Reduced BMP Signaling Results in Hindlimb Fusion with Lethal Pelvic/Urogenital Organ Aplasia: A New Mouse Model of Sirenomelia

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Abstract

Sirenomelia, also known as mermaid syndrome, is a developmental malformation of the caudal body characterized by leg fusion and associated anomalies of pelvic/urogenital organs including bladder, kidney, rectum and external genitalia. Most affected infants are stillborn, and the few born alive rarely survive beyond the neonatal period. Despite the many clinical studies of sirenomelia in humans, little is known about the pathogenic developmental mechanisms that cause the complex array of phenotypes observed. Here, we provide new evidence that reduced BMP (Bone Morphogenetic Protein) signaling disrupts caudal body formation in mice and phenocopies sirenomelia. Bmp4 is strongly expressed in the developing caudal body structures including the peri-cloacal region and hindlimb field. In order to address the function of Bmp4 in caudal body formation, we utilized a conditional Bmp4 mouse allele (Bmp4flox/flox) and the Isl1 (Isl1flox)Cre mouse line. Isl1-Cre is expressed in the peri-cloacal region and the developing hindlimb field. Isl1CreBmp4flox/flox conditional mutant mice displayed sirenomelia phenotypes including hindlimb fusion and pelvic/urogenital organ dysgenesis. Genetic lineage analyses indicate that Isl1-expressing cells contribute to both the aPCM (anterior Peri-Cloacal Mesenchyme) and the hindlimb bud. We show Bmp4 is essential for the aPCM formation independently with Shh signaling. Furthermore, we show Bmp4 is a major BMP ligand for caudal body formation as shown by compound genetic analyses of Bmp4 and Bmp7. Taken together, this study reveals coordinated development of caudal body structures including pelvic/urogenital organs and hindlimb orchestrated by BMP signaling in Isl1-expressing cells. Our study offers new insights into the pathogenesis of sirenomelia.

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Introduction

Sirenomelia, also known as mermaid syndrome, is a developmental malformation characterized by leg fusion and associated anomalies of pelvic/urogenital organs including bladder, kidney, rectum and external genitalia. Most affected infants are stillborn, and the few born alive rarely survive beyond the neonatal period. Despite the many clinical studies of sirenomelia in humans, little is known about the pathogenic developmental mechanisms that cause the complex array of phenotypes observed. Here, we provide new evidence that reduced BMP (Bone Morphogenetic Protein) signaling disrupts caudal body formation in mice and phenocopies sirenomelia. BMP4 is strongly expressed in the developing caudal body structures including the peri-cloacal region and hindlimb field. In order to address the function of BMP4 in the development of the caudal body such as hindlimb and pelvic organs is coordinated. Due to the striking leg fusion phenotypes in sirenomelia, most researchers have focused on understanding the pathogenesis of this feature. Clinical and anatomical studies in humans have resulted in two hypotheses for the pathogenesis of sirenomelia: vascular steal and a defect of blastogenesis. The vascular steal hypothesis posits that hindlimb fusion results from deficient blood flow and nutrient supply to the caudal mesoderm, while a primary anomaly in the process of the caudal mesoderm formation is attributed by defective blastogenesis [1], [3–5]. However, the molecular and developmental bases of the complex phenotypes have not been elucidated.
During mouse development, a transient embryonic cavity, the cloaca, forms at the caudal end of the hindgut and is subsequently divided into the urogenital sinus and the rectum by the urorectal septum (URS) [6–10]. The bladder and rectum are pelvic organs derived from the cloaca. Recently, we showed that the Peri-Cloacal Mesenchyme (PCM) is another essential tissue for development of the pelvic organs and external genitalia [11, 12]. Previous studies describe the PCM as infrapericloacal mesenchyme [13]. PCM region has been originally suggested as part of the Gsii, an indicator gene of hedgehog (HH) signaling, positive mesenchyme [11]. Previous lineage analysis suggests that PCM also includes the anterior part of cloaca [11]. To precisely define and discuss its role, the anterior part of the PCM region is hereafter designated as aPCM (anterior Peri-Cloacal Mesenchyme).

Shh, a major ligand of HH signaling for the aPCM formation, is expressed also in the anterior part of cloaca. Shh KO mice show the abnormal development of the aPCM-derived tissues such as external genitalia [14]. In addition to the roles of Shh in aPCM-derived tissue formation, the significance of the aPCM region to coordinate caudal organ formation is unknown.

Bone Morphogenetic Protein (BMP) signaling regulates a range of cellular processes and plays essential roles regulating the morphogenesis of many organs [15–19]. BMPs are members of the evolutionarily conserved Transforming growth factor-β (TGF-β) superfamily that signals via type I and type II receptors. Bmp7 KO mice die shortly after birth and display defects of kidney development [20], [21]. Although Bmp7 KO mice do not have defective caudal body formation, ablation of both Bmp7 and Tsg1 (which encodes a modulator of BMP signaling) results in hindlimb fusion [22]. Due to the functional redundancy of BMP genes, little is known about the role of individual BMPs in caudal body development. Therefore, it is necessary to address the role of BMP signaling using compound genetic analyses of Bmp mutant alleles. Intriguingly, Bmp4 is expressed in the caudal body region including the anterior cloacal mesenchyme (anterior mesenchyme adjacent to the cloaca before the formation of aPCM) and hindlimb field. However, Bmp4-null embryos die between embryonic day (E) 6.5 and E9.5, prior to caudal body formation [23]. Thus, determining the function of Bmp4 in the caudal body requires a conditional gene ablation approach.

A subset of hindlimb progenitors have been identified by fate mapping studies with an Isl1 (Islet1)-Cre mouse line generated by a knock-in of the Cre gene into the endogenous Isl1 locus [24]. Isl1, a LIM-homeodomain-transcription factor regulates the process of hindlimb initiation [25]. Isl1-Cre is expressed not only in the hindlimb field, but also in the cloacal regions [24]. We observed that Isl1-expressing cells contribute to the aPCM and subsequently to the caudal body structures including the bladder, rectum and external genitalia. To investigate the role of Bmp4 during caudal body formation, we analyzed the Bmp4 conditional KO mice utilizing Isl1-Cre driver strain. These Bmp4 conditional KO mice showed sirenomelia phenotypes including hindlimb fusion and also hitherto undescribed lethal pelvic/urogenital organ dysplasia. We show that Bmp4 is required to form the aPCM and to adjust hindlimb positioning during caudal body formation. Our study revealed a novel requirement of Bmp4 function and an essential population of progenitor cells for caudal body formation.

**Results**

**Disruption of Bmp4 function leads to the sirenomelia**

We found that Bmp4 is strongly expressed in the caudal tissues including the base of the umbilical cord and anterior cloacal mesenchyme (hereafter designated as peri-cloacal regions) and the hindlimb field at early staged embryo of E9.5 (Fig. 1 A, B and C, square). In order to address the function of Bmp4 in caudal body development, we utilized a conditional Bmp4 null allele (Bmp4<sup>flox/flox</sup>) and the Isl1-Cre mouse line [24]. Isl1-Cre is expressed in the caudal body regions, including the peri-cloacal regions and the mesenchyme of the developing hindlimb field at E9.5 (Fig. 1 D, yellow circle, E) [24]. Isl1CreBmp4<sup>flox/flox</sup> (hereafter designated as Bmp4 cKO) mutants possess hindlimb fusion similar to sirenomelia in humans (Fig. 1 G). Stocker and Heifetz classified sirenomelia into types I-VII, based on which skeletal elements are present and their relationship within the malformed extremity [26]. We therefore analyzed hindlimb skeletons of 22 mutant mice (Fig. 1 H–J). Based on the variants described by Stocker and Heifetz, 59% and 36% of the mutants had defects consistent with type III: loss of the fibula, and type I: abnormal medial location of fibula, respectively. The other 5% had type V sirenomelia limb phenotypes: loss of the fibula and fusion of the femur (Fig. 1 H–J, red arrowheads indicate the position of the ossified fibula).

Anomalies in the pelvic/urogenital organs are the major cause of lethality in human sirenomelia patients. Thus, we analyzed the pelvic/urogenital organs of Bmp4 cKO mice. Bmp4 cKO mice had bladder aplasia, hypoplastic kidney, hypoplasia of external genitalia and anal stenosis (Fig. 2 B, D, F). These results suggest that disruption of Bmp4 function in caudal body regions phenocopies all prominent phenotypes of sirenomelia observed in humans.

**Genetic analysis for tissue contribution of Isl1-expressing cells to the embryonic caudal body**

The above results prompted us to genetically analyze tissues contributing to caudal body formation with regard to Isl1 and Bmp4 expression domains. Isl1 mRNA was observed in the lateral plate mesoderm adjacent to the future hindlimb bud and at the base of the allantois at E8.5 (Fig. 3 A, E, bracket). Its expression was detected in the peri-cloacal regions (the base of the umbilical cord and anterior cloacal mesenchyme) and the hindlimb field at E9.5 (Fig. 3 B, F, square). Isl1 was expressed in the cloacal mesenchyme including aPCM, and URS (urorectal septum) at E10.5 (Fig. 3 C, arrow, G, square and arrowhead). Its expression in the hindlimb bud was decreased after E10.5, but maintained in the developing genital tubercle, an anlage of external genitalia, (hereafter designated as GT) at E11.5 (Fig. 3 D, arrow, H).

Previous fate mapping with Isl1-Cre and a R26R-lac<sup>Z</sup> reporter mouse strain [27], revealed that Isl1-expressing progenitors contribute to the hindlimb field [24]. In order to address the contribution of Isl1-expressing cells during caudal body formation, we used the Isl1-mER-Cre-mER allele in which sequences encoding a tamoxifen-dependent Cre recombinase were inserted into the Isl1 locus [28]. In order to identify Isl1-expressing cells at each embryonic stage, tamoxifen (hereafter designated as TM) was administered between E8.5 and E11.5 and the resulting β-galactosidase labeled cells in Isl1-mER-Cre-mER;R26R embryos were analyzed at E15.5 (Fig. 3 I–P). The TM-inducible labeling system marks cells within approximately less than a half day of TM administration [29], [30]. We observed that the majority of Isl1-expressing cells contribute to the posterior hindlimb (Fig. 3 I, J), dorsal GT (Fig. 3 I, J, inset) and the bladder (Fig. 3 M, N, arrow) with TM induction at E8.5 and E9.5. Induction at E10.5 and E11.5 resulted in labeled cells in both dorsal and ventral GT (Fig. 3 K, L, inset), bladder (Fig. 3 O, P, arrow), and in a restricted portion of the hindlimb (Fig. 3 K, L). TM induction at E11.5 labeled only some cells in the apical region of the bladder (Fig. 3 P). Previously, we reported that aPCM (anterior Peri-Cloacal
Mesenchyme) contributes to the dorsal GT and the mesenchyme of bladder [11]. The aPCM region has been originally described as part of PCM, which is located in the mesenchyme surrounding the anterior part of cloaca [11]. These observations prompted us to examine whether Isl1-expressing cells contribute to the aPCM formation. Thus we induced with TM at E8.5 and E9.5 and analyzed the LacZ labeled cells in Isl1-mER-Cre-mER;R26R embryos at E10.5. Predictably, Isl1-expressing cells were located in the aPCM at E10.5 (Fig. 4 B, C). Furthermore, we detected the labeled cells in the URS (urorectal septum) (Fig. 4 B, C, arrow). These results suggest that Isl1-expressing cells (between E8.5 and E9.5) contribute to the pelvic/urogenital organs, external genitalia, and hindlimb formations. Taken together, coordinated formation of caudal body may be orchestrated by Isl1-expressing cells.

Abnormal aPCM formation of Bmp4 cKO mice

We observed that Bmp4 was expressed in the peri-cloacal regions at E9.5 and thereafter in the aPCM at E10.5 (Fig. 1 C, Fig. 5 A). We also observed active BMP signaling in the aPCM based on anti-pSMAD immunohistochemical analysis (Fig. 5 B). This led us to investigate whether Bmp4 regulates the aPCM formation and to analyze expression of cloacal and aPCM marker genes (Shh, Gli1, Tbx4 and Tbx5) in Bmp4 cKO mice. HH (Hedgehog)-responding cells contribute to both the dorsal GT and the mesenchyme of bladder [11]. Of note, the expression of Shh, a critical ligand of HH signaling for the aPCM formation, was not altered in the cloacal endoderm of Bmp4 cKO mice (Fig. 5 G, I). Gli1 is an indicator of HH signaling and is expressed in the aPCM and mesenchyme of the URS (Fig. 5 E) [11]. Gli1 was expressed in these regions of Bmp4 cKO mice (Fig. 5 F). Tbx4 and Tbx5 are developmental genes for limb initiation and outgrowth. They were normally expressed in the aPCM of wild type (Fig. 5 G, I), but such expression was dramatically reduced in Bmp4 cKO mice (Fig. 5 H, J). In contrast, these expression remained in Shh KO mice (Fig. S1). Contrary to the case of aPCM, the Bmp4 cKO mice showed sustained expression of Tbx4 and Tbx5 in the limb bud (data not shown). BMP signaling was reduced in the mutant aPCM region based on pSMAD immunoreactivity (Fig. 5 K–N).

It has been shown that Isl1 is essential for hindlimb initiation [25]. Although Isl1 is expressed in the aPCM and URS at E10.5 (Fig. 3 G), the function of Isl1 in the development of pelvic/
Sirenomelia patients possess characteristic leg phenotypes including the fusion, abnormal medial rotation and defective skeletal formation of fibula [26]. Pattern formation is an essential process for hindlimb development. Limb patterning is established along three axes: proximal-distal (P-D: hip to toe), anterior–posterior (A-P: 1st to 5th digit; tibia to fibula) and dorsal–ventral (D-V: top versus plantar foot). To analyze the patterning of hindlimb in Bmp4 cKO mice, we performed gene marker analyses of the hindlimb bud. P-D limb development depends on a specialized epithelium at the distal tip of the limb bud, the apical ectodermal ridge (AER) [36–39]. Although Fgf8, the essential AER marker, was expressed normally in the AER, the location of hindlimb bud was closely apposed to one another in the Bmp4 cKO mice (Fig. 7 A, B). Wnt5a is expressed in the distal mesenchyme and essential for the proximo-distal limb outgrowth [40] (Fig. 7 C). Wnt5a expression was detected in the mutant mice in a bowl-shaped pattern at the ventral-caudal midline of the body (Fig. 7 D). The findings on Fgf8 and Wnt5a expression indicate that the P-D axis of the hindlimb bud is maintained in Bmp4 cKO mice. Shh is expressed in the posterior limb bud regulating the A-P patterning of the limb [41–43]. Shh was expressed in the mesenchyme of posterior limb bud of wild type (Fig. 7 E). Bmp4 cKO mice showed shifted location of Shh expression in the midline of the caudal body (Fig. 7 F, red arrow). These observations suggest that posterior hindlimb buds are fused in the Bmp4 cKO mice. However, there were some phenotype variations of Shh expression in the mutants. Some mutants showed the prominent reduction of its expression in the hindlimb bud (data not shown). In accordance with such variable expression pattern, digit phenotype was also variable (data not shown). In fact, several papers suggest various digit phenotypes in human patients of sirenemia [44], [45]. The transcription factor Lmx1b is expressed in the dorsal mesenchyme of the limb bud and is a primary regulator of dorsal limb identity [46], [47]. Its expression was detected in the mesenchyme of mutant mice at the ventral caudal midline of the body (Fig. 7 H, yellow arrow and inset). These results suggest that the hindlimb patterning is basically maintained in Bmp4 cKO mice. Taken together, these results suggest early hindlimb bud grows out normally but rather the midline tissue is missing. Thus the hindlimbs may be fused and abnormally rotated. Abnormalities in the aPCM of Bmp4 cKO mice may lead to such loss of midline structure in the mutant mice subsequently leading to the approximation of hindlimb to the midline with change of the Lmx1b expression. Recent reports indicate that Tbx4 regulates the formation of hindlimb skeletal elements such as fibula [48], [49]. Expression of Tbx4 was reduced in Bmp4 cKO mice (Fig. 7 I, J). These results suggest a possibility that the defective patterning of Tbx4 expression may cause the abnormal fibula formation in the mutant embryos.

**Discussion**

**Bmp4 cKO mice as mouse model of sirenemia with hindlimb fusion and lethal pelvic/urogenital organ aplasia**

We have identified Bmp4 cKO mice as a new mouse model for sirenemia. Unlike previous mouse models, the current model displays all the key phenotypes of sirenemia including hindlimb fusion, dysgenesis of pelvic/urogenital organs and hypoplasia of external genitalia. The kidneys and upper urinary tract are retroperitoneal organs whereas the bladder and urethra are caudal intra-peritoneal organs derived from the cloaca. Our study has
revealed a surprisingly wide range of abnormalities affecting the external genitalia as well as intra-pelvic organs and retroperitoneal organs.

Current tissue lineage analyses suggest that Isl1-expressing cells are essential population of cells for the caudal body formation including pelvic/urogenital organs and hindlimb. We found Isl1-expressing cells contribute to the aPCM formation. Tbx4 and Tbx5 are expressed in the aPCM region. Tbx4 is expressed in the umbilical cord, aPCM and also hindlimb bud. On the other hand, Tbx5 is not expressed in the hindlimb bud and its expression is more restricted in the aPCM. Thus, Tbx5 would be one of the appropriate markers for the aPCM. Bmp4 cKO mice show defective tissue formation such as bladder agenesis and GT hypoplasia, which are derived from the aPCM with reduced Tbx4 and Tbx5 expression. Tbx4-expressing cells contribute to the mesenchyme of the bladder and GT by lineage analysis with Tbx4-Cre R26R mice [50]. These results suggest that the aPCM is not formed in Bmp4 cKO mice. Hence, current observations indicate that the aPCM contains essential progenitors for pelvic/urogenital tissues. Normally, Bmp4 is expressed and BMP signaling is active in the aPCM during caudal body formation. Loss of pSMAD immunoreactivity in the aPCM region of Bmp4 cKO mice indicates that autocrine BMP4 action is required to form the aPCM. To investigate the role of BMP4 in the aPCM formation, we assessed apoptosis and cell proliferation by Tunel and EdU assay, respectively. Both conditions were not altered in Bmp4 cKO mice.

Figure 3. Tissue contribution of Isl1-expressing cells to the caudal body. (A–H) Expression pattern of Isl1 mRNA during caudal body development. (A, E) Isl1 is expressed in the lateral plate mesoderm adjacent to the future hindlimb bud and the base of the allantois at E8.5 (bracket in A and E). (B, F) Isl1 is expressed in the caudal body region and hindlimb bud at E9.5 (square in B). Its expression is detected in the peri-cloacal regions (square in F). (C, G) Isl1 expression is detected in the cloacal region at E10.5 (arrow in C). It is expressed in the URS (arrowhead in G) and cloacal mesenchyme including aPCM (square in G). Isl1 expression in the hindlimb bud is reduced at E10.5. (D, H) Its expression is maintained in the developing GT at E11.5 (arrow in D). Its expression is detected in GT mesenchyme and URS (arrowhead in H). Asterisk indicates cloaca. (I–P) The R26R-lacZ Cre reporter shows LacZ staining of caudal body regions in Isl1-MER-Cre-mER embryos at E15.5 after administration of tamoxifen at E8.5–E11.5. Whole-mount view of stained embryos of hindlimb and external genitalia (I–L) and pelvic organs (M–P). Isl1-expressing cells contribute to the hindlimb, external genitalia and bladder. Insets in I–L are high magnification of GT. Ventral GT is located at the bottom. t, tail; hl, hindlimb bud. doi:10.1371/journal.pone.0043453.g003

Figure 4. Isl1-expressing cells contribute to the aPCM. (A–C) Mid-sagittal sections of cloacal region at E10.5. The square indicates the aPCM (A). The Isl1-expressing cells contribute to the aPCM and URS (B, C). Arrows indicate the URS. u, URS; c, cloaca. doi:10.1371/journal.pone.0043453.g004
mice (data not shown). It has been shown that BMP signaling regulates the supply of mesenchymal cells during gastrulation [51]. Elucidation of another signaling crosstalks with BMP in the developing caudal body will greatly facilitate our understanding of molecular pathogenesis of sirenomelia. Further analysis is necessary to understand the function of BMP4 for the formation of the aPCM.

The current study indicates Bmp4 in the Isl1-expressing cells contribute not only to the aPCM but also in the URS formation. Cloacal septation by the URS is an essential process for the anorectal formation [9]. Previous study shows that Shh signaling is also essential for the anorectal formation. The expression of Shh in the endoderm was remained in Bmp4 cKO mice. These results indicate that BMP signaling is involved for the aPCM formation independently with Shh signaling.

An essentially important pathological feature of sirenomelia is leg fusion. The leg locates in the lateral body wall, which is supported by pelvic skeletons. Previous studies suggest abnormal formation of the leg is derived from the defective midline formation [52], [53]. The current phenotypes are associated with the aplasia of midline structure which is derived from the aPCM region. Although Shh KO mice show defective pelvic/urogenital formation, their mutants do not show the hindlimb fusion [14], [54]. Of note, the expression of the aPCM marker genes such as Tbx4 and Tbx5 remained in Shh KO mice. These observations suggest that proper formation of the aPCM is an essential process for regulating the location of hindlimb development. Although hindlimb bud was located at the ventral caudal midline of the body, the early pattern formation of hindlimb bud was basically not affected in the Bmp4 cKO mice. Taken together, the current results suggest that proper formation of the aPCM by BMP

![Figure 5. Defective aPCM formation of Bmp4 cKO mice.](image)

![Figure 6. Genetic interaction between Bmp4 and Bmp7 during the caudal body formation.](image)
signaling may be an essential process not only for the formation of pelvic/urogenital organs but also for the determination/allocation of hindlimbs bilaterally in the caudal embryos. In this aspect, the ventral midline of the lower body region between bilateral hindlimb bud develops coordinately with hindlimbs. It has been reported that Isl1 is expressed in the caudal lateral plate mesoderm during the caudal body development [55]. Isl1-expressing cells contribute not only to the pelvic/urogenital organs but also to hindlimb. One of the classifications of sirenomelia is the defect of skeletal elements in the leg such as fibula [26]. The limb is derived from two tissues sources, the lateral plate mesoderm and somatic cells. The lateral plate mesoderm of the hindlimb field gives rise to the cartilage, bone, perichondrium, tendons, ligaments, and connective tissues. In contrast, muscle and blood vessels arise from somatic cells that migrate into the limb [56]. Tbx4-expressing cells contribute to the lateral plate-derived tissues, particularly to the bone, tendon, and perichondrium [50]. Tbx4 plays a role in formation of the skeletal elements of the limb such as fibula [48]. In the current study, Bmp4 cKO mice display the defective fibula formation with reduced Tbx4 expression. The decrease of Tbx4 expression in the hindlimb bud by defective BMP4 signaling could be one of the causative factors for the defective skeletal elements in sirenomelia.

**Bmp4 is a major BMP ligand for the caudal body formation**

BMP genes are broadly expressed during embryonic development in a redundant manner [32], [33]. Furthermore, different degree of BMP signaling is required to regulate distinct developmental programs. The current compound genetic analyses of Bmp4 and Bmp7 have suggested a redundancy between BMP genes during caudal development. Bmp7 single KO mice do not show the sirenomelia phenotype. However, Bmp7/Twsg1 double compound mutants display the hindlimb fusion like a sirenomelia [22]. Twsg1 is a one of the modulators of BMP signaling and can act both to promote and inhibit BMP activities. Such observations imply that other BMPs play major roles for the caudal body formation. In the current study, Bmp4 heterozygous and Bmp7 homozygous double compound mutant mice, Hoxa3-Cre;Bmp4flox/+ Bmp7flox/flox, showed not only the hindlimb fusion but also a loss of bladder and anorectal malformations similar to the case of Bmp4 cKO mice. Thus the current series of compound genetic studies suggest that Bmp4 is a major gene and the presence of genetic interaction between Bmp4 and Bmp7 for the caudal body formation. Taken together, Bmp4 cKO mouse is a useful model to elucidate not only pathogenesis of sirenomelia but also to understand the developmental mechanisms of coordinated caudal embryonic formation.

**Materials and Methods**

**Embryo collections of conditional mutant mice**

The Bmp4flox/flox and Bmp7flox/flox conditional mutant alleles employed in this study were described previously [57], [58]. The Isl1-Cre, Isl1-mER-Cre-mER and Hoxa3-Cre, R26R-LacZ indicator mice strains were generated as described previously [24], [27], [28], [31]. For embryonic sampling, pregnant females were sacrificed between E8.5 to E18.5 and the embryos were examined. All animal experiments were approved by the animal study committee of Wakayama Medical University and Kumamoto University School of Medicine (Permit Number: 519 in Wakayama Medical University and A23-066, A23-069 and A23-073 in Kumamoto University). The tamoxifen (TM)-inducible Cre recombinase system removes the floxed sequence from the target genome [59]. TM (Sigma, St Louis, MO, USA) was dissolved in sesame oil at 10 mg/ml. 4 mg or 2 mg of TM per 40 g body weight was used to treat the pregnant mice. Under these conditions, no overt teratologic effects are observed in the hindlimb and pelvic/urogenital organs [11].

**Histology, immunohistochemistry and X-gal staining analysis and skeletal preparation**

The embryonic specimens were fixed overnight in 4% paraformaldehyde (PFA/PBS, dehydrated in methanol and embedded in paraffin. 6 μm serial sections were prepared for Hematoxylin and Eosin (HE) staining and immunohistochemistry. The tissue sections were stained with primary antibodies to pSMAD1/5/8 (Cell signaling) [60]. Immunostaining was visual-
ized using DAB against primary antibodies (WAKO). X-gal staining for the detection of Isl1-expressing cells was performed as previously described [11]. Bones and cartilage of E18.5 fetuses were stained with alizarin red and alcian blue as previously described [61].

In situ hybridization for gene expression analysis

Section in situ hybridization analysis was performed on 8 µm paraffin sections as previously described [62]. Whole-mount in situ hybridization was performed by standard procedures. For in situ hybridization, the following riboprobe templates were used: Bmp4, Shh, Gli1, Isl1, Tbx5, Foxa2, Wnt5a and Lmx1b (kindly provided by B.L. Hogan, N. Ueno, C. Shukunami, C.C. Hui, S. Evans, T. Ogura, TP. Yamaguchi, and RL. Johnson).

Supporting Information

Figure S1 Expression of Tbx4 and Tbx5 in Shh KO mice. (A) SEM (scanning electron microscopy) image of the mouse cloacal region at E10.5. The square indicates the aPCM region. (B, C) Whole-mount ventral view of LacZ-stained embryos of caudal body region. Isl1-expressing cells between E8.5 and E9.5 are located in the posterior mesenchyme of the hindlimb bud and in the aPCM region (B, C). The brackets indicate the aPCM region. (D-G) ISH for the expression of the aPCM marker genes such as Tbx4 and Tbx5. The expression of these genes remains in Shh KO mice at E10.5 (arrow in E and G). c, cloaca; t, tail; hl, hindlimb bud. (TIF)

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Author Contributions

Conceived and designed the experiments: KS GY. Performed the experiments: KS YA SN TN RH. Analyzed the data: KS GY. Contributed reagents/materials/analysis tools: NN MY SE DG AE. Wrote the paper: KS AM GY.

References

1. Garrido-Allegrup C, Haro E, Gonzalez-Lamuno D, Martinez-Frias ML, Bertocchini F, et al. (2011) A clinical and experimental overview of sirenomelia: insight into the mechanisms of congenital limb malformations. Dis Model Mech 4: 289–299.
2. Kallen B, Castilla EE, Lancaster PA, Mutchinick O, Knudsen LB, et al. (1992) The cyclops and the mermaid: an epidemiological study of two types of rare malformation. J Med Genet 29: 30–35.
3. Stevenson RE, Jones KL, Phelan MC, Jones MG, Barr M, et al. (1986) Vascular steal: the pathogenic mechanism producing sirenomelia and associated defects of the viscera and soft tissues. Pediatrics 78: 451–457.
4. Opunji JM, Zarni G, Reynolds JP Jr, Gilbert-Barness E (2002) Defects of blastogenesis. Am J Med Genet 115: 269–266.
5. de Santa Barbara P, Roberts DJ (2002) Tail gut endoderm and gut/entoderm/tail development: a new tissue-specific role for Hoxa13. Development 129: 531–561.
6. de Santa Barbara P, Roberts DJ (2002) Tail gut endoderm and gut/entoderm/tail development: a new tissue-specific role for Hoxa13. Development 129: 531–561.
7. Kimmel SG, Mo R, Hui CC, Kim PC (2000) New mouse models of congenital anorectal malformations. J Pediatr Surg 35: 227–230. discussion 230-231.
8. Pennington EC, Hutson JM (2003) The absence of lateral fusion in cloacal endoderm into hindgut is essential for normal enteric and urogenital development. J Pediatr Surg 38: 1297–1299.
9. Yamada G, Satoh Y, Baskan LS, Cunha GR (2003) Cellular and molecular mechanisms of development of the external genitalia. Differentiation 71: 445–460.
10. Yamada G, Suzuki K, Haraguchi R, Miyagawa S, Satoh Y, et al. (2006) Molecular genetic cascades for external genitalia formation: an emerging organogenesis program. Dev Dyn 235: 1738–1752.
11. Haraguchi R, Motomura Y, Sasaki H, Satoh Y, Miyagawa S, et al. (2007) Molecular analysis of coordinated bladder and urogenital organ formation by Hedgehog signaling. Development 134: 525–535.
12. Frigoletto FD, Harpur J, Lueth K, Dull R, et al. (2001) Unique functions of Sonic hedgehog signaling during external genitalia development. Development 128: 4241–4250.
13. Bertassoni JD, Zeller R (2009) Vertebrate limb development: moving from classical morphogen gradients to an integrated 4-dimensional patterning system. Cold Spring Harb Perspect Biol 1: a001339.
14. Luo G, Hofmann C, Bronckers AL, Sohocki M, Bradley A, et al. (1995) BMP-7 is an inducer of nephrogenesis, and is also required for eye development and skeletal patterning. Genes Dev 9: 2808–2820.
15. Zakin I, Reversade B, Kuroda H, Lyons KM, De Robertis EM (2005) Sirenomelia in BMP7 and Tsg compound mutant mice: requirement for Bmp signaling in the development of ventral posterior mesoderm. Development 132: 2419–2430.
16. Winner G, Blessing M, Labosky PA, Hogan BL (1995) Bone morphogenetic protein-4 is required for mesoderm formation and patterning in the mouse. Genes Dev 9: 2105–2116.
17. Yang L, Cai GL, Liu L, Qiang Y, Chung C, et al. (2006) Isl1Cre reveals a common Bmp pathway in heart and limb development. Development 133: 1575–1585.
18. Kawakami Y, Marit M, Kawakami H, Iou J, Quach T, et al. (2011) Isl1-mediated activation of the beta-catelin pathway is necessary for hindlimb initiation in mice. Development 138: 4463–4473.
19. Stocker JT, Heifetz SA (1987) Sirenomelia. A morphological study of 35 cases and review of the literature. Perspect Pediatr Pathol 10: 7–50.
20. Storino P (1999) Generalized Iaiz expression with the ROSA26 Cre reporter strain. Nat Genet 21: 70–71.
21. Laugwitz KL, Moretti A, Lam J, Gruber P, Chen Y, et al. (2005) Postnatal isl1+ cardioblasts enter fully differentiated cardiomyocyte lineages. Nature 433: 647–653.
22. Park EJ, Sun X, Nichol P, Saipoh Y, Martin JF, et al. (2008) System for tamoxifen-inducible expression of cre-recombinase from the Foxa2 locus in mice. Dev Dyn 237: 447–453.
23. Zuo J, Nakamura E, Nguyen MT, Bao X, Akiyama H, et al. (2008) Uncoupling Sonic hedgehog control of pattern and expansion of the developing limb bud. Dev Cell 14: 624-632.
24. Macatee TL, Hammond BP, Arenkirk BR, Francis L, Frank DU, et al. (2003) Ablation of specific expression domains reveals discrete functions of ectodermal- and endoderm-derived FGF8 during cardiovascular and pharyngeal development. Development 130: 6361–6374.
25. Goncalves A, Zeller R (2011) Genetic analysis reveals an unexpected role of BMP7 in initiation of urinary bud outgrowth in mouse embryos. PLoS ONE 6: e19370.
26. Solloway MJ, Robertson EJ (1999) Early embryonic lethality in Bmp5/Bmp7 double mutant mice suggests functional redundancy within the 60A subgroup. Development 126: 1753–1768.
27. Bandysopadhyay A, Tsuji K, Cox K, Harle BD, Rosen V, et al. (2006) Genetic analysis of the roles of BMP2, BMP4, and BMP7 in limb patterning and skeletogenesis. PLoS Genet 2: e216.
28. Bonilla-Claudio M, Wang J, Bai Y, Klysik E, Selever J, et al. (2012) Bmp signaling regulates a dose-dependent transcriptional program to control facial skeletal development. Development 139: 709–719.
29. Moon AM, Gacepchi MB (2000) Fgf10 is required for outgrowth and patterning of the limbs. Nat Genet 26: 453–459.
37. Benazet JD, Bischofberger M, Tiecke E, Goncalves A, Martin JF, et al. (2009) A self-regulatory system of interlinked signaling feedback loops controls mouse limb patterning. Science 323: 1050–1053.

38. Zakany J, Zacchetti G, Duboule D (2007) Interactions between HOXD and Gli3 genes control the limb apical ectodermal ridge via Fgf10. Dev Biol 306: 483–493.

39. Selever J, Liu W, Lu MF, Behringer RR, Martin JF (2004) Bmp4 in limb bud mesoderm regulates digit pattern by controlling AER development. Dev Biol 276: 268–279.

40. Yamaguchi TP, Bradley A, McMahon AP, Jones S (1999) A Wnt5a pathway underlies outgrowth of multiple structures in the vertebrate embryo. Development 126: 1211–1223.

41. Zeller R, Lopez-Rios J, Zuniga A (2009) Vertebrate limb bud development: moving towards integrative analysis of organogenesis. Nat Rev Genet 10: 845–858.

42. Zakany J, Knitz M, Duboule D (2004) A dual role for Hox genes in limb anterior-posterior asymmetry. Science 304: 1669–1672.

43. Bastida MF, Sheth R, Ros MA (2009) A BMP-Shh negative-feedback loop restricts Shh expression during limb development. Development 136: 3779–3789.

44. Browne M, Finchev P, Adley B, Crawford SE (2004) Sirenomelia with an angiomatous lumbosacral myelocystocele in a full-term infant. J Perinatol 24: 329–331.

45. Taori KB, Mitra K, Ghonga NP, Gandhi RO, Mammen T, et al. (2002) Sirenomelia sequence (mermaid): Report of three cases. Indian J Radiol Imaging 12: 399–401.

46. Chen H, Lun Y, Ovchinnikov D, Kokubo H, Oberg KC, et al. (1998) Limb and kidney defects in Lmx1b mutant mice suggest an involvement of LMX1B in human nail patella syndrome. Nat Genet 19: 51–55.

47. Loomis CA, Harris E, Michaud J, Wurst W, Hanks M, et al. (1996) The mouse Engrailed-1 gene and ventral limb patterning. Nature 382: 360–363.

48. Naiche LA, Papaioannou VE (2007) Tbx4 is not required for hindlimb identity or post-bud hindlimb outgrowth. Development 134: 93–103.

49. Ouimette JF, Jolin ML, L'Honore A, Gifuni A, Drouin J (2010) Divergent transcriptional activities determine limb identity. Nat Commun 1: 35.

50. Ono H, Ohto M, Ohto S, Tanaka K, Yamada G (2007) Cessation of gastrulation is mediated by suppression of epithelial-mesenchymal transition at the ventral ectodermal ridge. Development 134: 4315–4324.

51. Barr M Jr (1988) Comments on “Origin of Abnormality in a Human Simelian Foetus as Elucidated by Our Knowledge of Vertebrate Development”. Teratology 38: 487–491.

52. O'Rahilly R, Muller F (1989) Interpretation of some median anomalies as illustrated by cyclopia and synmelia. Teratology 40: 469–471.

53. Naiche LA, Arora R, Kania A, Lewandoski M, Papaioannou VE (2011) Identity and fate of Tbx4-expressing cells reveal developmental cell fate decisions in the allantois, limb, and external genitalia. Dev Dyn 240: 2290–2300.

54. Ono H, Ohto S, Tachibana K, Tanaka K, Yamada G (2002) Sirenomelia sequence (mermaid): Report of three cases. Indian J Radiol Imaging 12: 399–401.

55. Yuan S, Schoenwolf GC (2006) Id2-like-1 marks the early heart rudiments and is asymmetrically expressed during early rotation of the foregut in the chick embryo. Anat Rec 266: 204–207.

56. Chevallier A, Kieny M, Mauger A (1977) Limb-somite relationship: origin of the limb musculature. J Embryol Exp Morphol 41: 425–428.

57. Yuan S, Schoenwolf GC (2006) Id2-like-1 marks the early heart rudiments and is asymmetrically expressed during early rotation of the foregut in the chick embryo. Anat Rec 266: 204–207.

58. Chevallier A, Kieny M, Mauger A (1977) Limb-somite relationship: origin of the limb musculature. J Embryol Exp Morphol 41: 425–428.

59. Yuan S, Schoenwolf GC (2006) Id2-like-1 marks the early heart rudiments and is asymmetrically expressed during early rotation of the foregut in the chick embryo. Anat Rec 266: 204–207.

60. Chevallier A, Kieny M, Mauger A (1977) Limb-somite relationship: origin of the limb musculature. J Embryol Exp Morphol 41: 425–428.