The transformation suppressor gene Reck is required for postaxial patterning in mouse forelimbs

Mako Yamamoto1-2, Tomoko Matsuzaki1, Rei Takahashi3, Eijiro Adachi4, Yasuhiro Maeda1, Sachiyu Yamaguchi1-2, Hitoshi Kitayama1, Michiko Echizenya1, Yoko Morikoa1, David B. Alexander5, Takeshi Yagi6, Shigeyoshi Itohara7, Takashi Nakamura8, Haruhiko Akiyama8 and Makoto Noda1,*

1Department of Molecular Oncology, 2Global COE Program and 6Department of Orthopaedic and Musculoskeletal Surgery, Kyoto University Graduate School of Medicine, Sakyo-ku, Kyoto 606-8501, Japan
2Department of Pharmacotherapeutics, Faculty of Pharmaceutical Sciences, Doshisha Women’s College of Liberal Arts, Kodo, Kyotanabe, Kyoto 610-0395, Japan
3Department of Matrix Biology and Regenerative Medicine, Kitasato University Graduate School of Medical Science, 1-15-1 Kitasato, Minami-ku, Sagamihara, Kanagawa 252-0373, Japan
4Department of Molecular Toxicology, Nagoya City University Graduate School of Medical Sciences, 1 Kawasumi, Mizuho-cho, Mizuho-ku, Nagoya 467-8601, Japan
5KOKORO-biology group, Laboratories for Integrated Biology, Graduate School of Frontier Biosciences, Osaka University 1-3 Yamadaoka, Suita, Osaka 565-0871, Japan
6Laboratory for Behavioral Genetics, RIKEN Brain Science Institute, 2-1 Hirosawa, Wako, Saitama 351-0198, Japan

*Author for correspondence (mnoda@virus.kyoto-u.ac.jp)

Summary

The membrane-anchored metalloproteinase-regulator RECK has been characterized as a tumor suppressor. Here we report that mice with reduced Reck-expression show limb abnormalities including right-dominant, forelimb-specific defects in postaxial skeletal elements. The forelimb buds of low-Reck mutants have an altered dorsal ectoderm with reduced Wnt7a and Igf2 expression, and hypotrophy in two signaling centers (i.e., ZPA and AER) that are essential for limb outgrowth and patterning. Reck is abundantly expressed in the anterior mesenchyme in normal limb buds; mesenchyme-specific Reck inactivation recapitulates the low-Reck phenotype; and some teratogens downregulate Reck in mesenchymal cells. Our findings illustrate a role for Reck in the mesenchymal-epithelial interactions essential for mammalian development.

Introduction

Vertebrate limb patterning is controlled by two signaling centers, the zone of polarizing activity (ZPA) in the distal-posterior mesenchyme and the apical ectodermal ridge (AER) at the distal tip of the limb bud. The ZPA and AER produce signaling molecules (Shh and FgfS, respectively) that control the formation of anterior-posterior and proximal-distal limb axes in a cooperative manner (Saunders, 1948; Johnson and Tabin, 1997; Tabin and Wolpert, 2007; Mariani et al., 2008; Towers and Tickle, 2009; Zeller et al., 2009). Another signaling molecule, Wnt7a, produced by the limb bud dorsal ectoderm (DE) controls the formation of the dorsal-ventral limb axis and activates Shh-expression in the ZPA, thereby influencing anterior-posterior limb patterning as well (Parr and McMahon, 1994; Riddle et al., 1995; Yang and Niswander, 1995).

RECK is a membrane-anchored glycoprotein of 125 kDa which forms dimers and regulates a wide variety of extracellular metalloproteinases, including several MMP family members (e.g., MMP7, 9, and 14), ADAM10, and CD13 (Takahashi et al., 1998; Oh et al., 2001; Miki et al., 2007; Muraguchi et al., 2007; Omura et al., 2009). RECK is down-regulated in various cancers (Noda and Takahashi, 2007), and restored RECK expression in cancer cells suppresses their malignant behavior (Takahashi et al., 1998; Oh et al., 2001), suggesting the potential importance of RECK as a prognostic indicator and therapeutic effector.

Reck-deficient mice die around embryonic day 10.5 (E10.5) with reduced tissue integrity and defects in vascular and neural development (Oh et al., 2001; Muraguchi et al., 2007; Chandana et al., 2010), suggesting its importance in relatively early events in mammalian development. The embryonic lethality of the conventional Reck-deficient mice, however, limits our understanding of its roles in the later stages of development and adult homeostasis. In this study, we employed hypomorphic and conditional Reck mutant mice to circumvent this limitation and found an essential role for Reck in forelimb development.

Materials and Methods

Mice

All the animal experiments were approved by the Animal Research Committee, Graduate School of Medicine, Kyoto University and conducted according to the guidelines of Kyoto University. The mouse carrying the Reck allele (Acc. No. CDB0488K; http://www.cdb.riken.jp/arg/mutant%20mice%20list.html) was described elsewhere as an intermediate for obtaining the R2 allele (Chandana et al., 2010). The founder mouse was back-crossed four times with C57BL/6 mice before intercrossing with mice heterozygous for the Reck-null (R1.1) allele (Fig. 1A (3)) to yield Rlow/- (low-Reck) and Rlow/+ (control) mice. To generate mice...
carrying the R1 allele (Fig. 1A (4)), a targeting vector (pRCKO-2, 13.85 kb) containing 6 components [from 5’ to 3’; (1) 3.9 kb 3’-arm, (2) loxp site, (3) 0.99 kb exon 1-containing fragment, (4) PGK-neo cassette flanked by two loxp sites, (5) 1.9 kb 3’-arm, and (6) DT-A gene] was constructed using two vectors, loxp3-Neo and pMC1DTpA (Taniguchi et al., 1997), and a genomic DNA clone previously described (Sasahara et al., 1999). A mutant ES clone isolated after electroporation of linearized pRCKO-2 into E14 ES cells was used to produce a transgenic line following established protocols (Gomi et al., 1995), and the line was subsequently crossed with EIIa-Cre transgenic mice (Lako et al., 1996) to eliminate the PGK-neo fragment alone or to eliminate the fragment together with exon 1 to obtain the alleles R1/ and R1, respectively (supplementary material Fig. S1). The R1 allele contains two loxp sequences at the BssH1 and HindIII sites that flank Reck exon 1, and the R1 allele lacks the sequence between the two loxp sequences. The founder mice were back-crossed six times with C57BL/6 mice before intercrossing with various Cre-driver mice. DNA from adult mouse tail or embryonic yolk sac was used for genotyping by PCR under the conditions described in supplementary material Table S5. The Cre reporter mouse R26b was a gift from P. Soriano (Soriano, 1999). The Prxl-Cre (Logan et al., 2002) and Col2al-Cre (Ovchinnikov et al., 2000) transgenic mice were maintained and genotyped as previously described (Akiyama et al., 2002). The Reck-CreER allele contains the CreER coding sequence in-frame after exon 7 in Reck (T.M. et al., unpublished). In timed mating, the noon of the day of a vaginal plug was considered as E0.5.

Morphological examinations
Skeletal samples were stained with Alcian Blue 8GX (Chroma-Gesellschaft, 1A288) and Alizarin Red S (Chroma-Gesellschaft, 1F583) as previously described (McLeod, 1980). For scanning electron microscopy, E11.5 embryos (n=4 for each genotype) were immersed in Zamboni’s fixative for 12 h, and cut into two halves along the sagittal plane to identify right and left limb buds. The samples were post-osmificated, dehydrated, and immersed in t-butyl alcohol. They were then freeze-dried, coated with platinum, and examined using a scanning electron microscope (JSM 6360, JEOL, Tokyo, Japan). Ten images were recorded from each embryo. For histochemical analysis by light microscopy, tissue blocks were fixed in 4% PFA, embedded in paraffin, and sliced into 5 μm-thick sections which were subjected to hematoxylin and eosin (HE) staining following standard protocols. In situ TUNEL assay using ApopTag Plus Peroxidase In Situ Apoptosis Detection Kit (Chemicon, S7101) was performed as described previously (Smith and Cartwright, 1997). Whole-mount in situ hybridization (WISH) using digoxygenin-labeled RNA probes was performed as described previously (Conlon and Rossant, 1992; Parr and McMahon, 1994; Akiyama et al., 2002). To detect Reck mRNA, the 1092-bp Hind III fragment from a mouse Reck cdNA (Accession, NM_016678; position, 1669–2760) was used to generate an antisense ribo-probe. At least 3 embryos per group were examined, and the features common to the majority of samples were recorded. Experiments were repeated at least twice to confirm reproducibility.

Analyses of proteins and RNAs
Immunoblot assays using antibodies against Reck (5B11D12), Gapdh (Ambion, AM4300), and β-tubulin (Calbiochem, CP09) were performed as described previously (Oh et al., 2001). Densitometry was performed using ImageJ software. For qRT-PCR, total RNA was isolated from mouse tissues or cell lines using Trizol (Ambion) and subjected to qRT–PCR as described previously (Hatta et al., 2009) with additional primers newly designed for Wnt7a and Igf2 (supplementary material Table S5).

Cell culture
ATDC5 cells and their transfectants have been described previously (Atsumi et al., 1990; Shukunami et al., 1996; Kondo et al., 2007). MKE cells derived from newborn mouse kidney were maintained in chemically defined medium (T.M. et al., unpublished). Sources of cytokines and teratogens used to study their effects on gene expression in these cells: Shh (R & D Systems, 464-SH), Wnt7a (R & D Systems, 3008-WN), FgF8 (R & D Systems, 423-F8), IgF2 (R & D Systems, 792-MG), GcGl (nacalai tesque, 06613), acetazolamide (Sigma, A6011), thalidomide (Sigma, T144), and ethanol (nacalai tesque, 14713).

Fate mapping
R26R/R26R female mice were crossed with male mice carrying one Reck-CreERT2 allele, and 2 mg tamoxifen (TMX; Sigma, T5648; 20 mg/ml-corn oil) was injected intraperitoneally into the pregnant mice at a time point between post-coital day 7.5 and 11.5. The embryos were harvested at a time point between E10.5 and E12.5 and subjected to X-gal staining as described previously (Ahn and Joyner, 2004). To investigate the fates of Reck-expressing cells in the mutant mice, R1/;R1/;R26R/R26R female mice were mated with male mice with the genotype Reck-CreERT2;R26R/R26R, and the pregnant mice were treated with TMX. Offspring with the genotype of R1/; Reck-CreERT2;R26R/+ were analyzed.

Transcriptome analyses
ATDC5 cells stably transfected with a control vector (LXSN) or the vector expressing human Reck (Kondo et al., 2007) were plated at 6.5 x 10⁵ cells per 100-mm dish and cultured for 3 days. Total RNA extracted using RNeasy Kit

Fig. 1. Dermal and skeletal abnormalities found in low-Reck mutant mice. (A) Schematic representation of the 5’-terminal region of the five Reck alleles used in this study. (B) Immunoblot detection of Reck protein in whole E12.5 embryos. Each lane in the upper panels represents one embryo of the indicated genotype. Gapdh was used as a loading control. The lower bar graph summarizes the densitometry results (mean±SEM). (C) Body weights of control (R1/; n=12) and low-Reck mutant (R1/; n=6) mice at three time points after birth. (D) Gross morphology of typical cutaneous horns which developed on the dorsal surface of autopods in 4-week old R1/ mice. Scale bar, 1 mm. (E) Sections of cutaneous horn tissues stained with Haematoxylin and Eosin. Scale bar, 300 μm. (F) Dorsal views of a typical R1/+ (control) mouse and a R1/; mouse (homozygous mutant) mouse at day 3 after birth. Scale bar, 5 mm. (G) Skeletal morphology of the right forelimbs (panels 1-3 and 5-14) and fingertips (panels 4 and 15) of neonatal mice. One control mouse (R1/; panels 1-4) and four hypomorphic mutant mice with different degrees of severity (R1/; panels 5-15) are compared. Scale bars: mm in panels 1, 5, 7, 9, 12; 700 μm in panels 2, 3, 6, 8, 10, 11, 13, 14; 150 μm in panels 4, 15. The mutant sample shown in panels 12-14 has digit I, although this digit is unclear in these photographs. (H) The left-right asymmetry of the skeletal phenotype in R1/ mice. The number of animals (total 29) in which the indicated digits or zeugopod bones are affected (smaller and/or bent) or deleted was scored. No defects were found in the hindlimbs of these animals (data not shown). In contrast, cutaneous horns (D, E) and round porous fingertip bones (G, panel 15) are found in all extremities.
Biology Open

Results

Hypomorphic Reck mutant mice show limb abnormalities

To understand the roles of Reck in late-stage development (after E10.5) and adult homeostasis, we established several lines of Reck mutant mice (Fig. 1A). One of the intermediate products, which retains the selection marker PGK-neo in front of exon 2 (Fig. 1A (2)) (Chandana et al., 2010), was found to express Reck protein at less than one half the level of the wild type allele (Fig. 1B bar 4); we therefore call this allele R_{Low}, and animals hemizygous for this allele (R_{Low/-}) low-Reck mutants. Low-Reck mutants and their heterozygous littermates (R_{Low/+}; control) are comparable in their body weights (Fig. 1C). The low-Reck mutants, however, have remarkable limb abnormalities; they have multiple cutaneous horns on the dorsal side of their extremities (Fig. 1D,E) and, in some cases, one or both of their forelimbs are pointing backwards (Fig. 1F red arrow). Skeletal preparations of newborn low-Reck mutants show loss of postaxial forelimb structures with variable severity (Fig. 1G); forelimbs pointing backwards represent the absence of ulna (Fig. 1G panels 11 and 14). The skeletal phenotype tends to be more severe in the right forelimb (Fig. 1H). In contrast, the cutaneous horns occur in all limbs, albeit in varying severity, with almost 100% penetrance; the lesions in the forelimbs tend to be longer and pointed as compared to the lesions in the hindlimbs. In addition, fingertip bones are round and porous in all limbs of low-Reck mutants (Fig. 1G panel 15).

Reduced Reck expression affects some early events in forelimb development

Abnormal morphology of the forelimb buds of low-Reck mutants is evident as early as E12.5: the posterior side is hypoplastic (Fig. 2A panel 2 red arrow) and shows poor chondrocyte condensation (blue signals). Slices of mutant limb buds show altered cytoarchitecture, e.g., increased cell density in the central area and uneven, spongy distribution of cells in the marginal areas (Fig. 2B panel 2). Increased apoptosis, however, was not detectable around the hypoplastic area (Fig. 2C arrow). Forelimb bud hypoplasia can also be observed in scanning electron micrographs (SEM), especially in the posterior region of the mutant’s forelimbs (Fig. 2D panels 2 and 4 arrow). Interestingly, the AER, which is robust in the control samples (Fig. 2D panels 5 and 7 bracket), is flattened in the low-Reck mutants (Fig. 2D panels 6 and 8 arrows). Also, the DE cells in the mutant limb buds have a distinctly altered appearance (Fig. 2D panel 10).

Finally, the underlying mesenchymal cells and the ECM appear to have decreased integrity compared to those underlying the DE of the control limb buds (Fig. 2D panels 12 and 11; supplementary material Fig. S2).

Our previous study implicated Reck in chondrocyte differentiation in vitro (Kondo et al., 2007). To test whether reduced Reck expression in developing cartilage is responsible for the skeletal phenotype of low-Reck mice, we generated mutant mice (R1/-;Col2a1-Cre) in which Reck is inactivated selectively in the late-stage mesenchymal cells committed to the chondrocyte lineage. These mice, however, show no obvious defects in limb patterning (supplementary material Fig. S3).

We also generated another mutant line (R1/-;Prx1-Cre) in which Reck is inactivated in undifferentiated limb mesenchyme; these mice show skeletal defects (Fig. 2E) reminiscent of those in R_{Low/-} mice (Fig. 1G). Hence, Reck in undifferentiated limb mesenchyme seems to be critical for proper forelimb development.

Fates of Reck-positive cells in normal limb buds

How can we explain the abnormal cytoarchitecture found in the mutant forelimb buds (Fig. 2B)? To address this issue, we analyzed the fates of Reck-positive cells in mice carrying a Reck-CreER^{T2} allele (Fig. 1A (5)) and the ROSA26 reporter system (Soriano, 1999). Although the resulting mice (i.e., +/ Reck-CreER^{T2}, R26R) are normal in phenotype, the cells expressing Reck can be tagged by injecting tamoxifen (TMX) into the animal and staining with X-gal. In our first set of experiments, we injected TMX into pregnant mice at different time points and stained the embryos at E12.5 (Fig. 3A). Interestingly, the proximo-anterior regions of four limbs are among the most prominent areas of staining under these conditions. We also found that labeled cells were most abundant and widely distributed when TMX was injected at E8.5 (Fig. 3A panels 3 and 4); interestingly, the signals tended to be stronger in the hind- and left limbs (for expanded views, see supplementary material Fig. S4).

![Fig. 2. Morphology of forelimbs in hypomorphic or conditional Reck mutant mice. (A-D) R_{Low/+} (left panels) and R_{Low/-} (right panels) littermates were harvested at the indicated stages and analyzed by whole-mount Alcian Blue-staining (A), HE-staining of limb sections (B), whole-mount in situ TUNEL assay (C), and SEM (D). Arrows indicate hypoplasia. Bracket in D indicates robust AER. Scale bar: 200 μm in A and B; 100 μm in C and D panels 1–6; 10 μm in D panels 7, 8; 5 μm in D panels 9–12. Orientation: a, anterior; p, posterior. (E) Effects of selective Reck inactivation in early limb mesenchyme. Skeletal preparations of neonatal R1/- (Cre-negative control) and R1/-;Prx1-Cre mice are compared. Arrows indicate postaxial skeletal defects reminiscent of those in R_{Low/-} mice. Scale bar, 500 μm.](image-url)
In our second set of experiments, we injected TMX at E8.5 and stained the embryos at different time points (Fig. 3B). At E10.5, the Reck-positive cells were most abundant in the proximo-anterior region, forming a gradient toward the distal-posterior margin (Fig. 3B panels 1 and 2). At the later time points, the labeled cells were increased in number and distributed more widely along the anterior edge of the limb bud (Fig. 3B panels 3–6). In the dorsal view (Fig. 3C) and in cross sections of limb buds (Fig. 3D), signals are predominantly found in the mesenchymal tissue. Taken together, these findings suggest that the Reck-positive mesenchymal cells present in the proximo-anterior region of the limb buds at around E9 proliferate and spread toward the distal end.

Fates of Reck-positive cells in the mutant limb buds

Next, to ask whether Reck hypomorphism has any effect on mesenchymal cell behavior, we took advantage of the Reck-CreER<sup>T2</sup> allele which is null in terms of Reck gene function (Fig. 1A (5)). Namely, R<sub>Low</sub>/Reck-CreER<sup>T2</sup>;R26R mice (labeled R<sub>Low</sub> in Fig. 4) are low-Reck mutants which can be used to trace the fate of Reck-positive cells (detected as LacZ<sup>+</sup> cells). When these mice were treated with TMX at E8.5 and stained at E10.5, LacZ<sup>+</sup> cells were fewer and located more centrally in the limb bud (Fig. 4A panels 3 and 4) as compared to the control (+/Reck-CreER<sup>T2</sup>;R26R) (Fig. 4A panels 1 and 2). One day later (i.e., embryos stained at E11.5), the number of LacZ<sup>+</sup> cells increased, but their distribution was abnormal in the mutants: compared to the control, small clusters of LacZ<sup>+</sup> cells were scattered more widely and to the posterior side, and the signals tended to be less dense in the distal-anterior region (Fig. 4B panels 5–8). Hence, Reck hypomorphism has a significant impact on the spatial distribution of Reck-positive cells.

Signaling from the DE, ZPA, and AER is attenuated in the low-Reck mutant limb buds

To obtain more information regarding the mechanism of low-Reck limb abnormalities, we examined the expression of several genes involved in limb patterning in the mutant (R<sub>Low</sub><sup>−/−</sup>) and control (R<sub>Low</sub><sup>+/+</sup>) mouse embryos by whole-mount in situ hybridization (WISH). Known functional interections among the products of these genes during limb development are summarized in supplementary material Fig. S5 (Parr and McMahon, 1994; Riddle et al., 1995; Yang and Niswander, 1995; Johnson and Tabin, 1997; Niswander, 2003; McGlenn and Tabin, 2006; Tickle, 2006; Zeller et al., 2009). We found that the Lmx1b-positive area expanded and mis-located in the mutant forelimb buds (Fig. 5A panels 2, 4 and 6; supplementary material Fig. S6A panels 2 and 4). Of note, a similar phenotype has been found in mice with reduced Wnt7a-signaling (Adamska et al., 2005).

At E10.5, Shh expression is detectable but somewhat weaker than the control in the mutant right forelimb buds (Fig. 5B panel 2; supplementary material Fig. S6A panels 5 and 6). At E11.5, the area normally positive for Shh (i.e., the ZPA) is partially missing in the mutants (Fig. 5C panel 2). In addition, direct targets of Shh-signaling, e.g., Pth1 and Gli1, are also downregulated in mutant samples (Fig. 5B panel 10, Fig. 5C panel 4). Signals of another Shh target, Grem1, are decreased at E10.5 in mutant right forelimbs (Fig. 5B panel 6); at E11.5 the Grem1 signals are more aggregated in the central region than those in the control samples (Fig. 5C panel 6 vs. panel 5).

Two Fgf family genes (Fgf8 and Fgf4) expressed in the AER are downregulated in mutant samples at E10.5 (Fig. 5B panels 8 and 12); at E11.5, the AER (marked by Fgf8) is often truncated on both termini (anterior and posterior) and sometimes discontinuous near the posterior end (Fig. 5C panel 8; supplementary material Fig. S6B panels 3 and 4). Again, a similar AER truncation has been found in mice with reduced Wnt7a-signaling (Adamska et al., 2004). Hence, although these
expression of Shh, Lmx1b, and Fgf8 was also detected in these mice (Fig. 5D), further supporting the idea that reduced Reck expression in the early limb mesenchyme is responsible for the postaxial defects found in the forelimbs of low-Reck mutants.

Distribution of Reck and Wnt7a mRNA in E11.5 right forelimb buds

To verify the Reck distribution visualized in the fate-mapping study, we tried to directly detect Reck mRNA in E11.5 embryos by WISH (Fig. 5C panels 9 and 10), using a newly designed probe. In the control embryos, the Reck-positive areas overlapped with, but were somewhat larger than, the areas detected with the Reck-CreERT2;R26R system (compare Fig. 5C panel 9 to Fig. 4B panels 1 and 2). As expected, Reck signals were weaker in low-Reck embryos than in the control embryos (Fig. 5C panels 10 and 9), particularly in their right forelimbs (supplementary material Fig. S6C panel 4).

We also tried to visualize Wnt7a mRNA by WISH (Fig. 5C panels 11 and 12). Of note, there was considerable overlap between the areas of abundant Wnt7a signals and the Reck-positive areas in the forelimb buds in control animals, especially in their anterior region (compare Fig. 5C panel 9 and panel 11; supplementary material Fig. S7A). Importantly, Wnt7a signals were consistently lower in the mutant forelimb buds as compared to the control samples (Fig. 5C panels 12 and 11), especially in their right forelimbs (supplementary material Fig. S6D panel 4). These findings support the idea that Reck is required for the maintenance of Wnt7a production in the limb tissue.

Does Reck expressed in the limb mesenchyme play any inductive role in limb patterning? To test this model, we tried to ectopically express Reck in early limb mesenchyme (supplementary material Fig. S8A). A mouse expressing the Reck transgene in a posterior domain of its forelimb, however, showed no obvious abnormality in limb patterning (supplementary material Fig. S8B panel 2), suggesting that the action of Reck in this system may be supportive rather than inductive.

Gene expression in vitro

To better understand how Reck works in the limb tissues, we analyzed the effects of RECK over-expression on the gene expression profile of a chondrogenic cell line, ATDC5, which shares many properties with undifferentiated mesenchyme (Atsumi et al., 1990; Shukunami et al., 1996). RECK up-regulates genes involved in cell adhesion and the cell cycle while it down-regulates genes involved in pathways in cancer, focal adhesion, MAP-kinase signaling, regulation of the actin cytoskeleton, and chemokine signaling (supplementary material Tables S1 and S2; summarized in Fig. 6A). In addition, prominent induction of Igf2 (supplementary material Table S3) and down-regulation of several genes involved in cytokine/chemokine signaling (supplementary material Table S4) were also detected. On the other hand, Wnt7a mRNA was undetectable even after RECK expression in this mesenchymal cell line (data not shown), making it less likely that Reck induces Wnt7a-expression directly in the limb mesenchyme.

Igf2 is an insulin-related growth factor essential for normal fetal growth and development (DeChiara et al., 1990). Indeed, Igf2 mRNA is abundant in the right forelimb tissues from the control embryos at E12.5 but less abundant in the samples collected from low-Reck mice (Fig. 6C bottom panel bars 3 and
To test the simple model that Igf2 (probably produced by mesenchymal cells) promotes Wnt7a expression in epithelial cells, we used a mouse epithelial cell line, MKE, that does not express Igf2. We found, however, that no significant changes in the level of Wnt7a mRNA were detectable after Igf2-treatment of MKE cells (Fig. 6B bar 2). Instead, we found that treatment with exogenous WNT7A resulted in a dramatic down-regulation of endogenous Wnt7a mRNA (Fig. 6B bar 3), demonstrating tight, negative feedback regulation of Wnt7a expression in these cells.

We also analyzed the effects of several signaling molecules on Reck-expression in ATDC5 cells (Fig. 6D). Since our previous study with fibroblasts indicated that the level of Reck-expression is under the strong influence of cell density (Hatta et al., 2009), we treated the cells at two different cell densities. Reck was upregulated by WNT7A, Fgf8, and Igf2 (Fig. 6D bars 6, 8 and 10) but not by Shh (Fig. 6D bar 4).

Effects of teratogens on Reck-expression
Several teratogens induce right-dominant, postaxial ectrodactyly in mouse forelimbs (i.e., an almost perfect phenocopy of the RLow/- mutant) when the animals are exposed to these agents around E9.5 (Bell et al., 2005; Schreiner et al., 2009). Interestingly, some of the teratogens, e.g., cadmium and ethanol, down-regulate endogenous Reck in ATDC5 cells (Fig. 6E), implicating Reck as a target of these teratogens.

Discussion
For elucidating the physiological functions of Reck, conventional Reck-knockout mice have been of limited use, since they show embryonic lethality around E10.5 and provide little information on the functions of Reck in late-stage development and in the adult. One way to circumvent this obstacle would be to use mice with reduced Reck expression. The phenotypes of such animals may reveal biological processes heavily dependent on RECK. In the case of low-Reck mutants, the most obvious phenotype appears in their extremities (Fig. 1), and this is consistent with our finding that in mid-gestation embryos, limb buds are among the regions where Reck-positive cell are particularly abundant (Fig. 3). The limb phenotype may also reflect the lack of redundancy or the vulnerability in molecular circuitry regulating some critical step in limb development. We therefore decided to characterize these Reck hypomorphic mutant mice in detail, hoping to obtain fresh insight into the functions of Reck in vivo.

Morphological features found in the right forelimb buds of low-Reck mutants include (i) aberrant surface epithelium, (ii) hypoplastic distal-posterior margin, and (iii) a less well developed AER which is often discontinuous in its posterior region (Fig. 2D, Fig. 5C panel 8). These and other data (e.g., Fig. 5; see below) suggest that the Reck hypomorphic mice have defects in both the structure and function of three signaling centers, DE (producing Wnt7a), ZPA (producing Shh), and AER (producing Fgfs), essential for limb growth and patterning. Limb pattern abnormalities reminiscent of those in low-Reck mice have
been found in at least three systems: (i) mice with Wnt7a-deficiency either alone (Parr and McMahon, 1994) or in combination with Lrp6\textsuperscript{TM} mutation (Adamska et al., 2005), (ii) mice overexpressing a Wnt-signaling antagonist, SOST (Collette et al., 2010), and (iii) mice treated with certain teratogens (see below). On the other hand, mutations in the other signaling molecules or transcription factors involved in limb patterning, such as Shh (Chiang et al., 1996; Zhu et al., 2008), Lmx1b (Chen et al., 1998), Enl (Wurst et al., 1994), Grem1 (Michos et al., 2004), Bmp4 (Selever et al., 2004), and Fgf4/8 (Mariani et al., 2008), yield phenotypes distinct from those of low-Reck mice. Genetic evidence therefore suggests that deficiency in Wnt7a signaling is likely to be the immediate consequence of Reck-hypomorphism.

Our data in cultured cells (Fig. 6B) suggest the presence of an intrinsic negative feedback regulation that would allow homeostatic expression of Wnt7a from epithelial cells (Fig. 7C). In such a system, substantial reduction in Wnt7a expression (Fig. 5C panel 12, Fig. 5E; supplementary material Fig. S6D) is likely to result from a loss or malfunctioning of its source (i.e., limb bud DE). In the chicken system, removal of wing bud DE leads to the loss of Shh expression in the ZPA and the eventual loss of posterior components of the wing (Yang and Niswander, 1995), highlighting the importance of an intact DE for posterior morphogenesis in the limb. The aberrant DE in R\textsubscript{Low}/- limb buds (Fig. 2D; supplementary material Fig. S2) suggest that Reck is important for the health of limb bud DE and hence, for a continuous supply of Wnt7a.

Several lines of evidence indicate that Reck expressed in the mesenchyme, rather than DE, is critical for forelimb development. First, Reck expression is detectable in cultured mesenchymal cells (Fig. 6C,D) but not in epithelial cells (transcriptome data, not shown). Second, in our fate-mapping studies, most Reck-reporter signals are found in the mesenchyme (Fig. 3C,D). Third and most importantly, selective inactivation of Reck in early mesenchyme (using the Prx1-Cre system) was sufficient to induce postaxial limb abnormalities (Fig. 2E). These findings imply a non-cell-autonomous nature for Reck activity in this system.

How do the Reck-positive mesenchymal cells support the health of DE? Our fate-mapping studies indicate that Reck-positive cells are abundant in the anterior mesenchyme (AM) in the limb bud of mid-gestation embryos (Fig. 3). Previous studies in vitro demonstrated that Reck is critical for stable cell-substrate adhesion and, slow but persistent, directional migration of fibroblasts (Morioka et al., 2009). Some features of mutant limbs, such as distalo-posterior (i.e., ZPA) hypotrophy (Fig. 2), altered cytoarchitecture (Fig. 2B panel 2), flattened AER (Fig. 2D panels 6 and 8), and abnormal distribution of Reck-positive cells (Fig. 4) may well be explained by the failure of the cells to migrate properly under reduced Reck expression. It is, however, unclear whether the apparent mis-behavior and abnormal distribution of the cells are causative to the limb defects found in low-Reck mice. In fact, several lines of evidence seem to go against this possibility. First, in the fate mapping study, very few Reck-positive cells were found near the distalo-posterior domain in the wild type embryos at E11.5 (Fig. 4B panels 1–4); by this time, tissue hypotrophy is already evident in low-Reck mutants. Second, the posterior Reck-positive domain detected by WISH (see below) does not coincide with the Shh-positive region (i.e., ZPA; supplementary material Fig. S7B). Third, as alluded to above, the eventual limb phenotype of low-Reck mice is quite distinct from that of Shh-deficient mice [loss of many skeletal elements, resulting in the limb consisting of a stylopod, a single reduced zeugopod element, and a single reduced digit (Chiang et al., 2001; Kraus et al., 2001)] or a series of Fgf-deficient mice [loss of antero-distal elements to complete failure of limb formation (Sun et al., 2002; Mariani et al., 2008)]. We therefore speculate that ZPA hypotrophy and AER flattening may not represent the immediate consequence of Reck-hypomorphism.

In contrast, the Wnt7a-positive areas and Reck-positive areas largely overlap in the dorsal views of the control limb buds (Fig. 5C panels 9 and 11; supplementary material Fig. S7A), and the level of Wnt7a mRNA is greatly reduced in the mutant right forelimb buds (Fig. 5C panel 12; supplementary material Fig. S6D). These data, together with the genetic, morphological, and biochemical evidence discussed above, support a model that Reck expression in the underlying mesenchyme helps maintain the health of the DE and its production of Wnt7a, thus promoting subsequent tissue interactions required for the establishment of a robust AER and ZPA (Fig. 7A,B).

A previous study on the developing central nervous system in the mouse revealed a role for Reck in suppressing neuronal...
differentiation and expanding the neural precursor pool (Muraguchi et al., 2007). This mode of action fits with our findings that the mutant limb buds show hypotrophy without apoptosis (Fig. 2) and that fewer cells were labeled in the mutant limb buds in the fate-mapping studies (Fig. 4).

Unlike the fate-mapping data (Fig. 3), our WISH data showed strong Reck signals on the posterior side (Fig. 5C panel 9 green arrowhead; compare this panel with Fig. 4B panels 1 and 2), in addition to the AM. Whether this represents aberrant cross-reaction in hybridization probe, incomplete fidelity of Reck-CreER\textsuperscript{2} expression, post-transcriptional regulation, or is due to some other reason needs to be clarified in future studies. Nevertheless, the expression of Reck in the AM is consistent between the two techniques.

What would be the mediator(s) of the tissue interactions discussed above? Our transcriptome data indicate that Reck can alter the levels of a cohort of mRNAs in mesenchymal cells (supplementary material Tables S1–S4) and that Igf2 stands out among the up-regulated genes (supplementary material Table S3). Of note, Igf2 expression is quite high (\(\sim 200\)-folds of that in ATDC5 cells) in the right forelimb tissue of E12.5 control mice, while it is reduced to \(\sim 70\%\) in the mutant samples (Fig. 6C). The gene for Igf2 receptor, Igf1r, is expressed in both mesenchymal (ATDC5) and epithelial (MKE) cell lines, but the genes for its ligands (Igf1 and Igf2) are expressed only in the mesenchymal cell line (data not shown). Enhancement of Igf2 expression by Reck, probably in the mesenchyme, should have a major impact on the growth and survival of both epithelial and mesenchymal cells in developing limbs, although the exact extent of the contribution of Igf2 to the present phenomenon remains to be tested in future studies. Reck also modifies the expression of several components involved in cytokine signaling (supplementary material Tables S3 and S4); possible contributions of these molecules to the health of limb tissues also need to be tested. The exact mechanism by which Reck, a GPI-anchored protein devoid of a cytoplasmic domain, affects gene expression remains presently unclear; a plausible mechanism is that Reck interferes with the cleavage or interaction of cell surface or peri-cellular proteins involved in intracellular signaling that impinge on gene expression.

Igf2, WNT7A, and Fgf8 upregulate Reck in ATDC5 cells (Fig. 6C), suggesting the presence of three positive feedback loops (i.e., reciprocal activation and/or maintenance) in the limb bud system: [1] a loop within the anterior mesenchyme (AM) mediated by Reck and Igf2, [2] a loop between the AM and DE mediated by Reck and Wnt7a, and [3] a loop between the AM and AER mediated by Reck and Fgfs (probably with Wnt7a) (Fig. 7C).

What do we learn from the other limb phenotypes? The round porous fingertips in the Reck-hypomorph mice resemble several human conditions, including clubbed fingers, acro-osteolysis, and osteoporosis. These symptoms, however, have been correlated with diverse causes, and therefore the symptomatic resemblance is not very informative in elucidating its molecular basis. On the other hand, cutaneous horn is known as a rare precancerous skin tumor that may occur in various parts of the human body. Its exclusive occurrence on the dorsal side of extremities in Reck-hypomorphic mice may be another indication that reduced Reck impacts the limb bud DE. The precancerous nature of this phenotype is consistent with the activity of Reck to down-regulate several genes involved in “Pathways in Cancer” (Fig. 6A; supplementary material Table S2). It is unclear, however, why cutaneous horns occur in all extremities in the mutant mice while the postaxial bone defects occur exclusively in the forelimbs in a right-dominant fashion. This may reflect differences in the lineages and/or stages of target cells (e.g., keratinocytes vs. early limb bud mesenchyme/ectoderm) or in the levels of Reck required for the events (i.e., the cutaneous horns may arise with smaller decrease in Reck expression than the postaxial bone defects). It will be interesting to see whether RECK is down-regulated in human cutaneous horns and whether there is any correlation between the level of RECK and severity of the lesion. Further studies on the mechanism of cutaneous horn formation in the mutant mice may provide additional insights into how Reck works.

The reasons why the skeletal phenotype occurs only in the forelimbs in a right-dominant fashion in the low-\(\text{Reck}\) mice (Fig. 1H) is presently unclear. The observed difference in the levels of Reck expression among the four limbs (supplementary material Fig. S4) may provide a feasible explanation, although, other models such as temporal or spatial difference in the expression of functionally compensating genes, cannot be ruled out. Interestingly, Wnt7a-deficient mice show a similar, but milder, asymmetry in skeletal phenotype among the four limbs (Parr and McMahon, 1994), further supporting the relevance of Wnt7a-signaling to Reck’s action in this system. Besides being a system useful in exploring several aspects of signal transduction and tissue interactions, the low-\(\text{Reck}\) mutant may also be useful in studying some fundamental issues in developmental biology such as forelimb-hindlimb dichotomy and lateral symmetry.

A variety of teratogens, including cadmium, acetazolamide, and ethanol, are known to induce a common limb malformation in mice after treatment at E9.5 (Bell et al., 2005; Schreiner et al., 2009). This malformation (postaxial, right-dominant forelimb ectrodactyly) is almost a perfect phenocopy of \(\text{RLow/}\text{RLow}\) mice (Fig. 6E), implicating Reck as a target of these agents in teratogenesis. Other teratogens which cause this phenotype such as acetazolamide and thalidomide, however, did not affect Reck expression, suggesting that they may target at some points down-stream of Reck or may damage limb DE via a different mechanism(s). Artificial Reck expression in mice in conjunction with teratogen-treatment may be useful in testing these possibilities.

In conclusion, this study has revealed the importance of mesenchymal Reck in the maintenance/expansion of three signaling centers (DE, AER, and ZPA) essential for mammalian limb patterning and has shed some fresh light on the molecular and cellular interactions underlying limb development and patterning (Fig. 7). The newly emerging role of Reck in supporting tissue interactions has broad implications in understanding its roles in other processes in embryogenesis as well as in tumorigenesis.

Acknowledgements
We are grateful to Dr. Shin-ichi Aizawa for advice in generating mutant mice, Dr. Philippe M. Soriano for R26R mouse, Dr. Andrew P. McMahon and Dr. Matthew Scott for WISH probes, and Dr. Maja Adamska for technical advice. We also thank Aiko Nishimoto, Hai-Ou Gu, and Mari Kojima for technical assistance, Aki Miyazaki for secretarial assistance, and all members of the laboratories for their help and advice. This work was supported by JSPS Grant-in-Aid for
References


