Elongation of Müllerian ducts and connection to urogenital sinus determine the borderline of uterine and vaginal development

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In female mice, proximal, middle and caudal Müllerian ducts (MDs) differentiate into oviduct, uterus and vagina, respectively. The fate of female reproductive tract epithelia are determined by the mesenchyme. However, the mesenchymal fate determination system is still unclear. It is reported that presence or absence of retinoic acid (RA) signaling in MD mesenchyme induced uterine or vaginal mesenchyme, respectively. To analyze determination of the borderline, RA signal switching factors were found to play critical roles. Expression of a RA metabolizing enzyme, CYP26A1, was high in the epithelium of caudal MD and urogenital sinus, indicating that the enzyme causes the absence of RA signaling in the region. mRNA expression of some transcription factors regulating Aldh1a2, RA synthesis enzyme expressed in MDs, in other tissues was detected in MDs. When the transcription factor genes were overexpressed in a uterine mesenchymal cell line, C/EBPδ overexpression stimulated Aldh1a2 expression. Furthermore, C/EBPδ protein was strongly expressed in the proximal and middle regions of the MDs and bound to the Aldh1a2 promoter in vivo. Since C/EBPδ mRNA expression was maintained at the same level in proximal, middle and caudal MDs, we hypothesize that a high frequency of mitosis induces a lower level protein expression in MD mesenchyme. In fact, the mitotic activity was significantly high in caudal mesenchyme, and a mathematical model showed that a gradient of protein was induced by cell proliferation. Therefore, morphogenesis of MDs controls the fate of mesenchyme via RA degradation in urogenital sinus and a gradient of proteins involved in RA synthesis.

1. Introduction

In female mice, Müllerian ducts (MDs) initiate growth at the proximal region of mesonephros derived from mesoderm and elongate into urogenital sinus (UGS) derived from endoderm [1]. The proximal MD region of is adjacent to gonads and differentiates into oviducts, and the middle region distinguished from other tissues differentiates into uterus [2]. Although vaginal epithelium is derived from MD epithelium, vagina develops from caudal MD which are combined with UGS [3]. These female reproductive organs have their own morphology and function. Oviductal epithelium consists of ciliated cells and secretory cells [4], and uterine epithelium is composed of simple columnar luminal and glandular epithelial cells and vaginal epithelium develops into stratified cuboidal epithelium with cornification. Hetero-combinations of epithelium and mesenchyme between uterus and vagina demonstrated that the fate of MD epithelial cells was determined by the mesenchyme up to postnatal day 7 (P7) [5,6]. However, the mechanism of the fate determination of the mesenchyme itself has been unclear.

Homebox (Hox) cluster genes are most analyzed factors for development of MD mesenchyme. Hox9 is expressed at high levels in regions that will become oviducts, Hoxa10 is expressed in the development of uterus, Hoxa11 is expressed in the primordial lower uterus and cervix, and Hoxa13 is expressed in the cervix and upper vagina [7]. Hoxa10 deficiency induces an oviduct-like structure only in anterior part of uterus [8], and Hoxa13 deficiency causes hypoplastic urogenital sinus and agenesis of posterior part of MD, but not a genotype [9]. The Hox genes are also involved in adult functions of female reproductive tracts (e.g. implantation) [7]. These reports suggest that the Hox genes are mainly responsible for adult function in female reproductive tract, but they are not main factors that determined regional fate of MD mesenchyme.

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Retinoic acid (RA) is an essential component of cell-cell signaling during organogenesis [10]. Vitamin A deficient mice and RA receptor/retinoid X receptor mutant mice exhibits a complete absence of MDs, indicating that RA is essential for development of MDs [11–13]. RA is synthesized from retinol in an oxidation process catalyzed by alcohol dehydrogenases and aldehyde dehydrogenases [14,15]. RA is metabolized to hydroxylated forms by cytochrome P450, family 26, subfamily a, polypeptide 1 (CYP26A1) and CYP26B1 [16,17], and the metabolism of RA attenuated the activity of binding to RA receptors [18]. In MDs, expressions of retinol dehydrogenase 10 (RDH10), aldehyde dehydrogenase family 1, subfamily A2 (ALDH1A2) and RA signaling in the proximal and middle mesenchyme are higher than those in the caudal mesenchyme [2]. Furthermore, presence or absence of RA signaling is the fate-determining factor of MD mesenchyme into uterine or vaginal mesenchyme, respectively [2]. However, it is unclear why RA signaling disappears from the caudal MD mesenchyme and what induces RA synthesis enzymes in development of the mesenchyme.

In the present study, regulation mechanisms of RA metabolism and synthesis were analyzed in MDs. The expression profile of CYP26 in MDs was investigated, and candidates of transcription factors that could be upstream of Rdh10 or Aldh1a2 in MDs were searched. Roles of the candidates in Rdh10 and Aldh1a2 expression are then demonstrated.

2. Materials and methods

2.1. Animals

Female CD-1 mice (Sankyo Lab, Tokyo, Japan) were given a commercial diet and tap water ad libitum and kept at 22–24°C under 12 h light/12 h darkness by artificial illumination (lights on 08:00–20:00). Animals were maintained in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by our institutional Animal Care Committee. The presence of vaginal plugs indicated embryonic day 0.5 (E0.5). MDs at E12.5 were not selected for male or female. Spleens were dissected from adult mice.

2.2. Cell culture

P3US cells were established from uterine mesenchyme of p53−/− mice at P3 and cultured in 1:1 mixture of Dulbecco modified Eagle medium and Ham nutrient mixture F-12 without phenol red (Sigma-Aldrich, St. Louis, MO, USA) containing heat-inactivated FBS (Cell Culture Technologies, Waltham, MA, USA) at 10%, penicillin (31 μg/ml, Sigma-Aldrich) and streptomycin (50 μg/ml, Sigma-Aldrich) (10% FBS) in a humidified atmosphere of 5% CO2 at 37°C. The cells were passaged using 0.05% trypsin-0.02% EDTA (Sigma-Aldrich).

2.3. RNA isolation and reverse transcript (RT)-PCR

Total RNA was isolated from MDs and UGS at E12.5 and P0 (Fig. 1A) or P3US cells by acid guanidinium-phenol-chloroform extraction. RT was performed with ReverTra Ace qPCR RT Master Mix with gDNA Remover (TOYOBO, Osaka, Japan). PCR was carried out with AmpliTaq Gold PCR Master Mix (Applied Biosystems, Foster City, CA, USA) and specific primers (Supplemental table). β-actin was chosen as an internal standard. Ten-thirteen MDs or UGS were pooled in one sample.

2.4. Overexpression of transcription factors in P3US cells

RNA isolation and RT were performed from the middle of MDs at E16.5 as described above. ORFs were amplified with TksGflex DNA Polymerase (TaKaRa, Shiga, Japan) with specific primers (Supplemental table), and inserted into pcDNA 3.1 Hygro (+) plasmids using Ligation high ver.2 (TOYOBO). Each gene was sequenced using the BigDye Terminator v3.1 Cycle Sequencing kit and analyzed with 3130xl Genetic Analyzer (Applied Biosystems).

P3US cells in 60 mm dishes were transiently transfected with 1 μg of these plasmids by Screen Fect A (Wako, Osaka, Japan) according to the manufacturer’s instructions. Control cells were P3US cells transfected with pcDNA 3.1 Hygro (+) vector. After 48 h, gene expressions in these cell lines were analyzed by RT-PCR.

2.5. ChIP assay

Ten MDs at E14.5 were sampled in 1% Protease Inhibitor Cocktail (Wako; added to all buffer by chromatim extraction)/PBS, and cross-linked with 1.5% PFA. Fixation was stopped by addition of glycine. The samples were washed with PBS and homogenized. The pellets were lysed in 0.5% NP40 buffer (10 mM Tris (pH8.0), 10 mM NaCl), and then lysed in 1% SDS buffer (50 mM Tris (pH8.0), 10 mM EDTA). The samples were added with ChIP dilution buffer (50 mM Tris (pH8.0), 167 mM NaCl, 1.1% Triton X-100 and 0.11% Sodium Deoxycholate). The chromatin was sheared with a sonicator to an average length of 200 bp.

Thirty μl ChIP-grade protein G magnetic beads (Cell Signaling Technologies, Beverly, MA, USA) and 0.5 μg anti-RNA polymerase II CTD repeat antibody (Santa Cruz biotechnology, Santa cruz, CA, USA), anti-CEBPβ antibody (Santa Cruz biotechnology) or normal rabbit IgG (Santa Cruz Biotechnology) were mixed in RIPA buffer and incubated at 4°C overnight. After washing with RIPA buffer, the mixtures and the fragmented chromatin were mixed and incubated at 4°C overnight. Samples were then sequentially washed in RIPA buffer, RIPA buffer containing 500 mM NaCl, LiCl wash solution and TE buffer, and eluted in 0.5% SDS buffer (10 mM Tris (pH8.0), 300 mM NaCl, 5 mM EDTA) at 65°C for 8 h, then added with protease K at 500 μg/ml and incubated for 1 h at 55°C. Proteins were removed by phenol and chloroform and DNA were precipitated by ethanol. The DNAs were compared with inputs by quantitative PCR using KOD SYBR qPCR Mix (TOYOBO). Aldh1a2 TSS (−89 to +11), Aldh1a2 C/EβP8 predicted binding site (+2872 to +3987) and Aldh1a2 conserved region primer (−381 to +1, 25,794 to +26,539) were derived from Gene ID 19,378 (Supplemental Table).

2.6. Histology

To detect CYP26A1, female mice at E14.5, E16.5 and P0 were fixed in 4% PFA at 4°C for 3 h. The samples were embedded in paraffin and cut into 6 μm sections. To detect C/EβP8, female mice at E14.5 and spleens for positive control were embedded in O.C.T. Compound and frozen by liquid nitrogen. The cryosections (10 µm) were microwaved in 10 mM sodium citrate buffer (pH 6.0) for 10 min. The samples were em- bedded in paraﬁlm, and α-actin, CYP26A1, CEBPβ, C/EβP8 and nuclear actin were detected by immunohistochemistry. The samples were incubated with normal goat serum and 1% BSA for 1 h, then incubated with primary antibodies against CYP26A1 (1/200; ALPHALAB DIAGNOSTIC, San Antonio, TX, USA) in PBS containing 0.1% Triton X-100, 1% BSA and 0.1% nonfat dried milk, C/EβP8 (1/50; Abcam, Cambridge, UK) in PBS containing 5% goat serum and 1% BSA, and then incubated with Alexa488-conjugated anti-rabbit IgG goat antibody (1/250; Jackson Immuno Research, West Grove, PA, USA) for 1 h at 37°C. After that, the samples were mounted with Vectashield containing DAPI. To detect cell proliferation, pregnant mice at day of post coitus 14.5 were injected with 100 μg/0.1 ml of EdU (Thermo Fisher, Waltham, MA, USA). After administration for 1 h, female embryos were fixed in 4% PFA at 4°C overnight (n = 5–7). The samples were embedded in paraffin and cut into 6 μm sections. The sections were stained with Click-IT EdU Alexa Fluor 594 Imaging Kit (Thermo Fisher), ac- cording to manufacturer’s instructions. The number of EdU-positive cells and Hoescht-stained cells were counted manually. All of epithelial cells in the 1–3 pictures and 200 mesenchymal cells were counted in a
2.7. Mathematical modeling

A one dimensional cell-based MD ductal elongation model was generated. In this model, cells line one dimensionally and cell proliferation occurs from proximal to caudal region. (Fig. 4C). First, 10 cells have constant levels of mRNA or protein (the level indicated as "C"), and frequency of cell proliferation is set as 18, 24 or 30% in proximal (2/5 head region in all cells at the time), middle (2/5 following region) or caudal region (1/5 last region). mRNA or protein degradation levels are indicated as "d", and the synthesis levels are indicated as "s", and d and s are hypothesized at constant.

\[
C_n = \begin{cases} 
(1-d)C_{n-1} + s, & \text{not proliferated} \\
\frac{(1-d)C_{n-1}}{2} + s, & \text{proliferated} 
\end{cases}
\]

When the number of cells reaches more than 5000, C is averaged each in 100 cells and shows as dots graphically (n = 10).

2.8. Statistical analysis

Data were expressed as mean ± standard error. Two-tailed Student’s t-test or Welch’s t-test was used for single comparisons. For multiple comparisons, differences were estimated using ANOVA with appropriate post hoc tests. A statistically significant difference was defined as \( p \leq 0.05 \).

3. Results

3.1. Ontogenic expression of CYP26 mRNA and protein in MDs

The expression of RA metabolizing enzyme gene, Cyp26, was investigated in proximal, middle and caudal MDs and UGS at E16.5 and P0 (Fig. 1B). Cyp26a1 was mainly expressed in UGS and slightly expressed in proximal and caudal MD. Cyp26b1 was not detected in MDs and UGS at E16.5 and P0 (data not shown).

CYP26A1 protein expression was investigated in cross, sagittal or horizontal sections of MDs and UGS at E14.5, E16.5 and P0 (Fig. 1C). CYP26A1 was expressed in cytoplasm and not expressed in nuclei at all stages. CYP26A1 was mainly expressed in mesenchyme of MDs.
caudal MD and UGS, the enzyme expression was strongly detected in the epithelium at embryonic stages. The expression pattern of CYP26A1 was not changed at P0, but the expression levels were decreased. Therefore, RA metabolizing enzyme gene and protein were highly expressed at caudal MD and UGS at embryonic stages.

3.2. Analysis of ontogenic expression of transcription factors in upstream of Aldh1a2 mRNA in MDs by RT-PCR

Since it is hypothesized that a borderline of retinoic acid signaling is determined by RA synthesis and metabolization in MDs, ontogenic expression of RA synthesis enzymes, *Rd10* and *Aldh1a2*, and RA metabolizing enzyme *Cyp26a1* was analyzed in MDs from E12.5 to P0. At E12.5, MDs have grown to the caudal region of the developing gonad [1]; therefore, the middle region of mesonephros (Fig. 1A) contained little MD cells. However, *Rdh10* and *Aldh1a2* expressions were already detected in the middle region of mesonephros before development of MDs (Fig. 2A). *Aldh1a2* expression was decreased at the caudal region from E12.5 to P0, and *Rdh10* expression level was similar in all samples except for UGS at P0. *Cyp26a1* was mainly expressed in UGS at the embryonic stages as mentioned above. Therefore, in MDs, RA signaling may be mainly controlled by the gradient of *Aldh1a2* expression and *Cyp26a1* expression at UGS.

Transcription factors stimulating *Aldh1a2* promoter activity are reportedly *Meis1*, *Meis2*, C/ebpα, C/ebpβ, and *Wt1* in epicardium, *Sp1* in dendritic cells and *Foxc1* in somite of zebra fish [19–22]. Thus, ontogenic expression of the transcription factors was analyzed in MDs and UGS from E12.5 to P0 (Fig. 2A). *Meis1*, *Meis2*, C/ebpβ, C/ebpδ and *Wt1* were expressed in MDs from E12.5 to E16.5. Little *Wt1* expression was detected in caudal region at E14.5 and UGS at E16.5 and P0. *Sp1*, *Foxc1* and C/ebpα expressions were not detected in MDs at E12.5 and E14.5. These results suggest that transcription factors such as *Meis1*, *Meis2*, C/ebpβ, C/ebpδ and *Wt1* are candidates for activators of *Aldh1a2* promoter in MDs.

3.3. Effect of transcription factors on Aldh1a2 expression in P3US cells

The effects of the transcription factors on *Aldh1a2* expression was analyzed in P3US cells derived from uterus mesenchyme at P3 with overexpression, since RA signaling is localized in MD mesenchyme [2]. Expression of *Wt1*, C/ebpβ, C/ebpδ and *Meis1*, *Rdh10*, *Aldh1a2* and *Cyp26a1* expressions were analyzed by RT-PCR (B). (n = 3).
Aldh1a2 promoter in vivo.

Localization of C/EBPδ protein was investigated in cross sections of MDs at E14.5 by immunohistochemistry (Fig. 3C). C/EBPδ was expressed in the nuclei of MD epithelium and mesenchyme. Expression of C/EBPδ was higher in proximal region than that in caudal region. These results suggest that C/EBPδ is mainly localized in the nuclei of proximal MD mesenchyme.

3.4. Induction mechanism of gradient expression of protein in MD mesenchyme

Although C/ebpδ mRNA expression was maintained at the same level in proximal, middle and caudal regions (Fig. 2A), C/EBPδ protein was mainly localized in the proximal region (Fig. 3C). Half-life of protein (median = 46 h) is longer than that of the mRNA (median = 9 h) [24]; therefore, protein expression is reflect in various cell condition. Orvis and Behringer showed that MD epithelial cells highly proliferated at the tip of ducts [1]. If mitosis takes place in cells, the contents of proteins must be equally divided into daughter cells. Thus, we hypothesized that difference of mitosis frequency induces a gradient expression in MD mesenchyme. In fact, frequency of mitosis was significantly high in caudal mesenchyme compared with that in proximal mesenchyme at E14.5 by EdU staining (Fig. 4A, B). We then formalized a mathematical model of mRNA or protein level in MDs using parameter of frequency of mitosis (18, 24 or 30%/1 h in proximal, middle or caudal region) (Fig. 4C). When the value of $d$ is 1.1%/1 h (predicted by half-life of protein), frequency of mitosis induces a gradient of protein level, but not the due to the value of $s$, or $d = 5.6\%$ (predicted by 9 h half-life of mRNA). These results suggested that constant synthesized and degraded protein represent gradient expression in MDs.

4. Discussion

The present study demonstrated that CYP26A1 was expressed not only in caudal MDs but also UGS connected with MDs, and in MD elongation, high proliferative activity at caudal region caused dilution of proteins involving in RA synthesis (e.g. ALDH1A2 and C/EBPδ). Point mutation of lipoma HMGIC fusion partner-like 2 gene leads failure of the connection to UGS and vaginal agenesis [25], supporting that the connection to UGS is essential for vaginal development. Therefore, MD morphogenesis (i.e. elongation and connection to UGS) generates a borderline of RA signaling causing fate determination of uterine and vaginal mesenchyme.
that Cyp26a1 expression level was 168.7 ± 66.9 folds in UGS compared with middle MD, suggesting that CYP26A1 level was sufficiently high for degradation of RA in UGS as well as in adult uterus. Thus, CYP26A1 was involved in the fate determination of vaginal mesenchyme. In caudal MD, ALDH1A2 protein is slightly detected in the mesenchyme [2]. In caudal MD and UGS, CYP26A1 protein was mainly expressed in the epithelium. In adult uterus, RA is synthesized in the stroma, whereas RA is metabolized in the epithelium [27]. Therefore, in caudal MD, RA is synthesized in the mesenchyme and metabolized in the epithelium.

C/EBPβ stimulated Aldh1a2 expression, and it was directly bound to Aldh1a2 promoter in vivo, indicating that C/EBPβ directly regulates Aldh1a2 expression in MDs. However, the expression of C/ebp and Aldh1a2 showed reverse correlation in MD development (Fig. 2A), and female C/EBPβ knockout mice are viable and fertile [28], suggesting that C/EBPβ is one of the factors regulating Aldh1a2 expression. Aldh1a2 was already expressed in mesonephros at E7.5-E10.5 [29], and we also detected the expression at E12.5 before MD development. Furthermore, RA treatment to pluripotent cells induces differentiation into Osr1-expressing cells [30–32], which are mesonephric cells in mesoderm [33]. Collectively, RA synthesizing system including transcription factors regulating Aldh1a2 expression is essential for mesonephric development; therefore, in MDs, the system may be diverted to the mesenchymal differentiation.

In human, some disorders are reported for atresia and agenesis of vagina and cervix [34,35]. Although human vaginal development also include steps of MD elongation and connection of MD to UGS [36], vaginal epithelial origin is different between human in which vaginal epithelium is derived from UGS [36] and mouse in which it is derived MD [3]. Furthermore, in patient of Mayer-Rokitansky-Küster-Hauser syndrome, one of the major vaginal abnormality, the epithelium of closed vaginal antral mucosa are expanded to the entire vagina by surgery and treatment of fibroblast growth factor and differentiates into vagina-like estrogen receptor-expressed stratified epithelium [37]. Taken together, epithelial stratification of MD is necessary only for cervical development in human. Consequently, disorders of cervical abnormality with vaginal presence may be caused by elongation of MD or connection to UGS at development.

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Appendix A. Transparency document

Transparency data associated with this article can be found in the online version at doi:10.1016/j.bbrep.2018.10.013.

Appendix B. Supplementary material

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