caudal part of the uterovaginal canal may be a consequence of BMP4-induced EMT.

Although Hoxa13-induced BMP4 reshapes the Müllerian duct into the vaginal primordium, the cra-

nial part of the vagina is still lined by the columnar epithelium at birth. The Müllerian duct differentiates cranial-caudally, forming the oviduct, uterus, cervix and cranial part of vagina, which is completed by two weeks after birth (Yin and Ma, 2005). Thus, the cranial part of the vagina is not yet converted by BMP4 at birth, indicating that Hoxa13-induced BMP4 functions after birth and is not responsible for solidification of the caudal vagina before birth. Then, what causes the solidification in the caudal vaginal primordium?

The Müllerian duct reaches the urogenital sinus at E13.5. It is observed in normal mice (Bok and Drews, 1983) and rats (Sanchez-Ferrer *et al.*, 2006) that the caudal bifurcated Müllerian duct reaches the urogenital sinus and forms the Müllerian tubercle, on which the "sinus" vagina is formed. Actually, in the developing mouse BMP4 is abundantly expressed from E11.5-E13.5 in mesenchyma surrounding urogenital sinus (Brenner-Anantharam *et al.*, 2007). At birth, BMP4 still remains high in the urogenital sinus (Lamm *et al.*, 2001). Therefore, in the embryonic stage, extrinsic BMP4 produced from the urogenital sinus mesenchyma induces the EMT in the inserted caudal end of the Müllerian duct (Fig. 2A).

Next step, BMP4 induces c-myc expression (Paez-Pereda *et al.*, 2003), which is an important mediator for cell proliferation and migration (Biro *et al.*, 1993, Koster *et al.*, 1996). It also induces Rho GTPase activation (Theriault *et al.*, 2007), which plays an important role in cell migration (Raftopoulou and Hall, 2004). Therefore, BMP4 is involved in tissue migration. It is in agreement with the fact that the EMT plays a role in the disaggregation of epithelial cells and the reshaping of these cells for movement (Hay, 1995). For examples, 1) BMP4 is required for early mesoderm migration such as gastrulation (Winnier *et al.*, 1995). Most mouse embryos homozygous for a null mutation in BMP4 die around gastrulation
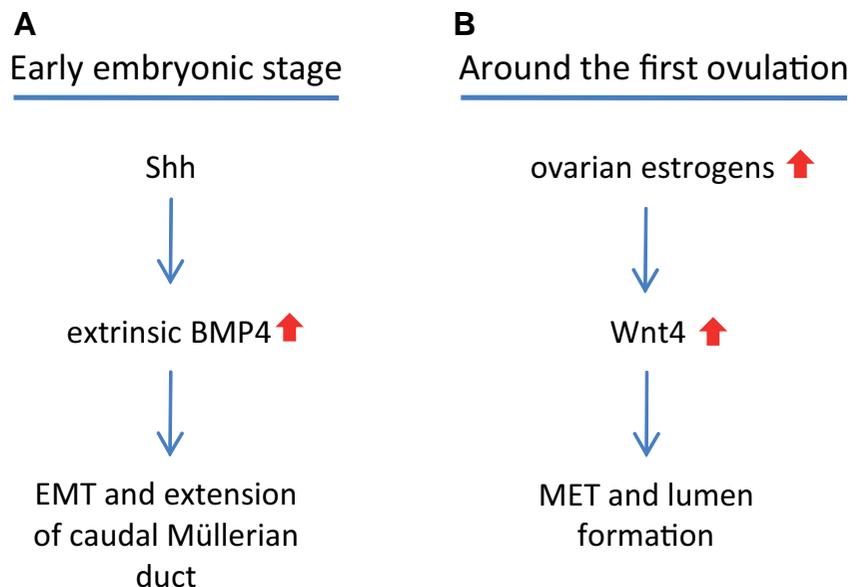
A

Early embryonic stage
_____

Shh

↓

extrinsic BMP4 🔺

↓

EMT and extension of caudal Müllerian duct

B

Around the first ovulation
_____

ovarian estrogens 🔺

↓

Wnt4 🔺

↓

MET and lumen formation

Fig. 2. Development of the caudal part of the vagina. (A) *The caudal end of the Müllerian duct inserts in the urogenital sinus wall and forms the Müllerian tubercle. During early embryonic stage, Shh induces BMP4 in the urogenita sinus mesenchyma. The extrinsic BMP4 induces the epithelial-mesenchymal transition (EMT) in the caudal end of the Müllerian duct to form the caudal vaginal primordium, and also guides it to extend caudally along the urogenital sinus wall.* **(B)** *Around the first ovulation, the ovarian estrogens enhance Wnt4 signaling, which induces the mesenchymal-epithelial transition (MET) and lumen formation in the caudal vagina.*

(Winnier *et al.*, 1995). 2) BMP4 also plays an essential role in elongation of the ureter bud (Miyazaki *et al.*, 2000, Cain and Bertram, 2006). Thus, BMP4 not only induces the EMT, but also leads to the caudal extension of the caudal Müllerian duct.

Then what direction does the caudal Müllerian duct follow to migrate along the urogenital sinus wall? In fact, BMP4 can serve as a chemoattractant for periureteral mesenchymal cells and induce locally the smooth muscle layer of the ureter at BMP4-expressing sites (Miyazaki *et al.*, 2003). Data from human samples show that BMP4 expression in the urogenital sinus mesenchyma proceeds in a cranial-to-caudal sequence (Jenkins *et al.*, 2007). In mice, diffuse BMP4 expression persists in the female urogenital sinus on embryonic day 18 and at birth (Lamm *et al.*, 2001). Thus, extrinsic BMP4 expressed in wall of the urogenital sinus guides the caudal end of the vaginal primordium to extend caudally (Fig. 3A and 3B).

The concept that the urogenital sinus guides caudal extension of the vaginal primordium via BMP4 signaling is supported by Sonic hedgehog (Shh) genetic mutation studies. The onset of the partition of the cloaca into the ventral urogenital sinus and dorsal anorectum is observed clearly by E10.5 (Mo *et al.*, 2001). Shh is essential for development of the urogenital organs (urethra and bladder) (Mo *et al.*, 2001, Haraguchi *et al.*, 2007). Shh induces BMP4 expression in the surrounding mesenchyma (Bitgood and McMahon, 1995). Under Shh induction, an urorectal septum forms in a cranial-to-caudal pattern. In the normal female, the vagina runs in the urorectal septum down to the perineum. In Shh-null animals, which exhibit improper urorectal separation (Mo *et al.*, 2001, Perriton *et al.*, 2002), the vagina never passes over the septum, although Hoxa13 can induce BMP4 in the caudal vaginal primordium. Therefore, Shh induces BMP4 in the urogenital sinus mesenchyma and BMP4 guides mesenchymal vaginal primordium to extend caudally.

The androgen antagonizes BMP4-mediated caudal extension of the Müllerian duct

The androgen down-regulates BMP4 signaling (Kuslak and Marker, 2007, Pu *et al.*, 2007). Extension of the caudal Müllerian mesenchyma is severely inhibited if the embryos are exposed to the androgen in early stage. The vagina thus fails to reach the perineum and can only open into the urethra. It is supported by animal studies, in which prenatal introduction of androgens leads to varied vaginal opening from the urethra at the level of the Wolffian duct opening to separate opening at the perineum. The inhibition effects of the androgen are dependent on timing and dosage of the androgen exposure (Lillie, 1917, Greene and Ivy, 1937, Yucel *et al.*, 2003). Therefore, BMP4 mediates caudal extension of the Müllerian duct to form the vagina.

Lumen formation in the caudal vagina
Generation of vaginal epithelium

The caudal vaginal primordium remains solid until the onset of sexual maturity. The origin of vaginal epithelium thus becomes a major concern. As discussed, solid evidence refutes the conventional concept that the urogenital sinus epithelium ascends to participate in vaginal organogenesis. Thus, a question arises if the solid vaginal primordium is able to generate the epithelium by itself.

The EMT can revert to generate the epithelium through the mesenchymal-epithelial transition (MET), which is crucial for vertebrate organogenesis. BMP4 expression is reduced in the urogenital sinus with gestation (Kuslak and Marker, 2007), indicating that its ability of EMT induction declines. On the other hand, Wnt4 is expressed in the vaginal primordium, and can be up-regulated by the estrogen (Hou *et al.*, 2004). One of key roles of Wnt4 in organogenesis is induction of the MET. Wnt4 positively regulates Rac1 and JNK pathways, which are involved in the MET (Osafune *et al.*, 2006). It is shown that Wnt4 is involved in epithelial generation in some mesoderm-derived organs, such as the kidney and the Müllerian duct derivatives. 1) Wnt4 is absolutely required for epithelialization in kidney development (Stark *et al.*, 1994, Kispert *et al.*, 1998). In Wnt4-deficient mice, the metanephric mesenchyma never enters the MET (Stark *et al.*, 1994). 2) Wnt4 is also essential for formation of the Müllerian duct. Wnt4-null animals lack the Müllerian duct (Vainio *et al.*, 1999). Adult vagina expresses Wnt4 in the epithelium only (Miller *et al.*, 1998). Therefore, vaginal epithelium can be generated through Wnt4-induced MET (Fig. 2B).

Lumen formation

The vagina of the mouse is closed at birth and does not open until the animal is 24 to 28 days of age, around the first ovulation representing the onset of puberty in female mice (Green, 1975). Vaginal opening is caused by the rise of estrogen levels, while the first ovulation caused by ovarian cyclic activity due to a gonadotropin surge. With estrogenic stimulation, the first vaginal cornification also occurs 24 to 120 hours after establishment of the vaginal opening (Green, 1966). Additionally, both prenatal exposure (Honma *et al.*, 2002) and postnatal introduction (Takasugi and Bern, 1962) of estrogens are able to induce precocious vaginal opening. Therefore, the ovarian estrogens around the first ovula-
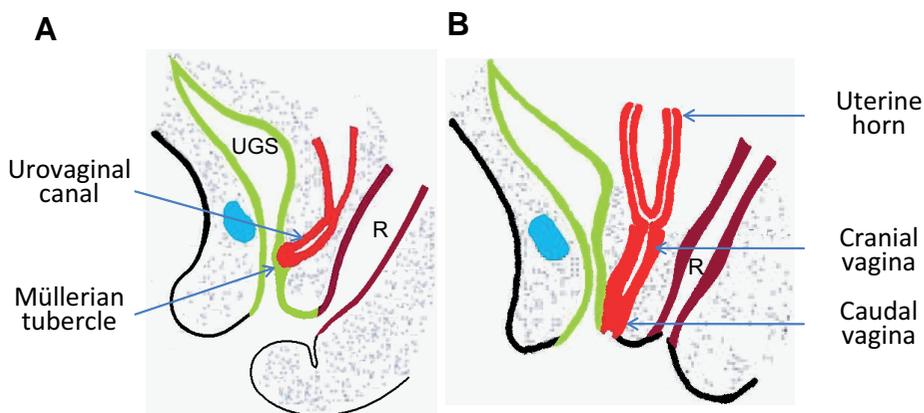

Fig. 3. The caudal vagina forms due to BMP4-mediated caudal extension of the Müllerian duct. (A) *The caudal end of the Müllerian duct inserts into the urogenital sinus (UGS) wall, in which BMP4 is strongly expressed. The caudal end of the Müllerian duct is solidified to form the Müllerian tubercle (the caudal vaginal primordium) via the extrinsic BMP-induced EMT. Furthermore, BMP4 expressed in a cranial-to-caudal sequence in the UGS wall guides the solid caudal vaginal primordium to extend caudally.* **(B)** *The solid caudal vaginal primordium reaches the perineum. R, the rectum; UGS, the urogenital sinus.*

tion induce vaginal canalization.

In cavitation, the lumen is generated by apoptosis of cells in the middle of the structure (Lubarsky and Krasnow, 2003). Animal studies show that the estrogen induces lumen-generated apoptosis in vaginal epithelial cells (Rao *et al.*, 1998). Two molecules, Wnt4 and β-catenin, are involved in vaginal lumen formation. 1) Wnt4 binds both canonical and noncanonical Frizzled receptors (Lyons *et al.*, 2004), which activate JNK-dependent pathway (Lisovsky *et al.*, 2002). Additionally, Wnt4 activates p38 mitogen-activated protein kinase (MAPK) in a novel noncanonical signaling pathway (Chang *et al.*, 2007). p38 MAPK has also been shown to play a role in tubulogenesis (Karihaloo *et al.*, 2005, Montesano *et al.*, 2007). Both JNK (Lei and Davis, 2003) and p38 (Choi *et al.*, 2005) are involved in induction of apoptosis. Consequently, Wnt4 is required for tubulogenesis in the kidney (Kispert *et al.*, 1998, Saulnier *et al.*, 2002, Kobayashi *et al.*, 2005, Itaranta *et al.*, 2006) and breast (Brisken *et al.*, 2000). 2) Nuclear β-catenin accumulation can induce activation of the p53-p21WAF1 pathway (Saegusa *et al.*, 2004, Chandar *et al.*, 2005, Buslei *et al.*, 2007) and overexpression of cyclin D1 (Shtutman *et al.*, 1999, Saegusa *et al.*, 2004), leading to suppression of cell proliferation or induction of cell senescence. It is consistent with apoptosis induced by overexpression of β-catenin (Kim *et al.*, 2000, Olmeda *et al.*, 2003). Both estrogen (Cardona-Gomez *et al.*, 2004, Chen *et al.*, 2005) and Wnt4 (Lin *et al.*, 2006) inhibit glycogen synthase kinase 3, leading to stabilization of β-catenin. It is shown that estrogen treatment causes an increase in cytosolic β-catenin followed by the accumulation of β-catenin in the nucleus (Chandar *et al.*, 2005, Rider *et al.*, 2006). Accumulation of β-catenin in the cytoplasm is involved in the formation of adherens junctions of mammalian epithelia where it links α-catenin to E-cadherin, while translocation of β-catenin to the nucleus is implicated in lumen formation and squamous differentiation.

Conclusions

An intense controversy persists on the vaginal development since 1830 when Johannes Müller traced and recognized development of the Müllerian duct. Previous morphological studies cannot resolve the controversy. Advances in molecular biology and genetics provide insights into the old topics on the vagina. The present review summarizes evidence from all morphological observations and genetic and molecular studies. Firstly, it shows that BMP4 reshapes the intermediate mesoderm-derived Müllerian duct into the vaginal primordium. This conversion makes the vagina acquire different features from the uterus, including stratified squamous epithelium and insensitivity to AMH. Secondly, the solid evidence shows that the "sinus" vagina is derived from the Müllerian duct rather than other tissues. The "sinus" vagina exhibits biological features that are unique to the Müllerian duct-derived vagina. Tfm mouse vagina forms because the vaginal primordium is insensitive to AMH induction and the androgen fails to repress vaginal morphogenesis. Lastly, extrinsic BMP4 from the urogenital sinus mesenchyma solidifies the caudal end of the Müllerian duct via the EMT, and further guides it (or the "sinus" vagina) to extend caudally along the urogenital sinus wall. Under estrogenic stimulation, the vaginal primordium reverts to generate the epithelium through the MET and eventually attains canalization. Therefore, the present review solves a century of controversy over the origin and organo-genesis of the vagina. The knowledge in the organogenesis determines understanding of clinical diseases, particularly congenital malformations and tumors (Bissell and Radisky, 2001). So, the present review will provide new insight in studies on diseases of the female reproductive tract.

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